Polymerised Liposomes As Intranasal Vaccine Adjuvants

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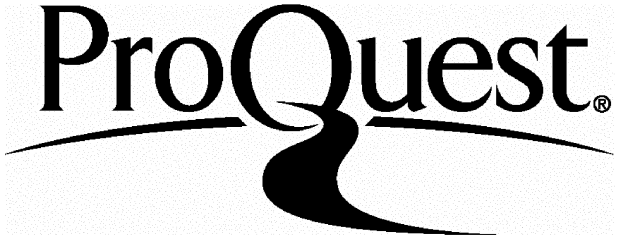

Abstract of thesis

The purpose of this thesis was to establish whether polymerised liposomes possessed adjuvant activity following intranasal administration and whether the inclusion of poloxamers or chitosan with such a formulation further potentiated the immune response when using tetanus toxoid (TT) as a model soluble protein antigen.

Early studies were based on the preparation, optimisation and characterisation of polymerised liposomes of a size appropriate for mucosal vaccination (with respect to attaining a high systemic and mucosal immune response), which was hypothesised to be between 0.3-1 μm in diameter. Such particle sizes were achieved through use of a freeze-thaw and extrusion procedure. Liposomes were prepared from a polymerisable phospholipid that was polymerised using UV light (254 nm), the extent of which was followed by measuring the decrease in UV absorption by the conjugated diene bond at 254 nm. Polymerised liposomes were shown to possess a less negative zeta potential and to be more hydrophobic (determined using the hydrophobic dye Rose Bengal) than non-polymerised liposomes.

A modified dehydration-rehydration vesicle (DRV) method was used for the post-polymerisation association of bovine serum albumin and TT to liposomes and hence avoided the exposure of the antigen to formulation processes such as UV light, which may have resulted in decreased protein immunogenicity. The effect of the association of TT to polymerised and non-polymerised liposomes using the modified DRV method was compared to polymerised liposomes with surface adsorbed antigen using *in vitro* release studies and also following intramuscular and intranasal administration to BALB/c mice. Polymerised liposomes were further modified by surface adsorption of poloxamer 331 (L101) and 401 (L121), chitosan chloride or chitosan glutamate for further investigation of intranasal adjuvanticity. The systematic addition of poloxamer or chitosan to liposomes was conducted to minimise vesicle aggregation and the levels of association were quantified using spectroscopic assays.

Animals immunised intramuscularly with TT associated to non-polymerised DRVs or polymerised liposomes with surface adsorbed TT produced a higher mean antigen-specific serum IgG antibody response compared to animals given free protein in solution. Unexpectedly, administration of TT associated with polymerised DRVs resulted in the lowest mean antigen-specific serum IgG antibody levels.

In comparison, intranasal administration of all liposomal formulations resulted in lower levels of TT-specific serum IgG titres for all formulations investigated. However, consistent with intramuscular administration studies, animals immunised with polymerised liposomes with surface adsorbed TT elicited the highest mean TT-specific serum IgG response amongst all liposomal formulations. In contrast, nasal administration of TT associated with non-polymerised DRVs showed a similar antigen-specific serum IgG response to that elicited by free TT. Further modification of polymerised liposomes by coating with L101 or L121 before surface adsorption of TT resulted in an enhancement of antigen-specific serum IgG antibody levels. Surprisingly, chitosan-coated polymerised liposomes did not enhance the systemic immune response markedly in comparison to non-coated polymerised liposomes. The high immune response elicited by L101-coated polymerised liposomes was also confirmed on analysis of cytokine production after antigen stimulation of mixed spleen cells of immunised mice, which secreted significantly higher levels ($p<0.05$) of IL-2, IFN-γ, IL-4, IL-6 and TNF-α than mice given L121-coated, chitosan-coated or non-coated polymerised liposomes.

The results reported in this thesis further emphasise the importance of the route of administration, which affects the type of immune response elicited to a particular vaccine, possibly due to differences in antigen uptake and subsequent processing by APCs. The overall findings indicated that the surface adsorption of TT to polymerised liposomes led to the highest immune response amongst liposomal formulations following intranasal administration and was further enhanced with the addition of L101 and L121.

Keywords: polymerised liposomes; nasal vaccine delivery; chitosan; poloxamer; TT

Every experiment proves something.

If it does not prove what you wanted it to prove, it proves something else.

Anon

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AIDS	Acquired immune deficiency syndrome
APC	antigen presenting cell
B cell	B lymphocyte
BCG	Bacillus Calmette-Guerin
BALT	Bronchus-associated lymphoid tissue
BSA	Bovine serum albumin
ChCl	Chitosan chloride
ChGl	Chitosan glutamate
CMC	critical micellar concentration
CMIS	Common mucosal immune system
CT	Cholera toxin
CTB	Cholera toxin subunit B
CTL	Cytotoxic T lymphocyte
DC-Chol	Dicetyl-cholesterol
DCP	Dicetylphosphate
DODPC	1,2-di (2,4-octadecadienoyl) phosphatidylcholine
DNA	Deoxyribonucleic acid
DPPC	L-α-dipalmitoylphosphatidylcholine
DRV	Dehydrated-Rehydrated Vesicle
DSPC	Distearoylphosphatidylcholine
EEA	*Euonymus europaeus*
ELISA	Enzyme linked immunosorbent assay
EPI	(World Health Organisation) Expanded Programme on Immunisation
FAE	Follicle-associated epithelium
FDEL	Freeze-dried empty liposomes
FT	Freeze-thaw
GALT	Gut-associated lymphoid tissue
GS I-B_4	*Griffonia simplicifolia* I isolectin-B_4
HEV	High endothelial venule
HIV	Human immunodeficiency virus
HLB	Hydrophile-lipophile balance
HSV-2	Herpes simple virus type 2
IFN-γ	Interferon-γ

IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgG_1	Immunoglobulin G_1
IgG_{2a}	Immunoglobulin G_{2a}
IgM	Immunoglobulin M
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-12	Interleukin-12
ISCOM	Immune stimulating complex
L101	Poloxamer 331
L102	Poloxamer 401
LPS	Lipopolysaccharide
LT	*Escherichia coli* heat labile enterotoxin
LTB	*Escherichia coli* heat labile enterotoxin subunit B
LUV	Large unilamellar vesicle
mwt	Molecular weight
M cell	microfold cell
MALT	Mucosa-associated lymphoid tissue
MDP	N-acetyl muramyl-L-alanyl-D-isoglutamine
MHC	Major histocompatability complex
MLV	Multi-lamellar vesicle
MMR	Measles/mumps/rubella
MPL	monophosphoryl lipid A
MVL	Multi-vesicular liposome
$^m/_m$	Mass *per* mass
$^m/_v$	Mass *per* volume
NALT	nasally-associated lymphoid tissue
NP DRV	Non-polymerised dehydration-rehydration vesicle
NP SAV	Non-polymerised surface adsorbed vesicle
ODN	Oligodeoxynucleotide
$^o/_w$	Oil in water
PAMP	Pathogen-associated microbial pattern
PBS	Phosphate-buffered saline

PBS-T	Phosphate-buffered saline/Tween® 20
PC	Phosphatidylcholine
PCLN	Posterior cervical lymph node
PE	Phosphatidylethanolamine
PG	phosphatidylglycerol
PS	Phosphatidylserine
P DRV	Polymerised dehydration-rehydration vesicle
PEO	poly (ethylene oxide
pI	Isoelectric point
PPO	poly (propylene oxide)
PRR	Pathogen-recognition receptor
P SAV	Polymerised surface adsorbed vesicle
REV	Reverse-phase evaporation vesicle
SA	Stearylamine
SAF	Syntax adjuvant formulation
SAV	Surface adsorbed vesicle
SCLN	Superior cervical lymph node
SD	Standard deviation
SE	Standard error
sIgA	Secretory immunoglobulin A
SIV	Simian immunodeficiency virus
SPB	Sodium phosphate buffer
SPC	Soya phosphatidylcholine
SPG	Soya phosphatidylglycerol
SUV	Small uni-lamellar vesicle
T cell	T lymphocyte
T_c	Phase transition temperature
T_c cell	T cytotoxic cell
T_h cell	T helper cell
TEM	Transmission electron microscopy
TNF-α	Tumour necrosis factor-α
TT	Tetanus toxoid
UEA	Ulex Europaeus Agglutinin I
UV	Ultraviolet
$^v/_v$	Volume *per* volume

$^{w}/_{o}$	Water in oil
WHO	The World Health Organisation

Preface

Edward Jenner (late 18th century) is the man that modern science will remember for his pioneering work in the field of vaccination. The influence of the Eastern practice of inoculation had already resulted in a decline in the incidence of smallpox in the West. Jenner, aware of the folk belief that dairymaids were protected from small pox, investigated the case of one such milkmaid presenting with the symptoms of cow pox (a disease in cattle which results in only mild symptoms in humans). Matter from a pustule on the finger of the milkmaid was used to infect a small boy. On regaining his health from this first exposure, this same boy was again subjected to small pox but this time he remained disease free. In 1798, Jenner published these results in his '*Inquiry into the causes and effects of the variolae vaccinae*'. The term vaccination was hence coined (from the Latin '*vacca*' - 'a cow').

In the following century, the German bacteriologist Robert Koch isolated and identified the bacillus that was found to cause anthrax and tuberculosis and the organism responsible for cholera. It was in recognition for his work on tuberculosis that he was awarded the Nobel prize for medicine in 1905. The connection between the 'germ' and disease led to Louis Pasteur's success in the development of the first vaccines for anthrax and tuberculosis. At around the same time, Emil von Behring and Hidesaburo Kitasato inactivated diphtheria toxin and used this in animals to protect them from diphtheria.

Such efforts of long-ago scientists coupled with the advancement of our understanding of immunology in the 20th century, has led us to our current vaccine position in which the threat of once major afflicting diseases has been ebbed. A mass immunisation campaign against small pox led to its global eradication and not one naturally acquired case has been reported since 1977 (Goldsby *et al.*, 2000; Dittmann, 2001). The World Health Organisation (WHO) is targeting poliomyelitis as the next major disease to be eradicated and this should be achieved in the next few years.

The current position of disease control however, is far from one in which we can be complacent. Records from of year 1998 show that 13.3 million deaths (25 % of all noted (53.9 million) deaths) in the world were caused by infectious diseases. This figure

does not account for those deaths attributable to infections but classified under cancer, respiratory or intestinal diseases. Death due to infectious diseases came second only to cardiovascular linked deaths (31%) (Dittmann, 2001).

Incidence of diphtheria, malaria and tuberculosis is increasing and some relatively new diseases such as acquired immune deficiency syndrome (AIDS) are yet not only without any vaccine but spreading at a rate of 16,000 new cases of Human Immunodeficiency Virus (HIV-1) *per* day (Goldsby *et al.*, 2000). Controlled infectious diseases have recently shown signs of re-emergence due to antibiotic resistance and other forms of 'self-defence' that the microorganisms have employed. Previously unexposed populations are now subjected to 'foreign' infectious pathogens due to increased global travel and migration. This may be due to the introduction of new infections to populations which have not had previous exposure to such pathogens (and hence no adaptive immunity to it) or exposure of the visitor to pathogens that are otherwise harmless to the native population (as a result of pre-existing immunity).

Patient acceptability and concordance towards immunisation is now an important factor to be accounted for when contemplating the future success of vaccination. Recent fears in Britain about the proposed linkage of the MMR (measles/mumps/rubella) triple vaccine to autism has led to many parents withdrawing their permission for their child to be vaccinated. Such actions have severe implications in terms of herd immunity. Current day vaccines (as with all other medication) will cause adverse reactions of varying extent but due to the vaccines own efficacy in disease reduction, there is a decreased awareness and fear of such infections and so a lower tolerance of such adverse events. Hence the benefits of vaccination in the developed world are not always at the forefront of people's minds.

The WHO Expanded Programme on Immunisation (EPI) was introduced in 1974 and seeked to promote the vaccination of all children against a minimum of six diseases (diphtheria, pertussis, measles, poliomyelitis, neonatal tetanus and tuberculosis) for which reasonably priced and safe vaccines were available. The result of the programme raised coverage of immunisation from 5 % at its conception to 80 % in 1990 (André, 2001). This statistic however, has reached a plateau at this figure due to a number of reasons including political and economic aspects. The magnitude of the impact of the vaccines in the EPI in terms of disease reduction is quantified by Clements and Griffiths

(2002). It is important to note that the supply for these 'basic' vaccines is fragile and there is a real risk of shortages. In many countries, this basic regime is further 'expanded' (to include for example: pneumoccocal, hepatitis B and yellow fever). Hepatitis B vaccination in particular, is hoped to be the next vaccine included with the current six in the EPI.

As a result of the EPI, babies and young children now face the prospect of a large number of vaccine doses within a relatively short period of time. Increasing numbers of routine vaccines results in more frequent distress for the patient (the majority of which are currently *via* injection). It has been reported (Jacobson *et al.*, 2001) that children (15-18 months and 4-6 years) experience serious distress to panic in about 20 % of cases prior to injection. During the procedure 92.7 % of the younger children (15-18 months) and 44.4 % of the older children (4-6 years) experienced serious distress to panic. The authors also reviewed situations by which the level of distress in children is reduced. The parent's attitude to and support for vaccination and reassurance to the child during the procedure as well as distraction with music or activity, the use of hypnosis and topical anaesthetics and improved injection technique have all decreased negative effects/responses to vaccination. The benefits of immunisation without injections using alternative technology and routes are highly recognisable in this regard. A review of adverse reactions to some commonly administered vaccines is given by Dittmann (2001).

The growing anti-vaccine movement (especially in the U.K., France and other Western European countries, USA, Japan and Australia) can be attributed to factors beyond the blame culture of current society (Poland and Jacobson, 2001). Anti-authority stances, conspiracy theories, infection in previously immunised individuals, fears about combined vaccine formulations, faulty batches and media propagation of accurate and inaccurate information will influence the concerns of 'expert' patients. The authors also review the outcomes of hasty vaccine withdrawals by authorities (prior to evaluation of sound scientific evidence) and the resultant increase in infection in non-immunised populations. Generally, people support vaccine research but there is a risk of this support being undermined due to insufficient public education on the area (Ritvo *et al.*, 2003).

One other crucial hurdle for vaccines is cost. In common with other pharmaceuticals, the investment necessary from discovery of a new product, through developmental stages and to licensing and commercial launching is enormous. Mahoney and Maynard (1999) estimated the figure at about 100-300 million US dollars, André (2001) at about 200-400 million US dollars and Masignani and colleagues (Masignani *et al.*, 2003) put the figure at 850 million US dollars. Though the discovery stages for new vaccines have reduced in time-span (to about 2-5 years), the developmental stages have increased to over 10 years. After the initial licensing (usually in a developed nation), further costs and time delays are incurred before the vaccine is licensed and available where maybe it's need is most: the public sector of developing nations. The high investment of products where the profitability is controlled (due to affordability constraints by those who would be the largest consumers) creates a major motivation sink for industries when deciding on resource allocation for new vaccines or improving existing ones.

Economic aspects at the consumer end (governments and similar being the largest faction) however, are in the favour of vaccination. Vaccination against *Haemophilus influenzae* type B, MMR and diphtheria, tetanus and acellular pertussis results in a saving double, 21 times and 24 times the cost of immunisation respectively (Masignani *et al.*, 2003). These figures only account for direct medical savings and do not include indirect gains related to a healthy workforce. The cost of immunisation against the six basic diseases in the EPI is US $15 but only $ 1 represents the cost of the vaccine (Martin, 1999). A saving of the other 93 % could be worked towards by decreasing costs associated with logistics (preserving the cold-chain) and personnel (necessity for trained individuals for vaccine administration).

Recent proposals to address the concerns of both suppliers (intellectual property rights and profitability of vaccine production) and consumers (standardisation of efficacy and quality) favour a system of 'tiered' pricing by which industries can profit substantially from supplies to developed nations to compensate for 'near-cost price' vaccine provision to developing nations (Mahoney *et al.*, 2004). Disease is linked to poverty, as the chicken is to the egg- it is hard to know which is the precursor for the other. Addressing the inequity in vaccine provision would lead to a reduction in disease burden, which it is anticipated will increase chances to escape poverty and thus foster conditions for development, stability and peace (Jimenez, 2001).

In 1990, the Children's Vaccine Initiative instigated an International Task Force for Vaccine Development (Alpar *et al.*, 2000) whose aim it remains to develop vaccines with the following attributes:

- Can be given earlier in life
- Single-dose (no need for booster doses)
- Non-parenteral administration
- Minimal storage requirements with a long shelf life

The WHO has pinpointed three communicable diseases (HIV/AIDS, malaria and tuberculosis) for concern and has proposed new guidelines to tackle them. One of the objectives of the Vaccine and Biological Department at the WHO is 'the continued research and development of new vaccines of public health importance' (Jimenez, 2001) with continuing efforts in the development of new administration technologies/approaches. This is further supported by the WHO, UNICEF, the World Bank and others (Global Alliance for Vaccines and Immunisations).

There is therefore much work to be done for the 'vaccinologist', not only in research and development to cover those diseases to which we remain unprotected but to improving existing strategies also so that healthy patients can be encouraged to continue with immunisation schemes. As summed up by Jaques-François Martin (1999) there is a good medical, economic and ethical case for the international community to improve equity in the access to healthcare.

1.0 Introduction

1.1 The immune system

The human response to infections can be broadly divided into two forms of defence: innate and acquired (adaptive) immunity.

1.1.1 Innate immunity

This is a pathogenic non-specific form of defence that we possess as a 'baseline' for which previous exposure to infection is not a prerequisite (table 1.1).

1.1.2 Adaptive immunity

Adaptive immunity is a pathogen specific mobilisation of the immune system comprising B lymphocytes, T lymphocytes and antigen presenting cells (APCs). Figure 1.1 depicts the cells of the immune system and figure 1.2 represents a summary of the main mechanisms of adaptive immunity.

B lymphocytes (B cells) contain membrane bound antibody molecules (glycoproteins specific to an antigen) which, on encounter with that specific antigen, will cause the B cell to divide and produce further B cells which may progress to become memory or effector B cells (plasma cells) that are currently thought to be either short-lived (half-life: 3-14 days) or long-lived (half-life: 3-4 months) (Crotty and Ahmed, 2004). B cells internalise specific antigens *via* receptor-mediated endocytosis by the membrane bound antibody before further processing of the antigen. The B cells are also responsible for the secretion of vast amounts of free antibody. Antibody facilitates the removal of antigen/pathogen by three methods:

- Several antigens linked to an antibody facilitate easier removal of the molecules by phagocytosis
- Activation of complement system and subsequent lysis of antigen/pathogen

- Action as a physical barrier preventing further binding of the antigen/pathogen to other cells

There are several antibody isotypes that are involved in mounting an immune response after antigenic stimuli. For the purposes of the studies in this thesis, we investigated IgM, IgG and IgA:

- Serum IgM production is an early indication of the activation of an immune response after the administration of an immunogenic agent (Playfair and Chain, 2001). IgM is found as a membrane-bound antibody on B cells which is secreted in response to an immunogenic stimuli as a pentameric structure which possesses a higher antigen-binding capacity than other antibody isotypes (Kuby, 1997). IgM (after antigen-binding) activates the classical complement pathway, which leads to the subsequent lysis of the antigen. IgG also activates the classical pathway after antigen binding but to a lesser extent than IgM.
- Serum IgG is generally observed after IgM in response to an antigenic stimulus. IgG, is found in serum in larger quantities than the other antibody isotypes. Unlike IgM, IgG is not also found as a membrane-bound antibody on the surface of mature B cells and activates complement to a lower extent. IgG (after antigen binding) has a high affinity for macrophage receptors which thus leads to the further processing of the antigen. Secreted IgG, being a monomeric unit, is also found in extravascular spaces. IgG can be further divided into subclasses having differing biological activities.
- Serum IgA is the second most abundant antibody found in the serum and secretory IgA (sIgA) (IgA plus a secretory component) is the main antibody found in mucosal and external secretions. Secretory IgA serves to bind pathogens and prevent their attachment to and subsequent invasion of host cells (see section 1.4.6.7.2 for further details about the role of secretory IgA in mucosal immunity).

T lymphocytes (T cells) like B cells are also derived from the bone marrow but are matured in the thymus gland. The T-cell membrane incorporates receptors: either CD4 or CD8 glycoproteins. The existence of the former promotes the T cell to function as a T helper (T_h) cell ($CD4^+$ cell) and with the presence of the latter the T cell functions as a T cytotoxic (T_c) cell ($CD8^+$ cell). $CD4^+$ T cells or $CD8^+$ T cells will bind to those APCs

(macrophages, dendritic cells and B lymphocytes) which possess cell membrane proteins called major histocompatability complex (MHC) molecules which are further subdivided into two types. $CD4^+$ cells will recognise MHC class 2 molecules (expressed due to APC processing of exogenous antigen) whereas $CD8^+$ cells will recognise MHC class 1 molecules (expressed due to APC processing of endogenous antigen) (McNeela and Mills, 2001). These two types of MHC molecules arise due to the process by which the antigen was previously dealt with by that cell. Antigen which would have entered the APC *via* phagocytosis will be processed *via* the endocytic pathway and be bound to MHC class 2 molecules and exported to cell surface. However, when the antigen was produced within the cell itself (cytosolic pathway), by viruses for example, then it will be bound to and expressed with MHC class 1 molecules prior to cell surface expression.

Activation of T cells following binding to MHC-APCs results in the formation of complex memory and effector cells (Crotty and Ahmed, 2004). When a T_h cell is 'activated' it secretes cytokines (growth factors), which in turn activate B cells, T_c cells and non-specific macrophages or natural killer cells (large granular lymphocytes) depending on the cytokines produced (reviewed by Rajananthanan *et al.*, 2000). Macrophages stem originally from monocytes circulating in the blood and are either fixed in tissues or circulating freely. When activated they have an enhanced phagocytic activity and secrete inflammatory and fever provoking mediators. Natural killer cells use cell receptors to recognise abnormal cell membrane configurations or certain antibodies which are conjugated to antigen.

The range of cytokines produced by immune cells influences the types of immune response seen (reviewed by Kaiser *et al.*, 2004). The T_h1 response predominantly encourages T cell and macrophage activation and inflammation whereas a T_h2 response activates B cells (reviewed by McNeela and Mills, 2001). Macrophages are activated by interferon-γ (IFN-γ), released from T_h1 cells whereas T_c cells are activated by the release of the cytokine interleukin-2 (IL-2) from T_h1 cells which then bind to Class 1 MHC-APCs and multiply into an effector cell known as a cytotoxic T lymphocyte (CTL) (Kuby, 1997). CTLs recognise and kill altered cells (such as virus infected and tumour cells) and have been shown to be important in the immune response against intracellular pathogens such as HIV (reviewed by Crotty and Andino, 2004).

Table 1.1 Summary of mechanisms involved in innate immunity (adapted from Kuby, 1997; Niedergang *et al.*, 2004)

Mechanism	Function
Physical barriers	Physical deterrent to invasion (e.g. skin)
Secretion of mucus	Prevents adherence of bacteria to epithelial cells Contains anti-bacterial and anti-viral components Ciliary movement, coughing and sneezing hastens removal of material entrapped in mucus Presence of lysozyme in mucus/tears cleaves the peptidoglycan layer of the bacterial cell wall
Microbial antagonism	Presence of a natural flora of organisms providing a competitive environment for any new challenging organisms
Fever response	Deterrent to the growth of infectious agents
Specific barriers	Low pH of stomach is a barrier to oral pathogens Virus infected cells produce and release interferon inducing a virus resistant state to cells in close proximity Bactericidal components in secretions (spermine and zinc in semen and lactoperoxidase in milk) Secretion of sebum from skin (containing lactic and fatty acids) provides non-optimal conditions for pathogens Rinsing function of tears and urine
Serum complement proteins	Certain complements are activated on encounter with microbial surfaces and *via* cascade mechanisms (alternate pathway) adhere to surface polysaccharides and enhance detection of such surfaces by phagocytic cells possessing receptors for such complements Certain complements increase the expression of anti-complement surface receptors on phagocytes Certain complement fragments combine to form a 'membrane attack complex' which is inserted into the microbial surface resulting in cell lysis due to infiltration of surrounding medium Complement proteins have a chemotactic effect and promote mast cell and basophil degeneration
Phagocytosis	Functionalised cells such as polymorphonuclear neutraphils and macrophages internalise organisms after initial attachment/association. This 'phagosome' then fuses with lysosomes to form phagolysosomes in which the engulfed material is subject to hydrolytic degradation by lysosomal enzymes. The degraded material is then associated with MHC molecules which are then presented to the outside of the cell for further processing (section 1.1.2).
Acute inflammatory response	Increased capillary permeation at site of infliction and release of chemotactic moieties accumulates phagocytes. Serum with a much higher than usual content of protein is presented (acute phase proteins). Decrease in capillary drainage increases local temperature due to erythema formation
Natural killer cells	Recognises and kills virally infected cells
Eosinophils	Damage parasitic membranes

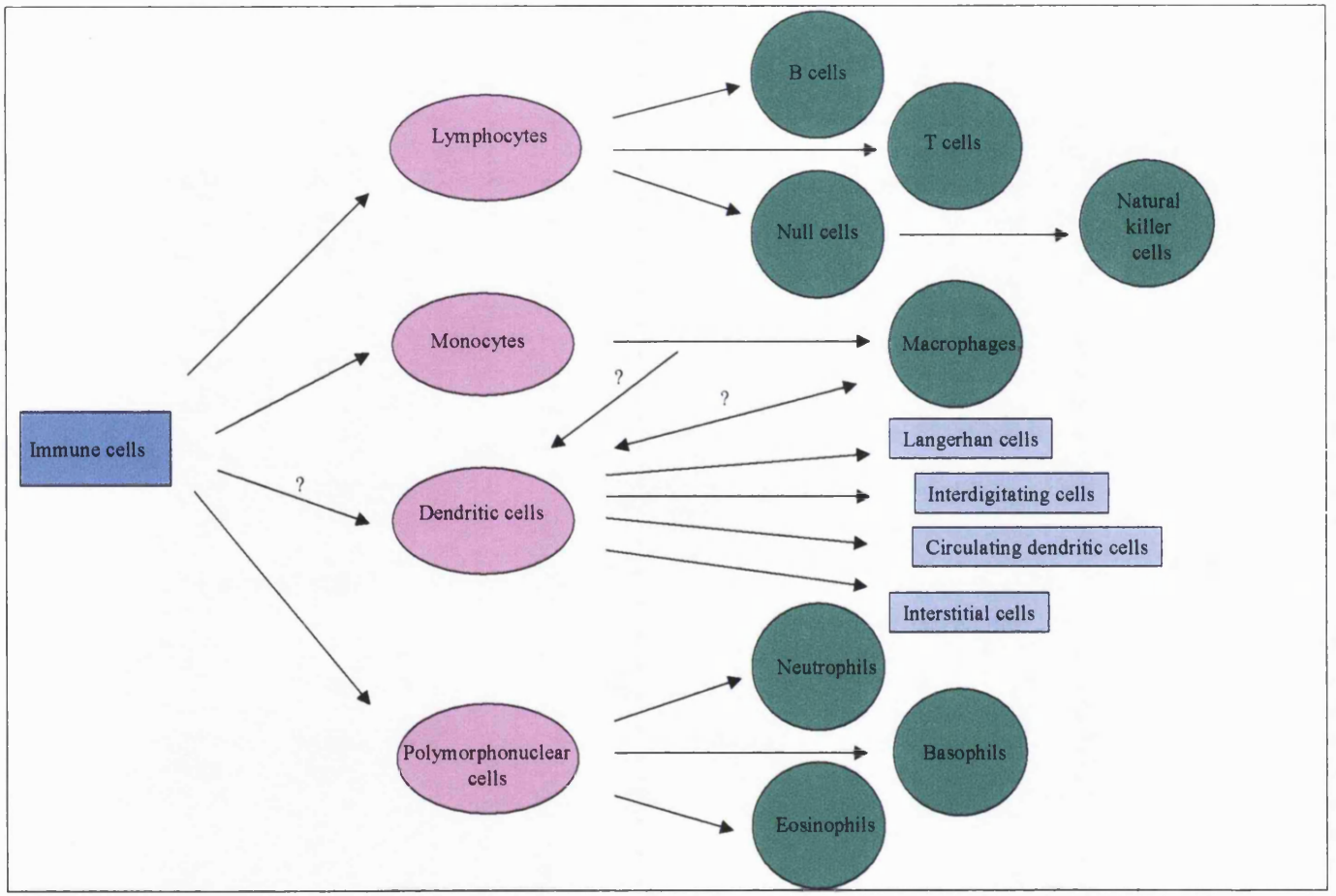

Figure 1.1: Cells of the immune system (Kuby, 1997; Beutler, 2004)
Note: The precursor cell pathway from which dendritic cells are formed remains unclear but may be either from an intermediate cell between monocyte to macrophage differentiation, an interconversion with macrophages or through a direct pathway from myeloid cells

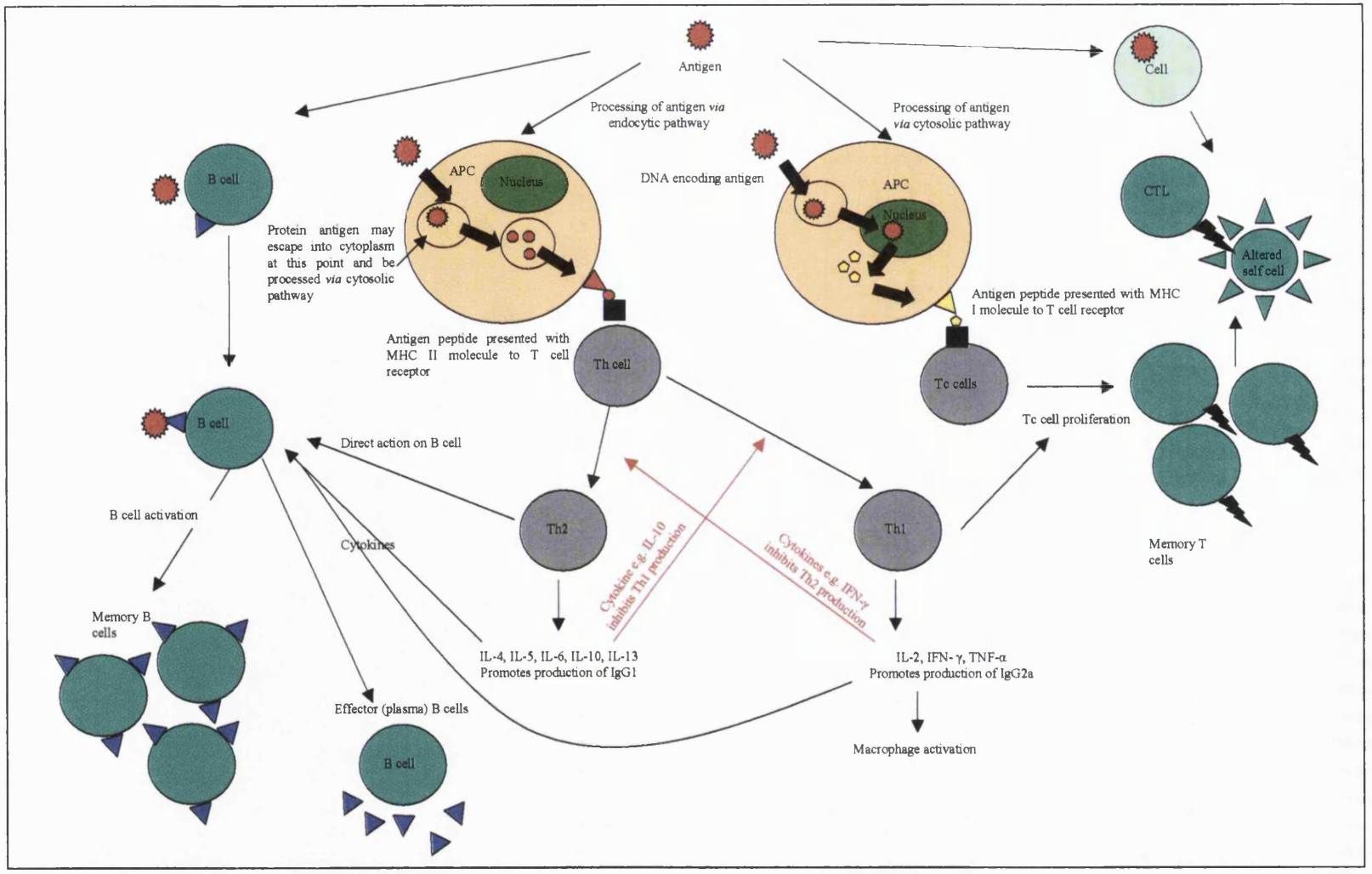

Figure 1.2 Overview of the immune response to antigenic stimuli

The above can be summarised as two branches of immunity. The B cell mediated branch is known as humoral immunity and CTL mediated branch as cell-mediated immunity. There are no clear-cut boundaries between the various weapons of immunity that we possess and an immune response is generally a fusion of many different elements. Thus, when developing a vaccine, we must bear in mind that not only is it important to elicit a memory response that will keep the patient protected beyond the subsidence of the primary response but also, as is necessary for many diseases, that both humoral and cell-mediated branches of the immune system are activated to ensure complete protective action.

1.2 Vaccines

1.2.1 Passive immunisation

This occurs naturally in the human life cycle in pregnancy, when antibodies are passed from the mother to the baby *via* the placenta. Continuation of such transfer occurs *via* breast milk when a mother chooses to breastfeed her baby. Antibodies can also be transferred to patients by injection of antibodies collected from animals or other humans. As there is only antibody transfer, passive immunisation provides only treatment not prophylaxis, as no memory response is elicited. There is also a great risk of hypersensitivity reactions to the 'foreign' antibody.

1.2.2 Active immunisation

This can occur either naturally as a memory response to previous infection or can be acquired due to priming with a vaccine. A vaccine can basically be of one of the following types:

1.2.2.1 Live attenuated vaccines

Live vaccines have been generally found to provide greater protection against infection than killed vaccines. This is probably due to the activation of both cell-mediated and humoral immune responses by live vaccines and the activation of a T_h1 type response, which is necessary for protection against intracellular pathogens (Norimatsu *et al.*, 2004). As the vaccine will replicate within the host cells, there is exposure of the

antigen to the recipient over some time hence possibly reducing booster requirements. However, as the vaccine is live there is a chance that the vaccine may revert back to its virulent form. Live vaccines are prepared by eliminating bacterial or viral pathogenicity by subjecting the microorganism to growth through different non-human hosts or unfavourable cell culture conditions. As the microorganism adapts to grow in the new environment, they lose their capacity to grow in the human host. Examples include the Bacillus Calmette-Guerin (BCG), Sabin polio vaccine and measles vaccine. The intranasal administration of a recombinant live attenuated poliovirus vector expressing antigens from the simian immunodeficiency virus (SIV) was recently reported (Crotty and Andino, 2004) to protect female cynomolgus macaques from infection after intravaginal challenge with $SIV_{mac}251$. The advantages of the use of a non-invasive (intranasal) route of administration (see section 1.4.6.7.1) in the study, such as eliminating the possibility of needle-stick injuries further adds to the promise of live recombinant vector vaccines.

1.2.2.2 Killed/inactivated vaccines

These are composed of microorganisms that are killed/inactivated after heat or chemical treatment to prevent replication (and return to virulence) in the host. There is however, a possibility of virulence if inactivation is incomplete but once it is assured there is no risk of reversion. Care must be taken to preserve the antigenic element during process and so chemical inactivation (with formaldehyde or alkylating agents) is preferred over heat treatment. Two examples of such vaccines are the Salk polio and pertussis vaccines. Killed/inactivated vaccines are less thermolabile than live attenuated vaccines and so costs associated with temperature regulation are reduced. However, as there is no replication of the organism within the host, only humoral immunity (due to a preferential T_h2 type response) is attainable and booster doses are often required due to an insufficient memory response. The use of a liposomal formulation of a formaldehyde killed whole cell *Yersinia pestis* vaccine (Baca-Estrada *et al.*, 2000) was reported to induce a higher systemic and mucosal response than intranasal administration of the commercially available (for invasive delivery) killed vaccine alone (following intranasal delivery to female C57BL/6 mice). Protection studies in the same publication showed that a higher number of animals primed subcutaneously with the killed whole cell vaccine and boosted intranasally with the liposomal killed whole cell vaccine survived intranasal challenge with *Y. pestis* than when animals were primed

(subcutaneously) and boosted (intranasally) with the killed whole cell vaccine alone. Hence, the inclusion of additional adjuvants such as liposomes, to existing vaccines may make the use of alternate delivery routes feasible and possibly provide a higher level of protection against certain pathogens due to the additional activation of a mucosal response.

1.2.2.3 Purified subunit (macromolecular) vaccines

There is no risk of virulence with these systems, which can be:

- the purified polysaccharide coat of the bacteria of interest which initiates the production of antibodies that will coat the vaccine and promote phagocytosis. However, cell-mediated immunity is not activated (Kuby, 1997).
- The antigenic surface proteins of pathogens such as the hepatitis B vaccine, which consists of the major surface antigen of the virus. The proteins can be produced by extraction or through using recombinant deoxyribonucleic acid (DNA) technology by cloning the protein into bacterial, yeast, insect or mammalian cultures.
- Toxoid (inactivated bacterial toxins) based; such as purified diphtheria and tetanus toxoid vaccines in which the toxins are inactivated with formaldehyde.

The use of subunit antigens for mucosal delivery usually requires the use of adjuvants to improve both the activation of systemic and mucosal immunity. Protective immunity against intranasal challenge with influenza virus in female ferrets was demonstrated (Lambkin *et al.*, 2004) following the intranasal administration of influenza virus haemagglutinin in a virosomal preparation (section 1.3.12). Protective immunity in female BALB/c mice against a low dose challenge with subcutaneous administration of *Yersina pestis* was shown (Eyles *et al.*, 2004) following transcutaneous immunisation with F1 and V proteins from *Y. pestis* when delivered with cholera toxin. Therefore, the use of subunit antigens provides a safer alternative to the use of live or killed vaccines and through the use of adjuvants can elicit protective immunity.

1.2.2.4 DNA vaccines

Following administration of plasmid DNA, host cellular mechanisms are used to express the encoded antigen(s) and so the protein produced will be processed as an intracellular pathogen. This leads to the activation of a T_h1 type of response and production of CTLs which may be necessary for protection against certain infections such as HIV (Crotty and Andino, 2004). A further advantage of DNA vaccines is that multiple antigenic components can be encoded for in one plasmid, resulting in the opportunity for vaccinating against a number of diseases in one formulation. If the antigen were to be expressed over a period of time, then such continuous exposure of the antigen to the host could lead to a greater memory immune response than if the antigen were to be delivered in a single dose (if not in a controlled release formulation). Other advantages of DNA vaccines would include lower costs of production, ease of modifying plasmid DNA to follow changing strains of pathogens and lower storage and transportation costs as plasmid DNA is heat-stable (McCluskie and Davis, 1999; Bergmann-Leitner and Leitner, 2004).

However, the immune response to naked DNA may be low in humans (Mollenkopf *et al.*, 2004)) and so high quantities of plasmid may need to be given. Thus, immune responses to plasmid DNA formulations often need to be optimised by modification of plasmid DNA, co-administration with adjuvants/carriers or delivery in live attenuated bacterial or viral vectors. The use of salmonella bacterium has been shown to produce cell-mediated and humoral responses against encoded antigens (Hormaeche and Kahn, 1996) as has the adenovirus vector (Perkus and Paoletti, 1996).

1.2.2.5 Synthetic peptide vaccines

These vaccines consist of peptides corresponding to B cell and T cell epitopes on protein antigens. For the induction of humoral immunity, the peptide would be made to be composed of B cell epitopes and in the case of activating cell-mediated immunity, T cell epitopes must be identified (Kuby, 1997). However, these systems still require optimisation as T cell epitopes vary in immunogenicity amongst different people due to the effect of MHC polymorphism within a species. Many proteins also contain suppressor amino sequences, which if removed would lead possibly to an enhanced immune response following administration.

1.3 Adjuvants

Adjuvants (from the latin 'adjuvare' – 'to help') are substances or formulations that are delivered with the antigenic component and work to further enhance the immune response. Cox and Coulter (1999) summarised five ways in which the mode of action of adjuvants can be categorised:

- Immunomodulation: A greater overall response and a modification of cytokine response (i.e. stimulation of either T_h1 or T_h2 cells).
- Presentation: The adjuvant preserves the natural configuration of the antigen for presentation to B cells and hence there being a higher proportion of neutralising antibodies.
- CTL induction: Antigen is preferably delivered *via* the cytosolic pathway and thus associated and displayed with MHC class I molecules, inducing a CTL response. The role of the adjuvant would be either in cell membrane-adjuvant association or direct antigen attachment to external MHC class 1 molecules.
- Targeting: The efficient delivery of antigen to immune effector cells by selective targeting using ligands, optimisation of parameters such as formulation size, surface charge and hydrophobicity and by decreasing antigen degradation/body clearance.
- Depot: The antigen is 'held' with the adjuvant and released in a continuous or pulsatile mode.

The mechanism of adjuvant action was also reviewed by Schijns (2000) and classified into five categories.

- Adjuvant action due to 'translocation of antigen from the site of administration to the draining lymph node' due to increased 'pull' of dendritic cells to the antigen or higher loading of antigen to APCs.
- Sustained (or pulsed) antigen presentation can be paralleled to quick succession booster regimes emulating a primary response due to initial antigen release followed by secondary antigen presentation.
- Crucial pathogen microbial structures (pathogen-associated microbial patterns (PAMPs)) different to human cell surface structures are recognised

by receptors (pathogen-recognition receptors (PRRs)) present on cells from innate immune system (macrophages and dendritic cells as well as epithelial cells) and are essential for the upregulation of co-stimulatory molecules (needed for naïve T cell-MHC activation). In this regard, some adjuvants may act to imitate microbial structures for PRRs.

- The adjuvant induces damage to local cells and the resultant release of chemotactic factors from such cells, attract APCs to the area of antigen administration.
- The use of co-stimulatory molecules or cytokines as adjuvants to directly influence immune response.

Adjuvants licensed for human use currently include aluminium and calcium salts, MF59, virosomes and exotoxins (Kenney and Edelman, 2003). A more thorough list of adjuvants is maintained by the National Institute of Allergy and Infectious Diseases and can be found at www.niaid.nih.gov/aidsvaccine/pdf/compendium.pdf.

1.3.1 Mineral compounds

Aluminium adjuvants in commercial vaccines are usually aluminium oxyhydroxide, aluminium hydroxyphosphate or alum ($KAl(SO_4)_2$) (Gupta *et al.*, 1995). Table 1.2 summarises the physico-chemical properties of such aluminium adjuvants.

Table 1.2: Physico-chemical properties of aluminium adjuvants (†Baylor *et al.*, 2002; ‡Hem, 2002; HogenEsch, 2002)

	Isoelectric point (pI)	Charge at pH 7.4
Aluminium oxyhydroxide	11†	Positive
	11.4‡	
Aluminium hydroxyphosphate	5-7†	Negative
	4.5-6.0‡	
Alum	0.3-0.6†	Negative

Aluminium adjuvants are thought to work through both a depot effect and immunostimulation. HogenEsch (2002) and Baylor (2002) reviewed the use of aluminium as a vaccine adjuvant and reported that the mode of action was still not clear and that many of the past trials with aluminium containing vaccines demonstrated

inconsistent findings. The WHO EPI uses vaccines containing aluminium to immunise against diphtheria, neonatal tetanus, pertussis and hepatitis B (Clements and Griffiths, 2002). Aluminium containing vaccines induce a stronger T_h2 response for many vaccine antigens, which is essential for vaccines against extracellular pathogens, bacterial exotoxins and helminth parasites. Aluminium has been used as an adjuvant for vaccination for approximately 70 years and has been administered to hundreds of millions of people. The general safety profile of aluminium adjuvants is satisfactory though severe local reactions have been reported including erythema, subcutaneous nodules, granulomatous inflammation and contact hypersensitivity (Gupta *et al.*, 1995).

Aluminium salts however, do not induce T_H1 type immune responses and so do not confer adjuvanticity in terms of cell-mediated and CTL responses, which would be a necessity for vaccines against intracellular pathogens such as viruses, mycobacteria and certain protozoa. The induction of an antigen specific IgE response evoked by aluminium adjuvants may result in allergic reactions to vaccine components. Antigen in aluminium-adsorbed vaccines (as opposed to alum-precipitated vaccines) cannot be lyophilised easily due to the aluminium lattice being susceptible to collapse on freezing leaving the antigen without support and/or the tendency for the desorption of antigen from aluminium and subsequent adsorption onto cryoprotectant/stabilisers if used (Clements and Griffiths, 2002; Gupta and Siber, 1995). This increases the complexity of the manufacture and transportation/storage of aluminium-adsorbed vaccines inevitably raising the end-cost to the user.

Even though aluminium salts tend to display the best immune potentiating activity and have been the most widely used, calcium phosphate (another mineral salt) has also been used in human vaccines. The adjuvant nature of other compounds such as cerium nitrate, zinc sulphate, colloidal iron hydroxide and calcium chloride have also been reported (Gupta and Siber, 1995).

1.3.2 Emulsions

1.3.2.1 Water-in-oil ($^{w}/_{o}$) emulsions

In such vaccines, the antigen is entrapped within either the internal or continuous phase of a water microdroplet-in-oil emulsion containing stabilisers. The oil phase is typically

squalene, squalane or mineral (paraffin) oil. Examples of such systems include Freunds complete adjuvant (mineral oil mixed with killed *mycobacteria*), the less toxic Freunds incomplete adjuvant (mineral oil without *mycobacteria* and Arlacel A as an emulsifier) and adjuvant 65, which however are not currently used due to safety concerns (Gupta and Siber, 1995). Water-in-oil emulsions evoke good antibody responses especially for hydrophobic antigens but display poor immunomodulatory activity (Cox and Coulter, 1997).

1.3.2.2 Oil-in-water ($^{o}/_{w}$) emulsions

Surfactants such as Tween® 80 or Span® 85 or co-block polymers are used to stabilise oil (squalene or squalane) droplets within the water phase. Such systems provide a good formulation for amphipathic antigen incorporation. MF-59 (O'Hagan *et al.*, 1997) is an example of an oil-in-water system containing squalene (a terpenoid cholesterol precursor), Tween® 80 (polyoxyethylenesorbitan monooleate) and Span® 85 (sorbitan trioleate). The intranasal administration of MF-59 with influenza haemagglutinin to mice displayed a systemic and a mucosal immune response (Barchfeld *et al.*, 1999) whereas this was not the case on intranasal administration to humans (Boyce *et al.*, 2001).

1.3.3 Muramyl dipeptide emulsions

N-acetyl muramyl-L-alanyl-D-isoglutamine (MDP), often used as a constituent in particulate carriers, is a component derived from the cell wall from Mycobacteria (see also section 1.3.2.1) and has been shown to be an effective systemic and mucosal adjuvant (Friedman and Warren, 1984). Cox and Coulter (1997) review the use of MDP hydrophilic derivatives as T_h2 inducers and hydrophobic derivatives displaying T_h1 activity. However, the application of MDP or derivatives as a vaccine adjuvant in humans has been held back due to the possibility of toxic effects.

1.3.4 Saponins

Saponins are triterpenoids extracted from the bark of the *Quillaja sapanaria* tree (Molina). Quil A is a partially purified component from the crude saponin mixture and has been used as a component of ISCOMs (section 1.3.5). QS21 has been isolated from

Quil A (amongst other components also possessing adjuvant properties) and elicits a T_h1 type response as well as inducing CTLs whilst exhibiting decreased toxicity compared to Quil A (reviewed by Gupta and Siber, 1995 and Kenney and Edelman, 2003). AS02® is an $^{O}/_{W}$ emulsion containing QS21 and monophosphoryl lipid A (MPL) (section 1.3.13) which has been shown to evoke both humoral and cellular responses leading to protection against malaria in humans (Garçon *et al.*, 2003).

1.3.5 Immune stimulating complexes (ISCOMs)

ISCOMs unlike liposomes (section 1.3.7) do not possess lipid bi-layers and are rather 'cage-like' structures about the size of a small virus (30-70 nm). They are generally constructed from phospholipids, cholesterol and the saponin mixture Quil A (section 1.3.4). Amphiphilic and hydrophobic protein antigens can be incorporated into ISCOMs but hydrophilic antigens may require prior modification. The use of ISCOMs for nasal vaccination has been reviewed by Hu and colleagues (Hu *et al.*, 2001) and Kersten and Crommelin (1995).

1.3.6 Micro/nano particles/spheres

Biodegradable polyesters such as poly-L-lactides and copolymers of lactide and glycolide amongst others have been used for vaccine delivery using a variety of routes (reviewed by O'Hagan, D.T., 1998). The intranasal administration of the recombinant proteins F1 and V from *Yersinia pestis*, encapsulated into poly-L-lactide microparticles (100 kDa) to male CBA mice, served to provide protection (following inhalational challenge with *Y. pestis*) in 66.6 % of mice whereas there were no survivors from the group of animals given F1 and V proteins alone (Alpar *et al.*, 2001). Therefore, the use of such carriers for the delivery of protein based antigens warrants further investigation especially in regard to mucosal routes of administration.

1.3.7 Liposomes

In essence, liposomes are vesicular structures composed of many amphiphilic molecules (a polar head group conjugated to two hydrophobic hydrocarbon tails) which after exposure to an aqueous environment are arranged into a closed spherical bi-layer structure. The central core remains an aqueous phase and can be surrounded by more

than one concentric bi-layer, each of which is further separated by an aqueous 'cushion'. Thus, material which is hydrophilic or hydrophobic/amphiphilic, regardless of size, can be encompassed into the structure. The liposome 'masks' the original attributes (hydrophobicity/hydrophilicity) of the incorporated drug/antigen and the formulation takes on the identity of the outer lipid bi-layer.

The adjuvant nature of liposomes was first reported by Allison and Gregoriadis (1974) who administered liposomally encapsulated diphtheria toxoid intramuscularly, intravenously and subcutaneously and found that liposomes composed of egg phosphatidylcholine (PC), cholesterol and phosphatidic acid or dicetyl phosphate (DCP) evoked a higher immune response than when free diphtheria toxoid was administered alone. Many studies have been conducted investigating the vaccine adjuvant activity of liposomes for the delivery of protein antigens and are reviewed by Gregoriadis and colleagues (Gregoriadis *et al.*, 1988; Gregoriadis, 1990); Childers and Michalek, 1994 and Rogers and Anderson, 1998). Cationic liposomes have also been used as adjuvants for the delivery of encapsulated plasmid DNA. Work by Perrie and co-workers (Perrie *et al.*, 2001) demonstrated that following intramuscular delivery to 6-8 week old female BALB/c mice, animals given plasmid DNA (encoding the small region of the hepatitis B surface antigen) encapsulated in cationic liposomes displayed a significantly higher antigen-specific humoral antibody response than animals given naked DNA alone.

1.3.8 Archaeosomes

Archaeans are prokaryotes distinct from bacteria due to differing cell membrane lipids and possess certain attributes more similar to eukaryotes than to those of bacteria. The polar lipids of Archaean membranes are used in the preparation of bi-layer vesicles (archaeosomes) and display greater physical and chemical stability compared to traditional liposomes (Patel *et al.*, 2000). Subcutaneous administration of ovalbumin-loaded archaeosomes was shown to elicit both a humoral and a CTL response in mice (Sprott *et al.*, 2004).

1.3.9 Cochleates

Cochleates are non-vesicular bi-layer structures in which cations (such as calcium) are intercalated with anionic bi-layers to produce a scroll type structure. There is little or no

internal aqueous volume (unlike liposomes) and thus cochleates are more suited for hydrophobic agents. Antibody and cell-mediated responses have been evoked to both protein and DNA cochleates administered *via* parenteral and mucosal routes (Gould-Fogerite *et al.*, 1998 and Gould-Fogerite *et al.*, 2000).

1.3.10 Niosomes/novasomes

These non-phospholipid liposomes are usually constructed using amphiphiles such as non-ionic surfactants to produce uni-lamellar vesicles. Rentel and co-workers (Rentel *et al.* (1999) prepared niosomes using cholesterol, DCP and sucrose ester surfactants and administered ovalbumin intragastrically to mice. Delivery of niosome encapsulated ovalbumin was shown to elicit a higher serum anti-ovalbumin IgG and higher salivary and intestinal IgA than administration of free ovalbumin. Gupta and co-workers (Gupta *et al.*, 1996) administered tetanus toxoid (TT) (subcutaneously) and diphtheria toxoid (intramuscularly) encapsulated within non-phospholipid liposomes (dioxyethylene cetyl ether, cholesterol and oleic acid) and reported elevation of antigen specific serum antibody titres to levels similar to that elicited using aluminium phosphate.

1.3.11 Transfersomes/elastic vesicles

Such carriers were designed to penetrate the skin to a higher extent than liposomes of the same size. Phosphatidylcholine 'ultradeformable' liposomes are prepared using 'edge-active' agents such as bile salts and other surfactants (e.g. Span® 80 or Tween® 80) to impart elastic properties to bi-layers compared to the more 'rigid' conventional liposome. Such elastic vesicles have been shown to pass through skin pores much smaller than the vesicle size (Cevc *et al.*, 1995 and van den Bergh *et al.*, 1999) most likely due to the influence of the transepithelial water gradient. Paul and co-workers (Paul *et al.*, 1998) administered transfersomes containing gap junction proteins (a highly lipophilic membrane component, mwt of monomer: 28 kDa, usually forms hexamers) transdermally (by applying formulation to the intact and manually trimmed skin of female NMRI mice) and showed that gap junction-specific antibody titres were similar to levels elicited when transfersomes, mixed micelles or liposomes containing gap junction proteins were administered subcutaneously. Thus, the use of transfersomes is a capable tool for the delivery of antigen through the skin, including the stratum corneum.

1.3.12 Virosomes

Virosomes are protein modified uni-lamellar liposomes constructed to a mean diameter of approximately 150 nm. Viral envelope glycoproteins (influenza hemagglutinin and neuraminidase) are incorporated as part of the vesicle structure which also consists of conventional phospholipids. Inflexal V® and Epaxal® are licensed prophylactic human virosomal vaccines against influenza and hepatitis A respectively. The use of Epaxal® was shown to be as effective as an alum based vaccine but associated with less side effects such as pain and swelling and Inflexal® was demonstrated to raise a superior immune response than other current influenza vaccines (reviewed by Moser and colleagues (Moser *et al.*, 2003)). An intranasal virosomal vaccine (with *Eschericia coli* heat labile toxin (LT)) against influenza was also licensed but has been withdrawn due to a possible association with Bell's palsy (Kenney and Edelman, 2003).

1.3.13 Lipopolysaccharide (LPS), lipid A and monophosphoryl lipid A (MPL)

Lipopolysaccharides from gram-negative bacteria are recognised by toll-like receptors (or PPRs), which lead to the activation of APCs (reviewed by Evans *et al.*, 2003). LPS initiates the release of a high number of inflammatory cytokines and the isolated lipid A fraction was shown to remain immunomodulatory yet is less toxic. MPL is a further derivative from lipid A that possesses even fewer side effects whilst still eliciting a potent T_h1 response (Reed *et al.*, 2003). MPL is included as a component to the AS02® adjuvant system (see section 1.3.4).

1.3.14 Cytokines

The use of cytokines as mucosal adjuvants has so far been restricted due to a lack of understanding in their complex multi-path role in the immune system. Advantages of the use of cytokine include that they are not foreign to the human body (unlike most other adjuvants) and so may possess fewer side effects and that cell-mediated and humoral responses can be generated depending on the choice of cytokine. Yuki and Kiyono (2003) review the investigation of IL-1, IL-6, IL-12 and IFN-γ in animal immunisation studies and Villinger (2003) review a number of clinical studies using cytokines as adjuvants for vaccines.

1.3.15 CpG oligodeoxynucleotides

CPG oligodeoxynucleotides (ODNs) present in bacterial DNA are found in lesser amounts in mammalian DNA. The incorporation of CPG ODNs into plasmid DNA vaccines or administration with antigenic proteins has been shown not only to increase the immune response but also to promote the production of T_h1 cells, $IgG2_a$ antibodies, IFN-γ and sIgA responses at mucosal sites (reviewed in Klinman, 2003). The intranasal administration of CpG DNA for vaccine adjuvanticity is summarised by Holmgren and colleagues (Holmgren *et al.*, 2003b). Frank and co-workers (Frank *et al.*, 2003) administered the major cystein proteinase of *Trypanosoma cruzi* (a parasite carried and transmitted by an insect which can cause Chagas' disease leading to heart complaints) intramuscularly to female 6-8 week old C3H/Hen mice. Animals were given the antigen plus CPG ODNs, antigen plus non-CPG ODNs or antigen plus alum. A higher IgG response was elicited against the antigen with CPG ODNs than the other two formulations. The CPG ODN formulation also elicited a T_h1 biased response unlike the other preparations.

1.3.16 Toxins and derivatives

The *Vibrio cholerae* cholera toxin (CT) and *Escherichia coli* heat labile enterotoxin (LT) are GM-1 ganglioside binding molecules which exhibit effective mucosal adjuvanticity inducing both humoral and cell mediated responses (T_h2 and T_h1 responses respectively) (Yuki and Kiyono, 2003). However, toxicities associated with use in humans such as severe diarrhoea and accumulation in olfactory tissue after nasal administration has limited their potential application in human vaccines to date. The identification of the toxic and immuno-stimulatory component (Subunits A and B respectively) of both CT and LT has resulted in the use of the less toxic CTB and LTB for mucosal adjuvanticity. Other 'mutant' derivatives (eliminating certain toxic effects whilst maintaining adjuvant properties) and chimeric mutants (association of the A subunit of CT or LT with the B subunits of the other have also been investigated (Holmgren *et al.*, 2003a, 2003b; Yuki and Kiyono, 2003). In the case of the latter, the cell-mediated response (T_h1 type) has been shown to be channelled in the direction associated with that of the B subunit origin.

1.3.17 Cationic lipid/DNA complexes

The *in vitro* and *in vivo* transfection of plasmid DNA has been shown to be enhanced by complexation with cationic lipids/liposomes prior to delivery (Sakurai *et al.*, 2000; Song and Liu, 1998). Work by Klavinskis and colleagues (Klavinskis *et al.*, 1997) showed that the intranasal administration of plasmid DNA encoding a luciferase reporter gene to mice elicited an antigen-specific serum IgA and IgG antibody response when the DNA was complexed with cationic liposomes but not when administered alone. The authors also reported that on analysis of vaginal fluids, animals given DNA/cationic liposome complexes displayed antigen-specific IgG and secretory IgA antibodies whereas animals given naked DNA did not. Thus, the use of liposomes as a mucosal adjuvant for the delivery of plasmid DNA has shown promising results and warrants further investigation.

1.3.18 Poloxamer surfactants

Poloxamers are non-ionic surface active agents which are amphiphilic in nature due to their triblock copolymers structure (figure 1.3). Poloxamers synthesised using propylene oxide and ethylene oxide are available commercially (most commonly as Pluronic® and Synperonic® brands) and are available in many different molecular weights of poly (ethylene oxide) (PEO) and poly (propylene oxide) (PPO), organised with the hydrophobic PPO flanked on either side with blocks of the hydrophilic PEO.

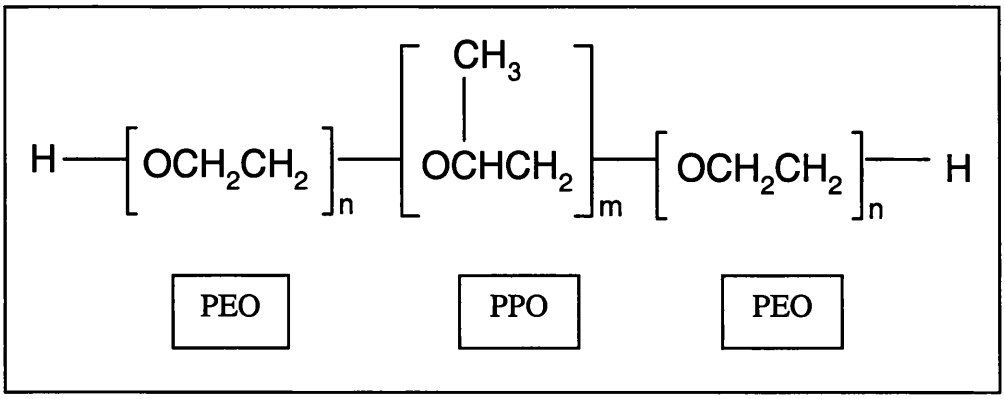

Figure 1.3: General structure of non-ionic block copolymers

1.3.18.1 Poloxamer nomenclature

There are two main recognised notations for the representation of block copolymers. The first stems from the original commercial manufacturers (BASF) who used a system of three digits prefixed with a letter. The prefixed letters F, L and P represent poloxamers that at room temperature are in flake, liquid or paste form respectively. The first one or two digits are series numbers related to the approximate molecular weight of the PPO block and the last digit (when multiplied by 10) is the weight percentage of the poloxamer that is PEO.

The second notational system was developed for use for non-proprietary (generic) poloxamers. The prefix letter is always 'P' (poloxamer) and the first one or two digits in this instance, when multiplied by 100, are direct approximations of the PPO molecular weight. The last digit (when multiplied by 10) remains the weight percentage of the poloxamer that is PEO.

1.3.18.2 Properties of poloxamers 331 and 401

In the course of the experimental investigations undertaken, P331 (L101) and P401 (L121) were used. Table 1.3 shows the molecular weight and the physicochemical properties of these poloxamers.

Table 1.3: Properties of poloxamers 331 and 401

	L101 (P331)	L121 (P401)
Physical state at room temperature	Liquid	Liquid
Approximate molecular weight of PPO hydrophobe	3250	4000
Percentage of PEO in molecule	10	10
Hydrophile-lipophile balance (HLB)	1.0	0.5

Poloxamers with a higher PEO content are more hydrophilic and hence are more soluble in water, which decreases with increasing temperature (Alexandridis *et al.*, 1995). At low concentrations, poloxamers will form single molecule micelles (with the hydrophobic PPO being shielded by the flanking hydrophilic PEO) and at higher concentration will form multi-molecule micelles where PPO groups are 'hidden' in the core leaving flanking PEO molecules to form the 'shield'. The critical micellar concentration (CMC) (the poloxamer concentration at which micelles spontaneously

start forming) varies for different block copolymers due to broad molecular weight distributions between the poloxamers. Unlike other surfactants, the CMC for PEO-PPO-PEO block copolymers decreases with increasing temperature (Alexandridis *et al.*, 1995). In general, as the PPO content is increased there is a decrease in the CMC for poloxamers. The hydrophobic block copolymers L101 and L121 are therefore soluble in isotonic buffers at temperatures lower than room temperature. Whereas the unimers are approximately 1 nm in diameter, block copolymer micelles are approximately 10 nm in diameter (aggregation number about 50).

1.3.18.3 Vaccination studies using poloxamers as adjuvants

Studies by Snippe and co-workers (Snippe *et al.*, 1981) demonstrated that poloxamers with an HLB value of 2 or less displayed adjuvanticity. L121 was shown to augment antibody formation against sheep red blood cells and dinitrophenylated BSA. L101 was found to be an adjuvant for the administration of the latter but not the former. Administration of Pluronic™ F68 (high mwt (8,350) but high PEO content (80 %)) and L31 (Low PEO content (10 %) but low molecular weight (1,100)), which have HLB values of 29 and 3.5 respectively, were shown not to possess vaccine adjuvant properties. Hunter and Bennett (1984) investigated a broader range of non-ionic block copolymers for adjuvanticity when administered with BSA after an $^{O}/_{W}$ footpad injection to mice. L101 and L121 as well as the octablock T1501 were shown to possess adjuvant properties and produced higher antigen-specific serum antibody levels compared to animals given L81, L92 or L122. A further study in the same publication using L121 showed a poloxamer dose-dependent increase in antibody titres. In contrast to *in vitro* studies where BSA association to poloxamers was low after 4 days (22 % and 31 % for L101 and L121 respectively), retention of the BSA in the footpad after 4 days was much higher (approximately 98 % for both L101 and L121). The authors concluded that as poloxamer molecular weight and hydrophobicity increases, there is also an increase in poloxamer adjuvanticity and that *in vitro* release studies using poloxamers do not necessarily reflect the *in vivo* release profile.

The poloxamer-$^{O}/_{W}$ emulsion concept was applied by the same group where haptenated liposomes replaced the $^{O}/_{W}$ component (Zigterman *et al.*, 1987). Intraperitoneal immunisation to 10 week old female BALB/c mice resulted in a higher humoral response following administration of all non-ionic block copolymers compared to

haptenated liposomes alone. However, in contrast to their earlier findings (Snippe *et al.*, 1981), higher molecular weight hydrophobic poloxamers (such as L101 and L121) displayed weaker adjuvant properties than lower molecular weight hydrophobic poloxamers such as L81. In the same way, L122 (high molecular weight) showed weaker adjuvant properties than L72 and L92 (lower molecular weights) even though the percentage of hydrophobic polymers (PEO) remained the same. Investigation into the release of carboxyfluorescein from liposomal formulations on addition of the poloxamers demonstrated that the preparations containing the weaker adjuvants (L101 and L121 and L122) displayed a faster release of the carboxyfluorescein than those containing the stronger adjuvants (L72, L81 and L92). The faster release of carboxyfluorescein from liposomes after addition of L101, L121 and L122 may be a possible reason for the lower adjuvanticity seen with these formulations. Therefore, when poloxamers are used with carriers like liposomes, adjuvanticity of the formulations may also be dependent on factors other than the molecular weight and HLB values of poloxamers.

The Syntex adjuvant formulation (SAF®) is an $^{o}/_{w}$ emulsion containing 2.5 $\%^{m}/_{v}$ Pluronic® L121, 5 $\%^{m}/_{v}$ squalene and 0.2 $\%^{m}/_{v}$ polysorbate 80 (Tween® 80) and has demonstrated the production of both humoral and cellular responses (Kenney and Edelman, 2003). A similar formulation (Provax®) consists of 15 $\%^{m}/_{v}$ squalene also with Pluronic® L121 and Tween® 80 and has been demonstrated as a potent inducer of CTLs (reviewed by Newman *et al.*, 1998a).

Immune response to ovalbumin in 8 week old female C57BL/6 mice following subcutaneous administration, in regards to T_h1 or T_h2 activation, was shown to be dependent on the PEO content of poloxamers (Newman *et al.*, 1998a; Newman *et al.*, 1998b). Formulations containing poloxamers with 5 % PEO (more hydrophobic) induced a mixed T_h1/T_h2 response whereas formulations containing poloxamers with a PEO content of 10 % (less hydrophobic) resulted in a predominantly T_h2 response (determined after antigen-stimulated *in vitro* cytokine production). Therefore, the type of immune response elicited (T_h1 or T_h2 type) can be adapted to depending on whether an intracellular or extracellular pathogen is being immunised against.

1.3.19 Chitosan

Chitosan is a polysaccharide derived from the partial deacetylation of chitin (found in the shells of crustaceans) and is a polymer of D-glucosamine and *N*-acetyl-D-glucosamine (figure 1.4). The degree of deacetylation of chitin determines the number of amino groups present (which become protonated at low pH (<6.5)) and hence determines the charge density of the polymer and related properties. Chitosan has found many applications in various fields from photography and cosmetics to potential for use in artificial skin, wound dressings, contact lenses, the water industry, for paper finishing and use in batteries (reviewed by Majeti and Kumar, 2000). The biodegradable and non-toxic qualities of chitosan make it's use in drug delivery also possible.

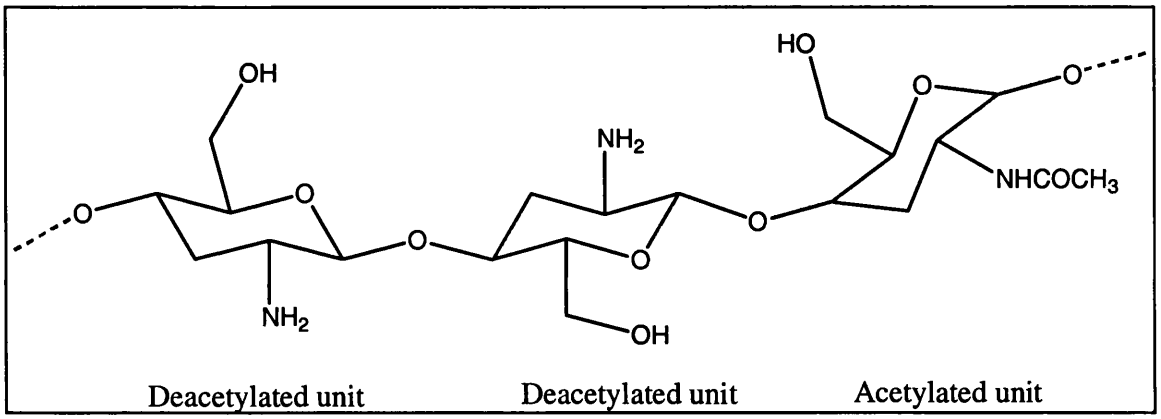

Figure 1.4 Structure of chitosan: containing randomly distributed β-(1→4)-linked D-glucosamine (deacetylated unit) and *N*-acetyl-D-glucosamine (acetylated unit)

Takeuchi and co-workers (Takeuchi *et al.*, 1996) coated anionic liposomes composed of L-α-dipalmitoylphosphatidylcholine (DPPC) and DCP with chitosan to investigate the presence of mucoadhesion *in vitro* to the intestine of male Wistar rats. It was found that chitosan-coated liposomes adhered to a higher extent to the intestinal lining than non-coated liposomes. The impact of this increased residence time of the coated liposomes on the intestinal lining was investigated *in vivo* by administering insulin loaded liposomes to male Wistar rats and observing blood glucose levels. It was found that glucose levels remained lower for the chitosan-coated liposomes than non-coated liposomes. Chitosan demonstrates mucoadhesive properties due to electrostatic interactions with sialic acid (net negative charge at physiological pH) in mucins found in mucus (Illum *et al.*, 2001). Studies on the human inferior turbinates *in vitro* (Aspden

et al., 1997) showed that chitosans with a higher molecular weight slowed the mucociliary transport rates the most and thus, have the highest mucoadhesive potential.

In addition to demonstrated mucoadhesive properties, the effect of chitosan on paracellular and transcellular absorption was investigated by Dodane and co-workers (Dodane *et al.*, 1999). Caco-2 cell monolayers were used to demonstrate a reversible decrease in transepithelial electrical resistance on chitosan addition. It was shown that chitosan affects tight junctions to increase paracellular passage as well as intracellular passage.

Seferian and Martinez (2001) also reviewed other properties of chitosan such as stimulating innate immunity by complement activation, stimulating antigen presenting cells and inducing cytokine production. The use of chitosan in nasal vaccine formulations has been reviewed by Illum and colleagues (Illum *et al.*, 2001) and van der Lubben and colleagues (van der Lubben *et al.*, 2001).

1.4 The nose as an alternate route for the delivery of vaccines

Delivery of biologically labile high molecular compounds such as proteins *via* the nose has been explored as an alternative to conventional methods such as parenteral administration. Arora and colleagues (Arora *et al.*, 2002) list a number of peptides, proteins, hormones and vaccines that have been administered nasally. There are many locally acting medications already licensed for nasal administration in the form of nasal drops, sprays and creams such as antibiotics, antifungals, sympathomimetics, steroids and antihistamines.

The physicochemical properties of the drug/antigen and excipients are an important factor for formulation permeability through the nasal mucosa. Molecular charge (depending on pK_a), hydrophilicity/hydrophobicity and capacity for hydrogen bonding are important factors for drug permeation. Drug size (in terms of both molecular weight and shape) plays a more dominant role in permeation when molecular weight is higher than 1 kDa, resulting in bioavailabilities generally below 10 % of administered dose (Donovan *et al*, 1998). This is supported by Hussain and colleagues (Hussain *et al.*, 1998) who stated that the permeability of compounds with a molecular weight less than 300 are not affected by their physicochemical properties such as lipophilicity and charge

whereas for larger compounds, size and hydrogen-bonding capabilities are the most important factors.

1.4.1 Anatomy of the human nose

The nose, nasopharynx, oropharynx, laryngopharynx and larynx together form what is known as the upper respiratory tract (figure 1.5). The nose is divided into two nasal cavities (by a cartilaginous septum ending at the nasopharynx). Inspired air enters predominantly through the external *nares* (Latin for nostrils) and is directed onwards past the vestibule (the space within the 'bendable' tissues of the nose containing course hair to strain out larger particles), through the atrium and to the nasal cavity. The air may then flow through one of three 'passages' within the nasal cavity to reach the nasopharynx. These passages are termed the superior, middle and inferior meatuses and are created due to the projection of the ethmoid, maxillary and inferior conchae/turbinate bones respectively. The meatuses increase the surface area of the nasal cavity and as they are a highly vascularised mucosal surface, they serve to warm and humidify inspired air. Air passing through these narrow 'grooves' is also brought into a more intimate contact with the mucosa, which results in a higher 'entrapment' of foreign matter.

Below the respiratory tract epithelium is the basal lamina and beneath that the lamina propria (containing mucus and serous glands). The lamina propria is continuous with the periosteum or perichondrium of bone or cartilage of the nasal cavity. Macrophages, lymphocytes, plasma cells and granular leucocytes are found in the lamina propria and lymphatic tissue is also to be found near the nasopharynx (Leeson and Leeson, 1981 and Krause and Cutts, 1981).

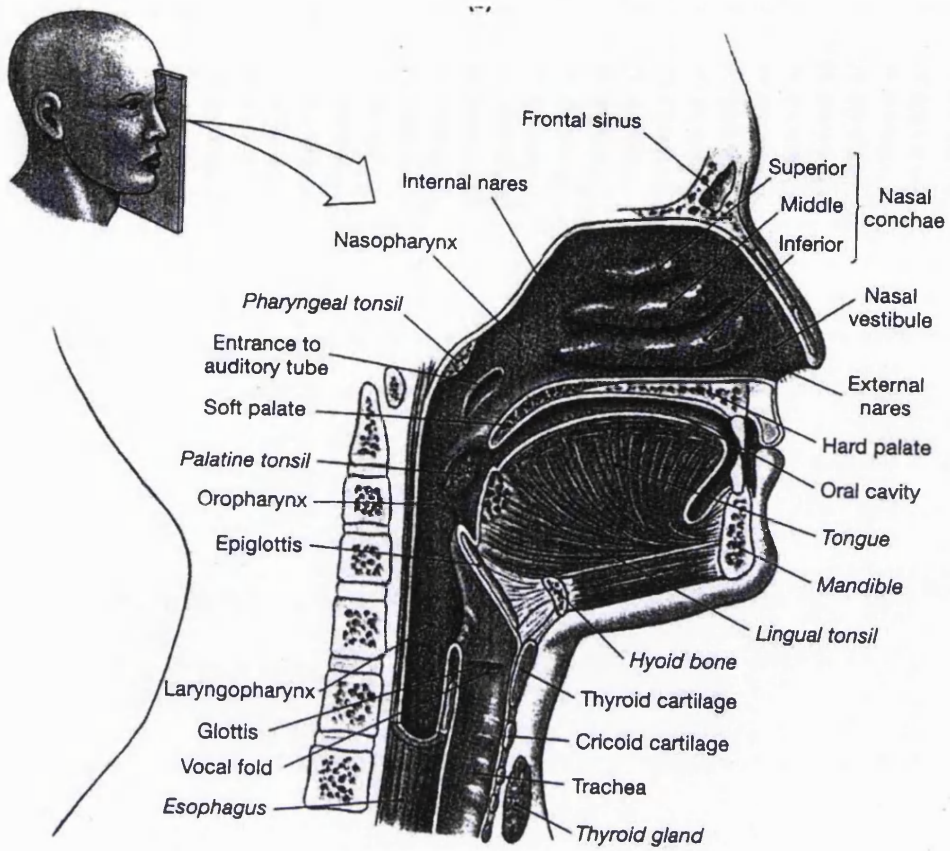

Figure 1.5: The anatomy of the human nose (reproduced from Martini, 2004)

1.4.2 Histology of nasal tissues and NALT (nasally-associated lymphoid tissue) in humans

The types of epithelial cells change from area to area in the human nose. The epithelium of the vestibule consists of stratified keratinised squamous cells that are resistant to dehydration and attack from environmental substances. The atrium contains both squamous cells and also pseudostratified columnar cells (with microvilli). The nasal cavity is composed of both pseudostratified columnar cells interdispersed by goblet cells and seromucus ducts (figure 1.6). Some of the columnar cells are ciliated whereas others are not. Basal cells are also present and these are 'precursor' cells, which will eventually differentiate to form other epithelial cell types. Together, the goblet cells and nasal glands serve to produce mucus, which not only protects the epithelium from drying, but also traps and 'washes' away small dust particles and microorganisms. Mucus is also secreted into the nasal cavity by the four pairs of paranasal sinuses (frontal, maxillary, ethmoid and splenoid) and fluid from the lacrimal duct.

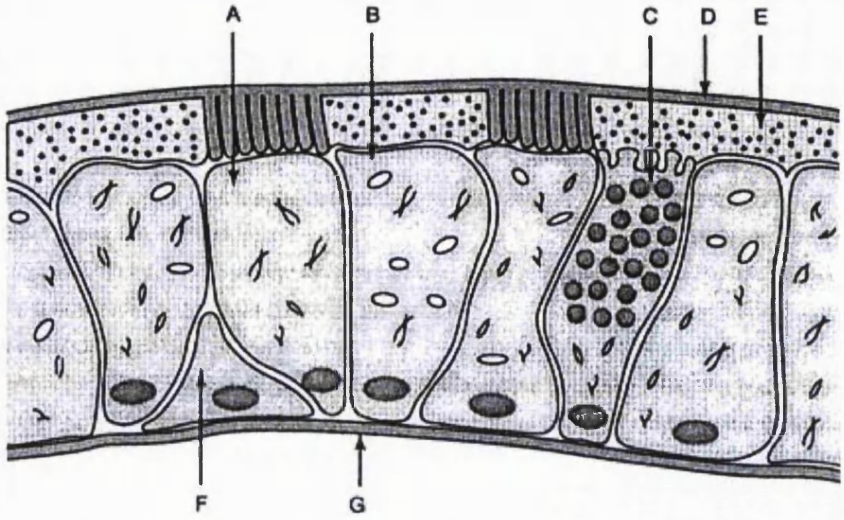

Figure 1.6: The various cell types found in nasal epithelium (reproduced from Ugwoke *et al.*, 2001). Figure shows ciliated cell (A), non-ciliated cell (B), goblet cell (C), gel-mucus layer (D), sol layer (E), basal cell and basement membrane (G)

Another epithelial cell type in the nasal cavity is that of the olfactory region, which is situated in the roof of the nasal cavity and extends over the superior turbinate. The epithelial cells consist of ciliated olfactory nerve cells which aid in smell perception. The observation of an initial absorbance phase in the central nervous system following the intranasal administration of radionuclides in rhesus monkeys (Gopinath *et al.*, 1978) and dextromethorphan hydrochloride in 9-10 weeks old male Sprague-Dawley rats (Char *et al.*, 1991) could indicate the presence of a more direct 'pathway' between the nose and brain. Both intracellular and extracellular pathways from the olfactory region in upper nasal passages to the central nervous system (bypassing the blood brain barrier) have been reported as well transport *via* the trigeminal neurons (reviewed by Thorne and colleagues (Thorne *et al.*, 2004)). The authors also reviewed that such pathways have been hypothesised as a possible route for invasion by viruses (to the central nervous system) and so the existence of such pathways results not only in toxicological implications for intranasally delivered vaccines (toxicity to central nervous system) but also presents advantages in terms of opportunities for vaccine delivery to prevent CNS invasion by pathogens *via* such pathways. Whereas in mice and rats the olfactory region represents approximately 50 % of the mucosal cavity, in humans it covers only 10 %, which however, is still equivalent to an area of 10 cm^2 (Popp and Monteiro-Riviere, 1985).

Beyond the nasal cavity, through the internal nares, inspired air enters the nasopharynx. Like the nasal cavity, the epithelium of the nasopharynx is composed of pseudostratified ciliated columnar cells but this changes in both the oropharynx and laryngopharynx to stratified squamous cells. Lymphoid tissues in the human pharynx are known as Waldeyer's ring (figure 1.7) and consist of the nasopharyngeal tonsil (adenoid) the pair of Tubal tonsils, the pair of Palantine tonsils and the Lingual tonsil.

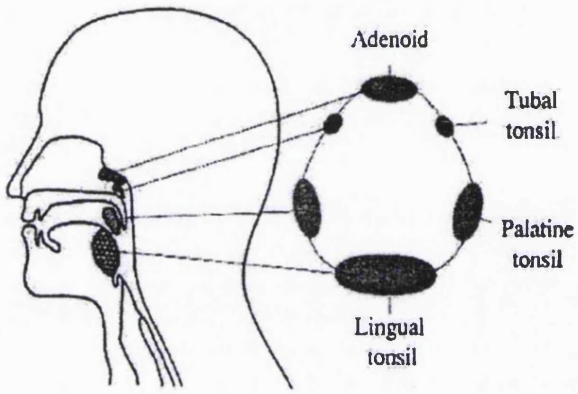

Figure 1.7: Lymphoid tissue of Waldeyer's ring (reproduced from Perry and Whyte, 1998)

Aggregates of lymphoid cells are found on the tonsils similar to the Peyer's patches in the intestine. Gebert and Pabsl (1999) in their overview on microfold cells (M cells) at locations outside the gut reaffirm that the epithelium of the human tonsils is heavily infiltrated with immune cells and give evidence for the presence of M cells. However, the authors also note papers in which the presence of M cells were not detected and conclude that the consensus on the presence of M cells on the tonsils remains divided. Fujimura (2000) examined human adenoid tissue and reported that M cells were found and that they were similar to those found in the Peyer's patches in terms of ultrastructure and functionality. Figure 1.8 is a diagrammatic representation showing a cross-section of the Peyer's patches of the gastrointestinal tract highlighting the presence of M cells as part of the follicle-associated epithelia (FAE).

In addition to the lymphoid structures of Waldeyer's ring, NALT was found throughout the nasal mucosa in 38 % of young children up to the age of two years old (Debertin *et al.*, 2003). Subepithelial lymphoid follicles were detected sometimes possessing germinal centres, lymphocytes in the overlying epithelium and with high endothelial venules (HEVs) (blood vessels possessing ligands for naïve lymphocytes which also

express certain cytokines which may also help to transport lymphocytes from the blood to secondary lymphoid tissues such as lymph nodes, Peyer's patches and the spleen (Miyasaka and Tanaka (2004))). Debertin and co-workers (Devertin *et al.*, 2003) also observed that lymphoid follicles were in some instances found in clusters resembling those found in the Peyer's patches in the intestine. NALT was mainly situated in the middle turbinate (26.4 %) and the upper nasal cavity (30.1 %). The authors also suggested further investigation regarding the extent of (or absence) of NALT in subjects and relation to the size of the tonsils to clarify any link that NALT may be a compensatory structure.

Therefore, there is good evidence that NALT occurs in aggregates in tonsils as well as being dispersed throughout other nasal areas and so is a potential target for mucosal vaccine delivery.

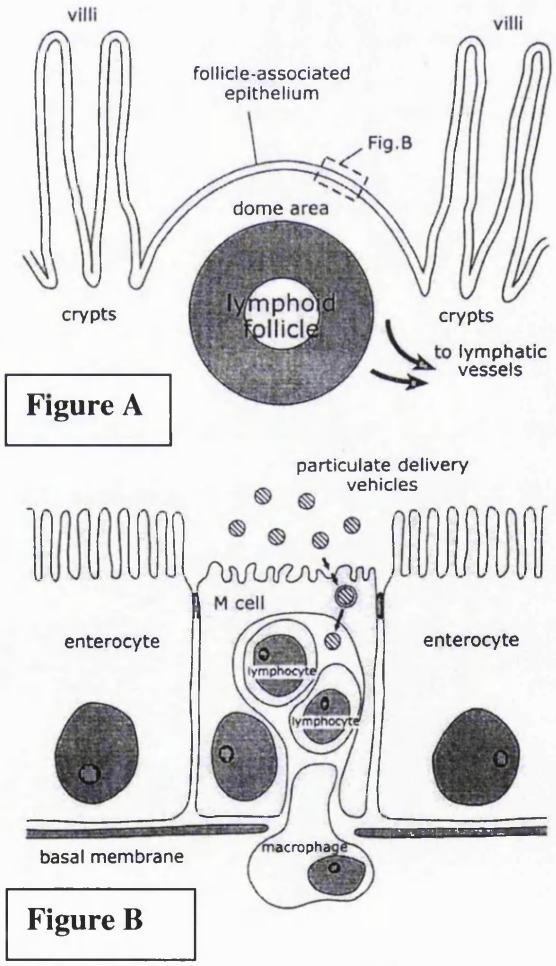

Figure 1.8: Diagrammatic representation of the transverse section of a Peyer's patch lymphoid follicle (figure A) and transport of particulates though M cells present on the overlying follicle-associated epithelium (figure B) (reproduced from Clark *et al.*, 2001)

1.4.3 Histology of nasal tissues and NALT in animals

Histological observations in 8-10 weeks old inbred male Lewis rats (Koornstra *et al.*, 1991) revealed paired lymphoid tissues in the naso-pharyngeal duct, which resembled the pharyngeal tonsils in man. The tissues were situated beneath the epithelium, the lymphoid follicles possessed germinal centres and there were seen to be interfollicular lymphocyte areas. M cells were seen to be present in the overlying epithelium. Drainage of the lymphatic tissue was detected to be mainly to the deep cervical lymph node but also to a degree, to the superficial cervical lymph node. Alike to the Peyer's patches in the intestines, the nasal lymphoid tissues were found to contain B cells and T cells (a larger quantity of the former). T cells were more of the T helper subset than of the cytotoxic type.

A previous investigation in 10-20 weeks old, male albino Wistar rats (Spit *et al.*, 1989) also revealed the presence of paired lymphoid tissue in the naso-pharyngeal duct. The tissues were described as dome-like and covered by an epithelium that was morphologically distinct from respiratory epithelium. Non-ciliated cells and ciliated cells were present (the former intermittently in clumps and the latter with fewer and shorter cilia than other areas of the epithelium) whereas there were very few goblet cells. Non-ciliated epithelial cells appeared to enclose lymphocytes and were composed of irregular apical membrane with microvilli type structures. The presence of possible immature precursor cells was also noted in the lymphoid tissue. The authors likened the these cells to the M cells (and their precursors) found in the FAE of the Peyer's patches in the intestine and concluded that the lymphoid tissues of the nose to be similar to that found as part of the gut-associated lymphoid tissue and to be part of the mucosa-associated lymphoid tissue (MALT) system. Beneath the epithelium, the lymphocytes were seen to be organised into two areas; a tightly packed region and a more diffuse area which contained many vessels including HEVs.

Thus, there is evidence for the existence of M cells (similar to the M cells of the Peyer's patches) in the NALT in rats. This reflects the situation in humans (section 1.4.2) and so the use of animals like rats for intranasal delivery may be an appropriate model for the administration of mucosal vaccines.

1.4.4 Antigen uptake, lymphoid tissue cell population and trafficking

Asanuma and co-workers (Asanuma *et al.*, 1997) determined the population of lymphocytes in NALT and total nasal tissues in 6-10 week old female BALB/c mice. NALT-associated lymphocytes accounted for 20-30 % of the total nasal lymphocyte count and the balance of B cells to T cells was found to be about equal. The T helper cell population was found to be 27.1 % and the T cytotoxic cell population only 7.2 %. The B and T cell distributions (including subtypes) were also similar amongst non-NALT nasal lymphocytes. Similar investigations of lymphoid tissue from the Peyer's patches revealed that B cell presence was far greater (75.1 %) than T cell presence (23.8 %). A previous study in 6 to 8 weeks old male ICR mice by Kawauchi and co-workers (Kawauchi *et al.*, 1991) showed the presence of lymphocytes, neutrophils and macrophages in nasal mucosa and showed that the pattern of immune cell presence in the nasal mucosa is linked with previous antigenic exposure.

Kuper and colleagues (Kuper *et al.*, 1992) reviewed the uptake and subsequent fate of nasally presented antigens. Particulate antigens escaping the mucociliary system are mainly 'sampled' by the M cells and presented to the underlying immune cells. The destination of the antigen thereafter lies predominantly to the posterior cervical lymph nodes. Soluble antigens however, will be exposed to both intraepithelial and submucosal immune cells after general transcellular/paracellular passage through the nasal epithelium. Thereafter, the immune cells migrate to the superficial cervical lymph nodes and then from there to the posterior cervical lymph nodes (figure 1.9). Processing at the posterior cervical lymph node is responsible for the activation of a secretory mucosal immune response whereas processing at the superficial cervical lymph nodes with the elicitation of a systemic immune response (Kuper *et al.*, 1992). Watanabe and co-workers (Watanabe *et al.*, 1980) used horseradish peroxidase (mwt = 40 kDa) in male Wistar rats to demonstrate the high level of capillary permeability of macromolecules through the nasal mucosa. Post intravenous injection of these materials showed that the permeability of venules in nasal mucosa was greater than that of venules found in the intestinal tract. The presence of horseradish peroxidase was higher in extracapillary tissues in the nasal mucosa than the intestinal mucosa. These results were attributed to the observation of loose endothelial junctions in nasal mucosa and that the tissues surrounding capillaries were also less rigid (compared to those of the intestine) allowing for the easier diffusion of horseradish peroxidase.

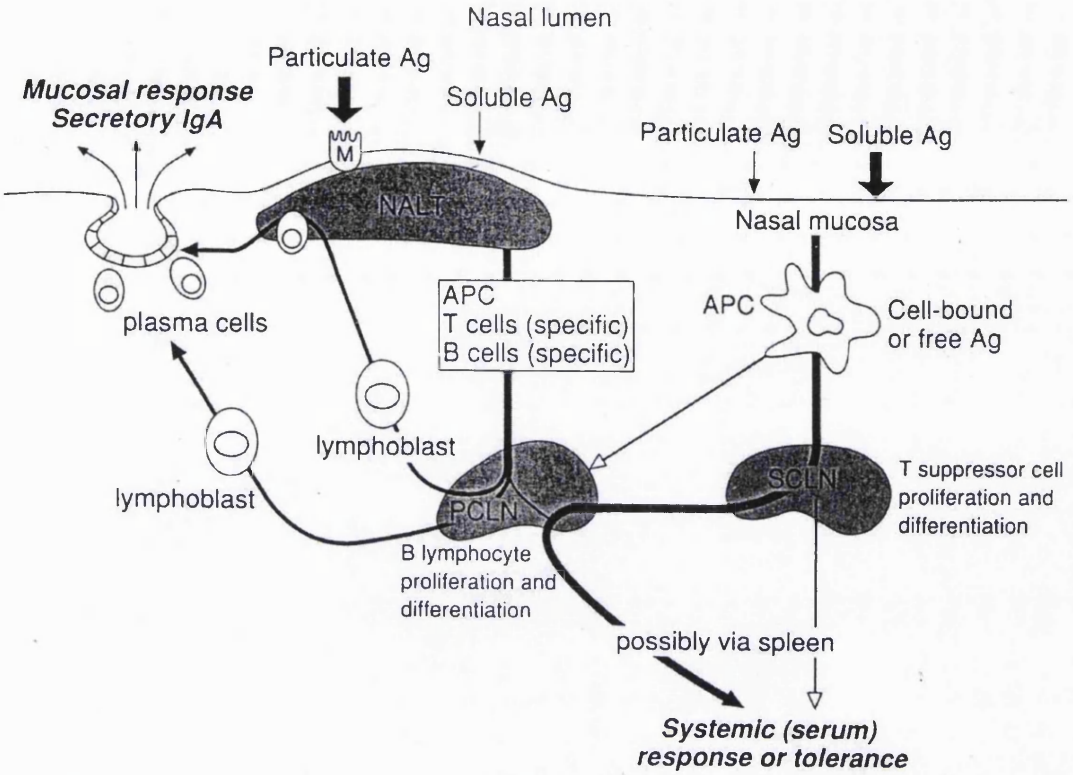

Figure 1.9: Formation of local mucosal and systemic immune responses after antigen uptake from nasal lumen (reproduced from Kuper *et al.*, 1992).

Key: APC: antigen presenting cell; M: microfold cell; NALT: nasally-associated lymphoid tissue; PCLN: posterior cervical lymph node; SCLN; superior cervical lymph node.

The differing roles of both the NALT and GALT in mucosal response was discussed by Kuper and colleagues (Kuper *et al.*, 1992) following observations made in Wistar rats. NALT lymphocytes were shown to migrate to and from cervical and mesenteric lymph nodes in greater numbers than GALT lymphocytes. T cells were also shown to adhere to NALT HEVs to a greater extent than B cells. In contrast, B cells adhered to GALT HEVs in preference to T cells. The authors concluded by suggesting that the 'NALT is more a T cell organ, while Peyer's patches are B cell organs'. In conclusion, the NALT is involved in a secretory IgA response through the posterior cervical lymph nodes but is also involved in systemic responses through lymphocyte migration.

1.4.5 Barriers to nasal delivery of vaccines

Mucus is secreted into the nasal cavity by submucosal mucus and serous glands and by goblet cells (as mucus granules which are subsequently hydrated in nasal secretions).

The lacrimal duct is the other source of secretions into the nasal lumen. The general composition of mucus is mainly water (95 %) and mucin (2 to 3 %) (an anionic glycoprotein at neutral pH) with the remaining constituents being electrolytes, proteins such as enzymes and antibodies and lipids (Ugwoke *et al.*, 2001 and Washington *et al.*, 2002). The normal luminal pH is 5.5-6.5 (Sayani *et al.*, 1996). Mucus serves to keep mucosal surfaces from drying, humidifies inspired air and also, due to it's viscous nature, helps to increase the adherence of foreign agents such as dust particles and pathogens to it. The normal mucociliary clearance value in man is usually approximately 8-20 minutes (Aspden *et al.*, 1997). Entrapped agents in the mucus are cleared by ciliated cells to areas where the mucus can be wiped away or swallowed. The composition of mucus and mucociliary clearance times are highly variable depending on concurrent illnesses, allergic reactions, tobacco smoking and in those people with airways diseases such as asthma.

Particles in inspired air more than approximately 10 μm are removed in the nasal cavity whereas those below 0.5 μm generally remain suspended in the air (Martini, 2004). In the same publication it was also stated that particles between 1-5 μm were retarded in the secreted mucus of the bronchioles or alveoli and were thus not cleared by the mucus escalator but could possibly be engulfed by alveolar macrophages. In support of the particle size effect on nasal deposition, Davis (2001) reported that particles greater than 5 μm in diameter were removed in the nasal cavity and Washington and colleagues (Washington *et al.*, 2002) that particles between 1-5 μm were also deposited where the air stream turned. Proctor and colleagues (Proctor *et al.*, 1973) reviewed factors affected the nasal deposition of particles such as mucociliary clearance and anatomical structure. Therefore, when delivering particulate carriers intranasally, depending on the end target (nasal or pulmonary), the particle size (aerodynamic size) needs to be tailored to minimise nasal clearance (< 5 μm) yet large enough for nasal deposition (0.5 μm).

1.4.6 Strategies for increasing particle uptake/retention after nasal delivery

1.4.6.1 Bioadhesives

The use of bioadhesives would serve to prolong the residence time of the administered substance to either the nasal epithelium or associated mucosa (mucoadhesive). In the case of the latter, the nasally administered substance would still be subject to

mucociliary clearance. Increased time of retention of antigens in the nasal cavity would possibly enhance epithelial absorption or in the case of particulate carriers, either increase the opportunity for M cell uptake/adherence or increase the half-life of the controlled release preparation. Chitosan (section 1.3.19) is an example of a mucoadhesive (Takeuchi *et al.*, 1996) which, after coating of anionic liposomes (DPPC and DCP) was shown to increase the retention of the liposomes to the intestinal lining of male Wistar rats compared to non-coated liposomes.

1.4.6.2 M cell targeting using lectins

Chen and co-worker (Chen *et al.*, 1996a) showed that Ulex Europaeus Agglutinin I (UEA-1) which binds to mouse M cells specifically, increased the uptake of polymerised liposomes from the gastrointestinal tract in comparison with non-coated polymerised liposomes in BALB/c mice after oral delivery. Wheat Germ Agglutinin coated polymerised liposomes showed increased binding to all epithelial cells (no cell specificity) and therefore there was a lower overall uptake compared to UEA-1 coated polymerised liposomes (though still a higher uptake compared to non-coated polymerised liposomes). Giannasca and co-workers (Giannasca *et al.*, 1997) investigated the binding of various lectins to the nasal M cell glycoconjugate receptors in tissue sections excised from 6-8 week old female Syrian hamsters. GS I-B_4 (*Griffonia simplicifolia* I isolectin-B_4) and EEA (*Euonymus europaeus*) were found to bind mainly to the FAE with very little binding to other respiratory endothelial cells. Intranasal immunisation with horseradish peroxidase (mwt = 40 kDa) conjugated to GS I-B_4 resulted in a higher horseradish peroxidase-specific serum IgG antibody response than the administration of non-conjugated horseradish peroxidase and GS I-B_4 or free horseradish peroxidase. Hence, the targeting of antigens to M cells using lectins is a promising approach for higher systemic (and possibly mucosal) immune responses.

1.4.6.3 Optimal particulate size for uptake by M cells of the NALT

Cremachi and co-workers (Cremachi *et al.*, 1998) studied the sub-epithelial tissue of the upper turbinate and the central septum of male New Zealand rabbits and observed the presence of three types of lymphoid tissues: isolated scattered cells, simple diffuse infiltrates with glands and thick lymphoid aggregates without glands. The authors reported that the extent of carbocalcitonin (mwt = 3,362) transcytosis was directly

related to the amount of thick lymphoid aggregates. No link of carbocalcitonin transcytosis to either isolated cells or simple infiltrates was found. Further studies by the same group (Ghirardelli *et al.*, 1999) on the same animal model, showed that after application of insulin coated fluorescent polystyrene nanoparticles (0.5 μm), nanoparticles were largely found bound to non-ciliated microvillar cells overlying lymphoid aggregates whereas none were found bound to ciliated cells.

The appearance of latex particles in the tail vein of Male Wistar rats or the ear vein of New Zealand White rabbits following intranasal administration indicated the transfer of particulates from the nasal cavity to the circulation (Almeida *et al.*, 1993). Particles with a mean diameter of 0.51 μm were administered in a 0.1 mL volume to rats and particles with a mean diameter of 0.83 μm in a 0.2 mL volume to rabbits. Blood collected 10 min after dosing showed that at this time-point 0.96 % and 1.9 % of particles administered to rats and rabbits respectively were detected. However, the mechanism and route of transfer of the particles cannot be ascertained from this study.

The intranasal administration of antigen in particulate form has been shown to evoke an enhanced immune response than when soluble antigen alone is given (see section 1.4.6.5) and the affinity of particles to lymphoid aggregates in the nasal mucosa over normal epithelium has also been shown (Ghirardelli *et al.*, 1999). However, complete understanding of the transfer of soluble and particulate matter across the nasal mucosa and the importance of each possible route in the immune process is as yet unclear. The effect of particle size on uptake by specific cells (e.g. nasal M cells) has still not been investigated fully in animal or human subjects. The nasal M cell could be likened to those present in the GALT on which more comprehensive studies have been undertaken (section 1.4.6.4).

1.4.6.4 Optimal particle size and charge for uptake by M cells of the GALT

The M cells found as part of the GALT have been investigated more intensely than M cells found in any other mucosal location. To further our understanding of M cells present in the NALT, it may be of use to compare with studies undertaken on the adsorption and absorption of particles to M cells of the GALT.

The inductive site for mucosal immunity gained in response to an orally administered antigen is thought to be the activity of the GALT (McCluskie and Davis, 1999) such as the Peyer's patches of the small intestine. Three possible mechanisms of exit from the lumen of the intestine to the underlying MALT have been established for particles and macromolecules. These are paracellularly, through enterocytes or through M cells (Florence, 1997; Mathiowitz, 2000). Antigen uptake is thought to occur *via* the latter two processes and also through capture and translocation by luminal macrophages. Gilligan and Li Wan Po (1991) describe M cells of the GALT as lacking microvilli and containing reduced amounts of lysosomes. They are located predominantly in the FAE, which overlies the lymphoid follicles of the tract. Enterocytes (which are found in great quantity along the length of the whole tract including the FAE) on the other hand are coated with microvilli and do contain lysosomes and therefore only small quantities of antigen pass through them. Only a few goblet cells are to be found in the Peyer's patches resulting in decreased mucus cover and thus a reduced barrier to particulate/macromolecule absorption.

The delivery of vaccines to M cells may elicit a mucosal response. This occurs following endocytosis of the antigen by M cells from the intestinal lumen (Neutra and Kraehenbuhl, 1992) and subsequent trancytosis (Jepson *et al.*, 1996) through the M cell to underlying 'pockets' in the basolateral plasma membrane which contain B cells, T cells and macrophages (Kuby, 1997). This interaction of the antigen with lymphoid tissues will result in multiplication of lymphocytes (IgA producing precursors for example) which then migrate to the mesenteric lymph node *via* efferent lymphatics, on to the thoracic duct and from there will enter the systemic circulation (Gilligan and Li Wan Po, 1991). From this wide spread circulation, these antigen-sensitised precursors of IgA plasma cells then enter the mucosal 'effector' sites (lamina propria and glandular tissues) (Mestecky, 1987) from where secretory IgA can be released (Kuby, 1997). Thus, immunity can be achieved at all mucosal sites even those distant from the point of primary infection.

Childers and co-workers (Childers *et al.*, 1990) showed by transmission electron microscopy (TEM) that liposomes composed of DPPC and cholesterol ranging in diameter from 20-210 nm (mean diameter = 72 nm) were taken up by M cells in the Peyer's patches in the jejunum of 16-20 week old Fischer CDF(F-344)CrlBR rats *in vivo*. Jani and colleagues (Jani *et al.*, 1990) showed that latex particles of 3 μm were

not detected in the liver, spleen or blood after oral administration in 15-20 week old female Sprague Dawley rats whereas smaller particles (50 nm, 100 nm, 300 nm, 500 nm and 1000 nm) were detected. Histological evidence demonstrated that particles of all sizes were found predominantly in the Peyer's patches and mesentery network.

Eldridge and colleagues (Eldridge *et al.*, 1990) administered a range of coumarin loaded polystyrene microspheres (1-100 μm) orally to 8-12 week old mixed sex BALB/c mice and showed that microspheres above 10 μm were not absorbed on the Peyer's patches whereas particles below this size were. The authors also demonstrated that the Peyer's patches adsorbed the more hydrophobic microspheres to a higher extent than the more hydrophilic ones. Microspheres composed of poly(DL-lactide-*co*-glycolide) copolymers of various sizes were shown to be predominantly captured within macrophages in the Peyer's patches and those less than 5 μm were detected in the mesenteric lymph nodes and spleen. Larger particles (5-10 μm) remained in the dome macrophages under the Peyer's patches. Hence, the authors proposed that particles less than 5 μm could evoke a systemic immune response as well as a mucosal one whereas those between 5-10 μm would sustain a mucosal response predominantly due to their retention in the domes under the GALT.

Howard and co-workers (Howard *et al.*, 1994) administered fluorescent latex microparticles of two sizes (0.11 μm and 0.94 μm) intraduodenally to adult male white and Netherland Dwarf rabbits and observed that the larger particles (0.94 μm) remained mainly on the surface of the domes (above the lymphoid follicles) whereas the smaller particles (0.11 μm) were found mainly at the serosal surface. The larger particles were detected to a higher extent in the mesenteric lymph than the smaller particles. The authors also reviewed that particles between 0.5-1 μm displayed a greater absorption than larger or smaller particles. The overall extent of absorption was low yet it was higher than that demonstrated in adult male Wistar rats.

Ermak and Giannasca (1998) give a review of work on microparticle uptake by M cells and conclude that for systemic access, a particle size lower than 500 nm is desirable whereas a particle size of 0.5-2 μm is suitable for lymphoid tissue targeting.

Many studies have been conducted investigating the effect of liposomal surface charge on its uptake by the Peyer's patches but reports have been inconsistent. Tomizawa and

co-workers (Tomizawa *et al.*, 1993) demonstrated that negatively charged liposomes were preferentially taken up from the Peyer's patches. They administered liposomes composed of PC, phosphatidylserine (PS) and cholesterol intraluminally to male Wistar rats and showed using fluorescent microscopy, that two hours following administration, 6-carboxyfluorescein encapsulated liposomes containing higher amounts of PS (and hence with a higher negative zeta potential) were taken up to a higher extent in the Peyer's patches. The authors also showed that two hours following administration, liposomes with mean diameters of 374 nm and 855 nm possessed a higher uptake in the Peyer's patches than smaller liposomes with a mean diameter of 162 nm. In the same article it was reviewed that macrophages preferentially took up negatively charged liposomes and that studies on the rabbit ileum showed that DCP containing liposomes (negatively charged) were absorbed better (across the ileum) than neutral or positively charged liposomes. However, *in vitro* studies (Iwanaga *et al.*, 1997) have shown that insulin encapsulated liposomes with a neutral charge (composed of DPPC and cholesterol) are more stable in intestinal fluids than positively charged liposomes (containing DPPC /cholesterol/stearylamine (SA)), yet following oral administration to rats, the positively charged liposomes showed a greater hypoglycaemic effect than the delivery of liposomes with a neutral surface charge.

Therefore, the effect of particle surface charge on gastrointestinal uptake of liposomes remains unclear, though conclusions from the studies cited above in regard to particle size and attainment of both systemic and mucosal immune responses, point to an optimal particulate size of about 0.3- 1 μm for vaccine delivery which may possibly vary depending on the hydrophobic nature (amongst other characteristics) of the particulate.

1.4.6.5 Immune response studies using particulate antigens delivered nasally: effect of particle size and volume of administration

A number of studies have been conducted investigating the effect of particle size on immune responses following intranasal delivery. Almeida and co-workers (Almeida *et al.*, 1993) administered TT adsorbed poly(L-lactic acid) microspheres with a mean diameter of 0.8 μm (range from 0.1-1.6 μm) intranasally in a 0.2 μL volume to female Dunkin-Hartley guinea-pigs. TT-specific serum IgG was found to be higher than when free antigen was administered alone.

Intranasal administration of BSA loaded poly(D,L-lactic-co-glycolic) acid particles with diameters of 0.2 μm, 0.5 μm and 1 μm to 6-8 week old female BALB/c mice resulted in a higher BSA-specific antibody response for particles with a diameter of 1 μm followed by particles with diameters of 0.5 μm and the lowest BS-specific antibody levels for particles with a diameter of 0.2 μm (Gutierro *et al.*, 2002). However, the volume of administration was 50 μl and so the correlation between particle size and elicited immune response will not be solely linked to delivery of particles to the NALT but also due to oral and pulmonary delivery through swallowing and inhalation respectively.

Jung and co-workers (Jung *et al.*, 2001) administered TT adsorbed sulfobutylated poly(vinyl alcohol)-graft-poly(lactide-co-glycolide) nanoparticles with mean diameters of approximately 0.1 μm, 0.5 μm and 1.5 μm intranasally in a volume of 20 μL to 7-9 week old female BALB/c mice. Particles with a mean diameter of 0.1 μm and 0.5 μm evoked similar TT-specific serum IgG and IgA titres whilst the larger particles (1.5 μm) did not induce TT-specific serum antibody levels above that of free TT in solution.

Investigations on the effect of larger particles (of micron size) on intranasal vaccine delivery to 6-8 week old female BALB/c mice in a volume of 10 μL was carried out using polystyrene based particles by Higaki and colleagues (Higaki *et al.*, 1998). Influenza virus haemagglutinin was administered mixed/adsorbed with anionic sodium polystyrene sulfonate particles of various diameters (< 20 μm, 20-45 μm, 45-105 μm and > 105 μm). Initial haemagglutinin adsorption to the particles was 58.3 %, 58.3 %, 6.7 % and 6.7 % respectively. Particles with diameters < 20 μm and 20-45 μm resulted in the highest level of haemagglutinin-inhibiting antibodies compared to larger particles (45-105 μm and >105 μm). Animals given particles with diameters of 20-45 μm resulted in the highest nasal antigen-specific sIgA titres. The authors relate this to either the higher initial antigen adsorption to the smaller particles (< 20 μm and 20-45 μm) or because the larger particles (45-105 μm and >105 μm) were cleared away faster from the nose following delivery. Unlike studies using far smaller particle sizes, this study probably indicates the role of particles in creating a depot for the controlled release of antigen in the nasal passages and not transportation of the carrier through nasal epithelia (however such histological examination was not undertaken). The use of particulate carriers such as starch, dextran, albumin or hyaluronic acid microspheres as a tool to increase the bioavailability of intranasally administered drugs due to increased nasal

residence times and opening of tight junctions between epithelial cells is reviewed by Pereswetoff-Morath (1998).

Alpar and colleagues (Alpar *et al.*, 1992) demonstrated the immunoadjuvant nature of liposomes when administered intranasally to guinea pigs. Uni-lamellar liposomes composed of equimolar distearoyl phosphatidylcholine (DSPC) and cholesterol were loaded with 30 μg of TT and administered intranasally in a volume of 200 μL on weeks 1, 2 and 4. Liposomal TT gave not only higher anti-TT IgG titres compared to free TT (of same TT dose and also of double dosage i.e. 60 μg/dose) but also achieved a longer-lived response (week 11).

Eyles and co-workers (Eyles *et al.*, 1998) administered *Y. pestis* subunits V and F1 co-encapsulated into poly-(L-lactide) microspheres (mean diameter of 5.6 μm) intranasally to 6 week old female BALB/c mice in a volume of 50 μL. Anti-F1 and anti-V serum IgG antibody levels were higher following the administration of the co-encapsulated microsphere formulation compared to co-administration of antigens without microspheres. An administration volume of 50 μL is likely not to have been delivered just to the nasal cavity and pharynx but also possibly to the gastrointestinal tract and the lower respiratory tract and therefore a relationship between the size of the microspheres (5.6 μm) and efficacy of vaccine delivery to nasal immunocompetent cells is not directly possible.

The effect of the intranasal administration volume on the immune response elicited was investigated by the same group (Eyles *et al.*, 1999) in 6 week old female BALB/c mice. Poly-(L-lactide) microsphere (mean diameter of 1.8 μm) encapsulated TT was administered in volumes of 10 μL and 50 μL. The smaller administration volume (10 μL) resulted in a lower TT-specific serum IgG antibody response than the larger volume (50 μL). Scandium-46 labelled styrene-di-vinyl benzene microspheres (mean diameter of 7 μm) administered in volumes of 10 μL or 50 μL showed that a smaller volume of administration results in greater deposition in the nasal passages than the lungs and conversely that a larger volume of administration leads to greater lung deposition. Irrespective of administration volume, comparative quantities of microspheres could be detected within the gastrointestinal tract.

The volume effect was again confirmed in 6 week old female BALB/c mice (Eyles *et al.*, 2001) using fluorescent polystyrene carboxylate microspheres (diameter of 1.1 μm). Microspheres could be detected in NALT and the posterior cervical lymph nodes for both 10 μL and 50 μL administration volumes. The larger administration volume (50 μL) however, led to the deposition of microspheres in the lung and there was evidence of the translocation of particles to the mediastinal lymph node and to the spleen whereas this was not the case for the smaller administration volume (10 μL).

The importance of the secretory IgA response elicited locally following intranasal immunisation may be evident in the study by de Haan and co-workers (de Haan *et al*, 1995a). The intranasal administration (in 50 μL) of influenza virus subunit (aggregates of viral haemagglutinin and neuraminidase derived from H1N1, A/Chile/83 virus and H3N2) co-administered with liposomes (composed of PC, cholesterol and DCP) to 8-10 week old female BALB/c mice conferred a higher protective state on challenge with influenza A (H1N1) compared to the intramuscular administration of the free antigen alone. The use of a 50 μL volume for vaccine administration indicates that the immune responses seen were due to total respiratory tract deposition of the antigen. Results from the same laboratory (de Haan *et al.*, 1995b) showed that when whole inactivated measles virus was administered intranasally to 8-10 week old female BALB/c mice mixed with liposomes, that the use of a smaller total volume of administration (25 μL) did not result in a detectable antigen-specific serum IgG or secretory IgA response whereas a larger volume (50 μL) did. In more recent studies the authors (de Haan *et al.*, 2001) showed that a protective immune response (following intranasal challenge with influenza virus) was elicited when influenza subunit antigen (from influenza strain A/Puerto Rico/8/34) was administered with the B subunit of LT intranasally to 6-8 week old female BALB/c mice in a 20 μL dose.

Thus, the nasal route of administration has been shown to be highly effective at achieving not only high systemic and mucosal immune responses but also protective cover and that this may be optimal when administering the antigen in liposomes with a submicron particle size distribution (between 0.3-1 μm), though a more thorough study investigating the immune response to both submicron and micron-sized particulates needs to be conducted. The fate of the delivered vaccine depends on, amongst other factors, the administered volume and restricting dosing volumes to no more than about 20-25 μL in mice (total volume to be divided between nostrils) should ensure that

vaccine delivery is predominantly to the nasal cavity. However, delivery of the vaccine in a larger volume (50 μL total volume in mice) may further enhance the immune response due to pulmonary and oral delivery of vaccine also.

1.4.6.6 Animal models for studying intranasal vaccine delivery

The conclusions made after investigations in animal models and the subsequent projection of them to the human is subject to the appreciation of many factors. The occurrence of a multi-layered epithelium in areas of the hamster's nasal cavity and a lower number of ciliated cells and goblet cells is a limitation in the use of this model for nasal delivery studies (Ugwoke *et al.*, 2001). The guinea pig is a good model for nasal delivery studies so long as the formulation is applied to the nasal cavity (as the vestibule is covered with squamous cells) and the use of rabbits is suitable for preparations where larger volumes have to be administered (for formulation purposes) (reviewed by Gizurarson, 1993). The rat and mouse provide good models for initial immunisation and transport studies. However, the presence of the septal window in mice, rats and guinea pigs needs to be considered if delivery through only one nostril is desired (Ugwoke *et al.*, 2001).

Apart from large anatomical differences, the sites and extent of lymphatic tissue distribution, epithelium structure and composition and mucociliary clearance in the nasopharynx is also highly variable between species. Thus, the main considerations in the choice of animal model lies mainly with lower husbandry costs, wider availability of reagents for antibody analyses and ease of animal care concerns.

In their review of adjuvants, Kenney and Edelman (2003) suggest that due to a high variation in immune response in mice that either the guinea pig or rabbit model should be utilised. When mice are used, two strains of mice (with different haplotypes) should be investigated followed up by studies in a non-rodent mammal. The authors also propose that toxicity as well as immunogenicity studies be undertaken (using doses and protocols comparable to that useable in humans.

1.4.6.7 Nasal immunisation strategy

1.4.6.7.1 Patient and administration benefits

Unlike parenteral injections, administration by the nasal route carries no risk of the transmission of blood borne diseases *via* needle stick injuries or the reuse of needles. This is of marked advantage especially in developing countries where mass immunisation schemes aim to tackle a large population in short time periods. The administration of intranasal formulations would not require highly trained personnel and due to simpler storage requirements (if the need for cold-chain were to be eliminated) would increase cost efficiency. It has been recognised that only 12 % of the total cost of vaccination is due to the price of the medication itself, the rest being attributable to the expense of maintaining the cold chain and the salaries of medical staff (O'Hagan, 1994b). Another advantage of intranasal vaccine delivery is that a larger population could be 'netted' at the same time. The nasal route is one which is more acceptable to the patient and drop-out rates in relation to booster doses may fall (currently estimated at 20 % after each booster in developing countries (Goldsby *et al.*, 2000)). Nasal delivery devices that may be suitable for the self-administration of vaccines include unit-dose containers for delivery of nasal drops, metered-dose pump sprays, dry powder inhalers, and pressurised metered-dose inhalers (Kublik and Vidgren, 1998). OptiNose™ (yet to enter clinical testing) is a nasal delivery device that decreases the occurrence of pulmonary delivery. The patient places the nozzle of the device into one nostril and exhales into a mouthpiece (exhaling against a resistance closes the soft palate and separates the oral and nasal cavities). The resultant airflow leads to the drug/vaccine entering the nostril *via* the nosepiece and subsequently leaving through the other nostril. The use of the device with submicron particulates remains to be investigated.

1.4.6.7.2 Systemic and mucosal immunity

In addition to the advantages outlined in section 1.4.6.7.1, a further important benefit of administering a vaccine *via* the nasal route is that the induction of mucosal as well as systemic immunity can be achieved (reviewed by Mestecky, 1987 and McGhee and colleagues (McGhee *et al.*, 1992). Current vaccines for humans are predominantly administered parenterally, which results in a systemic response but not a mucosal

response (unless previously immunised mucosally) and hence would not hinder the invasion of organisms at mucosal surfaces. The systemic immune system comprises of bone marrow, spleen and lymph nodes whilst the mucosal portion is associated with all mucosal surfaces and external secretory glands. Mucosal responses (like systemic responses) are comprised of a humoral and cell-mediated component (see section 1.1.2). The principle antibody produced in response to mucosal immunisation at inductive sites such as the MALTs, where antigen is presented to B and T cells is secretory IgA (sIgA). MALT includes the GALT (see section 1.4.6.4), NALT (see section 1.4), bronchus-associated lymphoid tissue (BALT) and genitourinary-associated lymphoid tissue amongst others (McCluskie and Davis, 1999). Antigen presentation to B cells and T cells at these inductive sites leads to their migration to effector sites (such as intestinal, bronchal and genito-urinal lamina propria regions and exocrine secretory glands) where precursor IgA-B cells mature into IgA plasma cells (aided by T cell and cytokine stimulation). This cell migration pathway is known as the Common Mucosal Immune System (CMIS) and links the inductive sites to the effector sites. IgA thus produced at the effector sites, is joined to a secretory component and released into external secretions. The action of IgA in the immune response (reviewed by Tlaskalová-Hogenová and colleagues (Tlaskalová-Hogenová *et al.*, 2004); O'Hagan (1994b) and McGhee and colleagues (McGhee *et al.*, 1992)) includes preventing the entry of the pathogens from mucosal sites (due to binding of the sIgA to organisms) and detection of those that have gained entry (through recognition of organisms by intraepithelial IgA). There are many infectious agents that are introduced to the host *via* the mucosal surfaces (orally, *via* nasal and pulmonary mucous membranes or genital mucous membranes) and hence mucosal immunity can prevent such successful invasion (McGhee, *et al.*, 1992; O'Hagan, 1994b)

The role of IgG as a protective antibody in mucosal secretions has also been investigated by Parr and co-workers (Parr *et al.*, 1997). The authors passively transferred serum IgG from female BALB/c mice previously immunised with Herpes Simple Virus Type 2 (HSV-2) intraperitoneally to naïve mice and confirmed the detection of specific IgG in vaginal secretions in these mice 48 h later. Vaginal challenge with HSV-2 in these mice resulted in reduced vaginal infection and illness scores. The concentration of shed virus protein was also shown to be lower in the vagina of animals with passively transferred IgG. The removal of vaginal mucosa prior to challenge resulted in the increase in concentration of shed protein after challenge.

Studies by de Haan and co-worker (de Haan *et al.*, 2001) also provide evidence for the role of mucosal IgG in eliciting protection in 6-8 week old female BALB/c mice against influenza virus infection using LT as an adjuvant. Mice immunised intramuscularly or intranasally were protected against infection. Intranasal administration resulted in both an antigen-specific serum IgG and mucosal sIgA antibody response unlike intramuscular administration in which no antigen-specific sIgA antibodies were detected in the nasal washings. However, there was a stronger serum IgG response and also the presence of mucosal IgG. On intramuscular administration of the subunit antigen alone, mucosal IgG was not detected and protection was incomplete. The authors consider the mechanism by which IgG becomes to be in nasal secretions and propose that either it is by transudation (augmented by a 'stimuli') or through local production and secretion.

1.5 Liposomes as vaccine adjuvants

In essence, liposomes are amphiphilic phospholipids (composed of a polar head group and two hydrophobic hydrocarbon tails) which after exposure to an aqueous environment are arranged into a closed spherical bi-layer structure (figure 1.10).

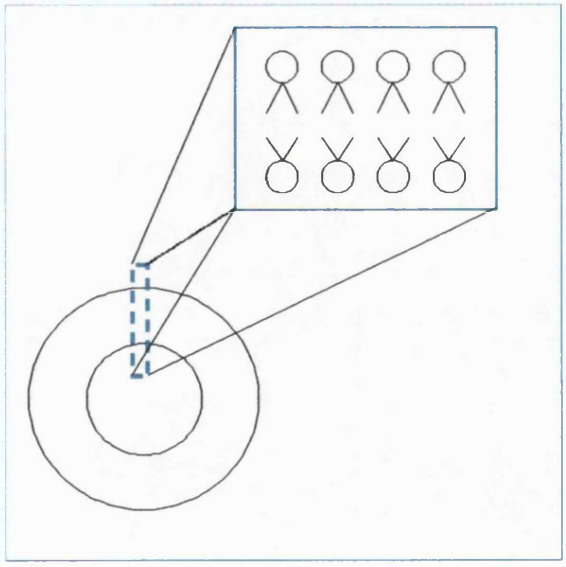

Figure 1.10: 2-D structure of an amphiphilic bi-layer within a liposome. Phospholipid molecules are arranged so that the hydrophilic head groups (circles) are exposed to the aqueous environment and the two hydrophobic hydrocarbon tails are shielded from the aqueous environment by closing into a bi-layer.

The central core remains an aqueous phase and can be surrounded by more than one concentric bi-layer, each of which is further separated by an aqueous 'cushion'. Thus, material that is hydrophilic, hydrophobic or amphiphilic, regardless of size, can be encompassed into the structure. The liposome 'masks' the original attributes of the incorporated drug (such as charge) and the formulation takes on the identity of the outer phospholipid layer. The membranes of this biological 'cell' like structure are usually composed of a variety of phospholipids (natural or synthetic) plus various other components such as cholesterol or functionalising moieties depending on the purpose of use. Vesicles can be made in a broad range of sizes from 20-25 nm to many tens of microns. Other parameters such as liposome structure and composition are detailed in section 1.5.1.

1.5.1 Liposomal structure

1.5.1.1 Phospholipids: structure, phase transition temperature and charge

Phosphatidylcholine (PC) (lecithin) is the most commonly used phospholipid for the preparation of liposomes. The molecule is composed of a glycerol bridge that links the hydrophilic phosphocholine head group to two hydrophobic acyl hydrocarbon chains. PCs extracted from plant sources (e.g. soya) contain a higher degree of unsaturations in the acyl chains than of that extracted from animal sources (e.g. egg). Phospholipids often used include PC and phosphatidylethanolamine (PE) which are both neutral and phosphatidylserine (PS) and phosphatidylglycerol (PG) which are both negatively charged.

The phase transition temperature (T_c) of a phospholipid membrane is the temperature at which the bi-layer membrane passes from a tightly ordered gel/solid phase to a liquid 'crystal' phase (figure 1.11). Factors such as the degree of saturation in the phospholipid chains, length of lipid chains and polarity and size of head group define the T_c for different phospholipids. The addition of other phospholipids or molecules such as cholesterol into a liposomal formulation will change the T_c of the vesicle and so the T_c of the new vesicle would need to be re-determined.

The change in state has very important consequences for factors such as membrane permeability. Leakage of entrapped molecules is greater when the temperature is above

the T_c of a particular composition of phospholipids due to looser packing of membranes. The fatty acid chains of the phospholipid glycerol backbone in positions 1 and 2 are in the trans conformation state, but as temperature increases the fatty acids take on the gauche conformation which expands the volume occupied by the chains (and thus the membrane) and at the same time, bi-layer thickness decreases (both changes due to increased chain tilts).

As a result of this changing of state, numerous packing defects arise (point defects, line defects and grain boundaries) in the membrane. This is especially so at the T_c due to the increased number of interfaces as both phases (solid and fluid) co-exist in large amounts. Inevitably, this results in a higher membrane permeability at this temperature. A lower permeability is seen when only one phase is in existence.

The importance of the phospholipid T_c has been demonstrated using liposome stability studies conducted at human body temperature (37 °C). It has been shown that certain phospholipids are more stable in the gastro-intestinal environment than others. Phospholipids with a T_c above 37 °C should be used in the preparation of liposomes, as this will decrease the release of the encapsulant at body temperature. Phospholipids with a T_c near to 37 °C should not be used for preparation of liposomes where fast release of encapsulant is not desired, as at body temperature, the liposomal membrane would possess many 'point defects' due to parts of the membrane being in fluid phase and parts still being in solid phase, leading to increased membrane permeability.

Liposomes composed solely of DSPC (T_c = 58.0 °C) or DPPC (T_c = 41.5 °C) were shown to be more stable (*in vitro*) in varying pH's, 10 % bile and pancreatin than PC/cholesterol liposomes or PC/PS/cholesterol liposomes (T_c for PC =-15 to –7 °C) (Aramaki *et al.*, 1993). The addition of pig pancreas phospholipase A_2 to liposomes composed solely of dimyristoylphosphatidylcholine (T_c = 23 °C), DPPC (T_c = 41.5 °C) or dilauroylphosphatidylcholine (T_c = 0 °C), demonstrated that there was a greater hydrolysis of the phospholipid membranes (and thus the leakage of entrapped agents) at the T_c (Op Den Kamp *et al.*, 1974). However, at temperatures above and below the T_c there was very little increase in liposome permeability. This may be due to the PC in these liposomes being unapproachable by the phospholipase A_2.

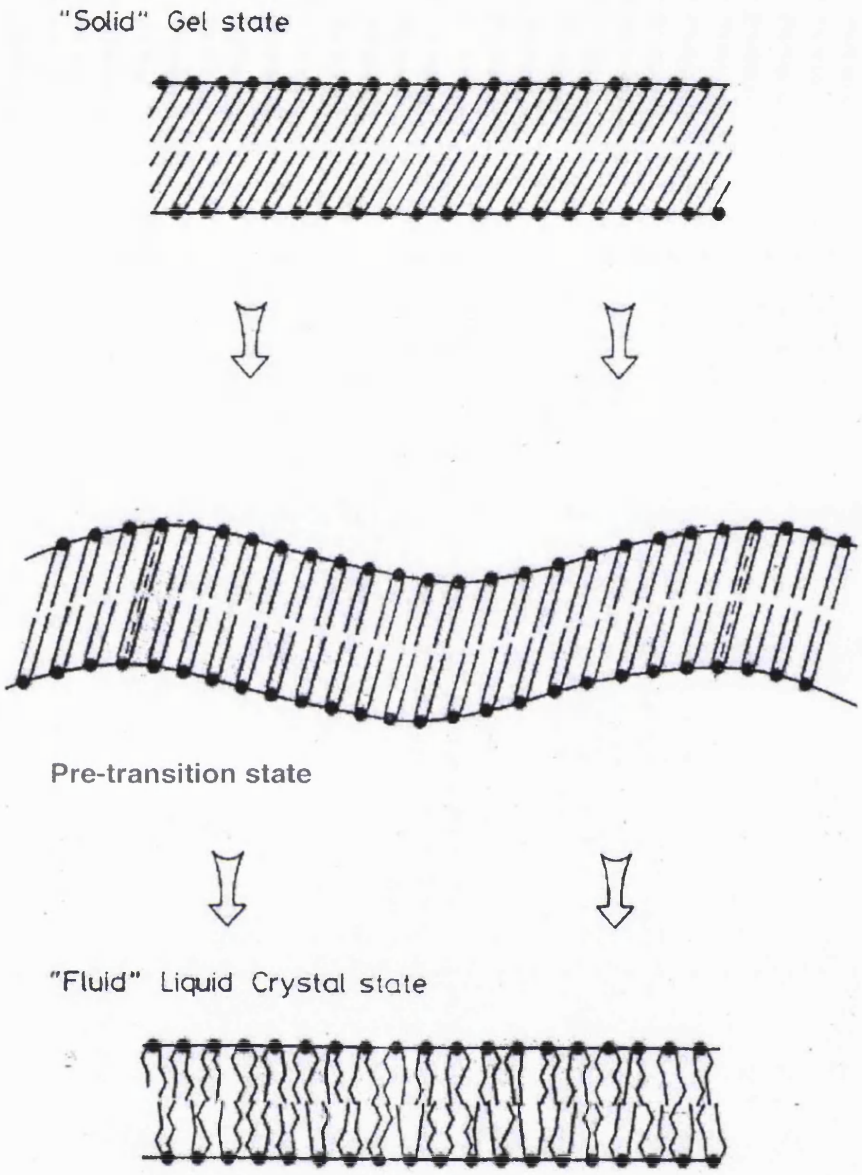

Figure 1.11: The transition of bi-layers from a solid state (below transition temperature) to a fluid state (above transition temperature) (reproduced from New, 1990)

Sterols such as cholesterol are found in many natural membranes (molar ratio to PC varying from 0.1 to 1) and their incorporation into the liposomal membrane (as it is amphiphilic) affects phospholipid chain tilting. The fluidity of membranes below the T_c is increased due to the addition of sterols whilst the membrane becomes more rigid on incorporation of sterols when liposomes are above the T_c (Qi *et al.*, 1996). The addition of cholesterol (or other sterols) to the membranes of liposomes should therefore be only

after consideration of whether the system would be above or below the phase transition temperature when in use (usually 37 °C).

Therefore, we can see that we can improve the bioavailability of the drug/carrier system by giving careful consideration to the composition of the bi-layer membrane of the liposome.

1.5.1.2 Liposomal size and lamellarity

Liposomes can be classified according to their size/diameters and also according to the number of bi-layers they possess (table 1.4).

Hydrophilic drugs would be captured into the aqueous compartments of the liposome and so structures which have large aqueous to lipid ratios are beneficial e.g. large or intermediate size uni-lamellar liposomes, which have a large internal aqueous core. However, these liposomes may not provide a good barrier to water and due to osmotic intake, rupture may occur. An oligo-/multi-lamellar structure may provide a more stable liposome (to osmotic rupture and also gastro-intestinal conditions) and would also result in a more gradual release of entrapped material (which may or may not be required). Through changing drug/antigen characteristics such as aqueous solubility, the entrapment/association of these agents into/onto liposomes can be increased and subsequent release from the carrier after administration modified.

Hydrophobic drugs will associate themselves within the phospholipid membranes and therefore a large phospholipid to total liposomal volume ratio, such as in multi-lamellar vesicles should increase entrapment values. Incorporating charged phospholipids into membranes results in electrostatic repulsion between bi-layers and thus aqueous compartments increase in size and hence hydrophilic drug entrapment should increase (New, 1990).

Table 1.4: Summary of liposome structures: size and lamellarity

Type	Main characteristics
Small uni-lamellar vesicle (SUV)	25-100 nm Single bi-layer
Large uni-lamellar vesicle (LUV)	>200 nm Single bi-layer
Multi-lamellar vesicle (MLV)	No defined size range >5 bi-layers (>1 but <5 bi-layers is termed oligo-lamellar)
Multi-vesicular liposome (MVL)	No defined size range Bi-layers not arranged in concentric spheres but more like aggregates of several uni-lamellar vesicles

1.5.2 Methods for the preparation of liposomes

There are many methods documented for the production of liposomes (New, 1990). In essence, the first step in all protocols is the solvation of phospholipids and other constituents (including encapsulate if hydrophobic) into an organic medium to ensure their homogenous mixing. The following sections describe the most common methods used and a brief account of their main advantages and disadvantages.

1.5.2.1 Classical thin film method

The organic solvent (usually a blend of chloroform and methanol) is removed using a rotary evaporator resulting in the deposition of the dissolved phospholipid as a thin film onto the holding vessel (usually a round-bottomed or pear-shaped flask). After further drying (using nitrogen gas or attachment of flask to a vacuum pump) the film is rehydrated using an aqueous solution (in which the drug to be encapsulated, if hydrophilic may be dissolved) and shaken to allow the deposited phospholipid to 'swell' and form liposomes. The resulting vesicles are multi-lamellar and above micron size with a broad range of particle sizes. The encapsulation of hydrophilic agents is generally poor whilst that for hydrophobic agents is very high.

1.5.2.2 Lyophilisation of phospholipids as an alternative to thin film drying

Some phospholipids do not deposit satisfactorily as a thin film after rotary evaporation of the solvent. For such phospholipids an alternative method involves using a lyophiliser compatible solvent like tertiary butanol to dissolve the phospholipids in. The solvent is then removed using lyophilisation leaving the phospholipid as a finely divided solid in the vial. The addition of the aqueous medium then allows for an efficient hydration of the phospholipids to allow liposome formation.

1.5.2.3 Pro-liposome method

In the preparation of pro-liposomes (Song *et al.*, 2002), the solvent (containing phospholipids) is removed in the rotary evaporator and the phospholipids (and other membrane constituents) deposited onto finely divided 'supports' such as powdered sodium chloride or polysaccharides such as sorbitol. This results in dried phospholipids dispersed in such a way to increase their surface area which allows for a faster hydration on addition of an aqueous solution. This method produces MLVs generally of a smaller size than those produced by the classical thin film method. The phospholipid-support intermediate can be stored and the liposomes produced on hydration at the patient end.

1.5.3 Solvent dispersion methods

Methods such as ethanol injection, ether injection, double-emulsion and reverse-phase evaporation are grouped under one category as the organic solvent (including dissolved phospholipids) is mixed directly with the aqueous phase to form an emulsion followed by the removal of the organic phase, which allows for liposomal formation.

1.5.3.1 Ethanol injection method

The rapid injection of ethanol (containing phospholipids) through a needle into an aqueous phase (containing encapsulate) results in the dispersion of phospholipids throughout the solution and subsequent formation of SUVs. Pevaryl® Lipogel is a commercial liposomal product containing econazole that is produced in batches of several hundred kilograms using an ethanol injection method (Naeff, 1996) showing that such a method is feasible.

1.5.3.2 Ether injection method

The slow injection of ether (containing phospholipids) through a needle into an aqueous phase (containing encapsulate) maintained at a high temperature (e.g. 60 °C) results in the evaporation of the ether and consequent formation of LUVs (Deamer and Bangham, 1976).

1.5.3.3 Double-emulsion method

The aqueous compartment (containing encapsulate) is mixed into the organic phase (containing phospholipids) to create a water-in-oil emulsion stabilised at the interface by the phospholipids. The emulsion is then manipulated using various shear forces or sonication (section 1.5.4). The inner mono-layer (entrapping the aqueous volume) of each liposome is thus created. The addition of this system to a second water phase results in the creation of a water-in-oil-in-water system. The removal of the inner organic phase leads to the formation of bi-layers around each inner aqueous droplet and the formation of uni-lamellar vesicles with very high encapsulations (New, 1990). Whether the outer phospholipid layer of the bi-layer is formed from excess phospholipid in the system or the collapse of other 'droplets' will affect the end encapsulation efficiency, as break down of liposomes would result in loss of encapsulate into free solution. The construction of MVLs is also possible using this technique (Ramprasad *et al.*, 2003).

1.5.3.4 Reverse-phase evaporation method

A water-in-oil emulsion is created as in section (1.5.3.3) and modified to the required size (section 1.5.4). The organic phase is then removed by evaporation bringing each water-in-oil droplet closer together (and hence the hydrophobic lipid tails of the mono-layer of different droplets). This technique differs to that of the double-emulsion method in that a second aqueous phase (which becomes the suspending medium for the liposomes) is not added but is derived from the collapse of some of the water-in-oil droplets (due to vortexing) at this stage and results in the formation of LUVs (termed reverse-phase evaporation vesicles (REVs)) with large internal aqueous cores. This method originally developed by Szoka and Papahadjopoulos (1978) leads to the loss of

50 % of the encapsulate as it necessary that 50 % of the water-in-oil droplets collapse to form the outer coat of the bi-layer for the remaining 50 % of droplets.

A modification of this method (Pidgeon *et al.*, 1986) involves using a large quantity of phospholipid with a small volume of aqueous phase. The resultant droplet is coated with multiple layers of phospholipid so that when the outer layer is transferred to another droplet the donor droplet also forms liposomes with the addition of a small amount of 'exogenous' water. The resultant vesicles not only have large internal encapsulate captures but also possess multiple bi-layers, possibly increasing stability of liposomes.

1.5.4 Procedures for size modification

Shear forces such as that provided by homogenisation, micro-emulsification (microfluidiser), extrusion or French press are essential tools for the reduction of particle size, altering liposomal structure and for the production of liposomes with a monodisperse particle size distribution. Ultrasonication is also another tool that imparts high energy to the sample to produce SUVs. There are two methods: bath sonication for larger dilute volumes or probe sonication for more concentrated and smaller samples.

The size distribution of a population of liposomes can also be altered through the use of freeze-thaw (FT) cycles (Castile *et al.*, 1999). The authors found that the mean diameter of PC liposomes increased after the vesicles were subjected to cycles of freezing and thawing. They reasoned this to be due to the formation of internal ice crystals during the freezing stage (rupturing and fragmenting liposomal bi-layers) and the formation of new liposomes during the thawing stage (due to rehydration effect on phospholipid membranes and fusion of fragmented liposomes). The authors review that faster rates of cooling (such as immersion in liquid nitrogen) results in greater ice crystal formation and would lead to increased liposomal rupture than slower rates of cooling (such as with the use of a freezer). The effect of lyophilisation (without cryoprotection) increases mean liposome diameter and internal trap volume due to disruption of lamellae packing. Both encapsulation and encapsulate distribution throughout the liposome is affected due to the rupture and fusion of liposomal membranes during freezing and thawing.

If the liposomes are pre-loaded with encapsulate before any of the above methods (homogenisation, micro-emulsification, extrusion, French press, ultrasonication, freeze-thaw cycles) then the encapsulated drug/antigen will be exposed to any denaturing effects of the processes. There will also be release of the encapsulate by the carrier (or further loading into the carrier) when liposomal membranes are ruptured by the processes mentioned above, driven possibly by equilibration of encapsulate throughout the sample.

1.5.5 Procedures for encapsulation of drug/antigen post-liposome construction

Two similar methods have been used (Kirby and Gregoriadis, 1984; Yachi *et al.*, 1996) for the post-production encapsulation of drugs/antigens. Such a method is advantageous due to the minimal exposure of the drug/antigen to the production procedure. In the preparation of polymerised liposomes (section 1.6) where the liposome is polymerised (either by oxidation by UV irradiation or radical initiation) post-construction, the encapsulate will also be exposed to such oxidation which could be highly detrimental, especially for protein vaccines. Thus, the loading of liposomes after this step would ensure optimal protection for the protein.

Figure 1.12 shows the main steps in the production of Dehydration-Rehydration Vesicles (DRVs) (Kirby and Gregoriadis, 1984; Gregoriadis *et al.*, 1999) and Freeze-Dried Empty Liposomes (FDELs) (Yachi *et al.*, 1996 and Kikuchi *et al.*, 1999). In both cases the liposomes were first prepared without addition of the drug. The next step used the observations from section 1.5.4 and the liposomes were 'freeze-thawed' with an intermediate step of drying between the 'freezing' and the rehydration (thawing). Thus the liposome membranes were first ruptured due to the ice crystal formation during the freezing stage (Castile *et al.*, 1999) and then preserved in such a state due to the sublimation of the water (ice) in the lyophiliser not allowing for rehydration of membranes (and reformation of 'closed' liposomes) at this stage. When required, the liposomes are rehydrated with water so that the vesicles reform closed spherical structures.

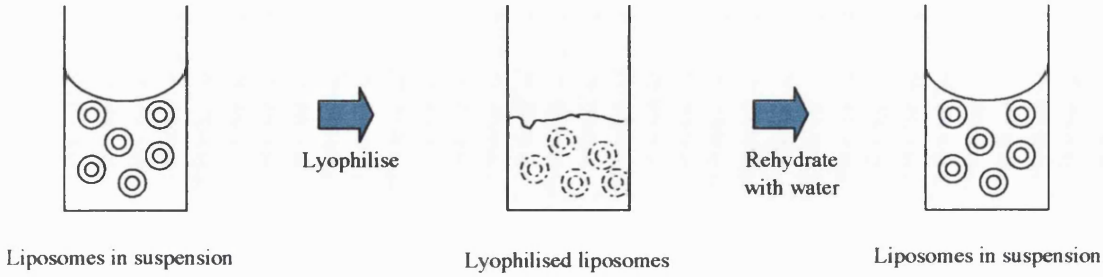

Figure 1.12: Basic steps of post-production drug/antigen encapsulation into liposomes using either DRV or FDEL method

The main difference between DRVs and FDELs is that DRVs are produced without the use of a cryoprotectant in the lyophilisation stage whereas FDELs are produced using a cryoprotectant such as sucrose. The DRV method has been shown to encapsulate a higher percentage of protein when the protein is added before the freezing stage and when the liposomes are of the SUV type. FDELs however, were only produced from MLVs and when the drug is added with the re-hydration solution only. The encapsulation of DRVs has been shown to be greater with incremental rehydration yet the FDELs were rehydrated with one instant step only. Both methods ensure that the temperature of the rehydration solution is higher than that of the phase transition temperature of the membrane phospholipids.

The loading of polymerised liposomes by either the DRV or FDEL method has not been reported and a modified technique would make such a procedure possible. However, polymerised liposomes should theoretically possess more rigid bi-layers which are less likely to be (reversibly) disrupted by the freezing process. Juliano and co-workers (Juliano *et al.*, 1983) photopolymerised a di-polymerisable PC analogue and showed that there was no size change on freezing and thawing (unlike non-polymerised liposomes) and that vesicles were not subject to aggregation or fusion. If lyophilisation of polymerised liposomes does not result in a change in liposomal structure, then it may not be possible to load protein into liposomes using a modified DRV/FDEL method, as without bi-layer membrane disruption, there will probably be no protein encapsulation. Protein association to vesicles may be limited to surface adsorption only and this possibility can be investigated by preparing polymerised liposomes where protein could only be surface adsorbed (SAVs) for comparison purposes (figure 1.13).

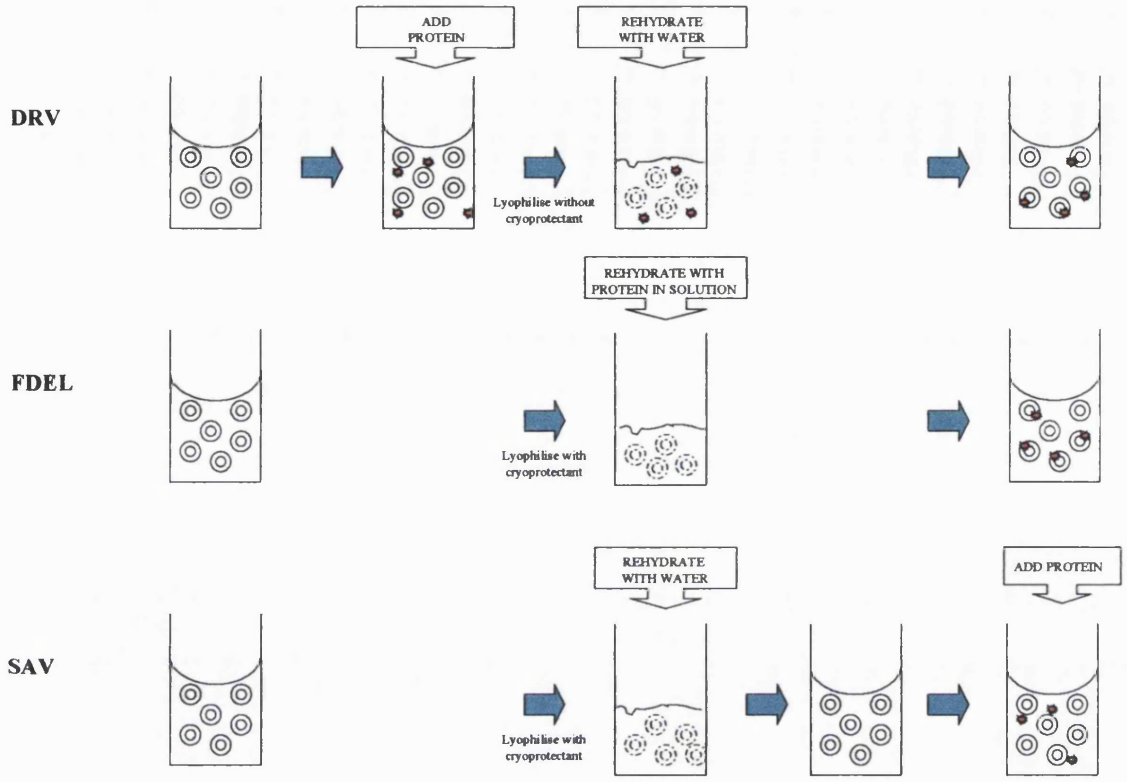

Figure 1.13: Schematic of procedures for preparation of DRVs, FDELs and SAVs

1.6 Polymerised liposomes as an alternative to conventional monomeric liposomes for vaccine delivery: increased stability

Common problems associated with the use of conventional liposomes include aggregation and fusion of phospholipid bi-layers on storage (in suspension) and loss of encapsulate due to permeability of membranes. Polymerised liposomes have been projected as more robust carriers for proteins/macromolecules than non-polymerised liposomes and can be manufactured with the polymeric backbone running either through the centre of the phospholipid bi-layer, through the phospholipid chains themselves, through the polar head groups, across a bi-layer from one monolayer to the other or concentrically outside of the bi-layer externally and internally without joining to the phospholipid or head group at all (figure 1.14) (reviewed by Regen, *et al.*, 1984 and Regen, 1987). Thus, the formation of these covalent bonds between phospholipids increases their stability in the biological environment (table 1.5).

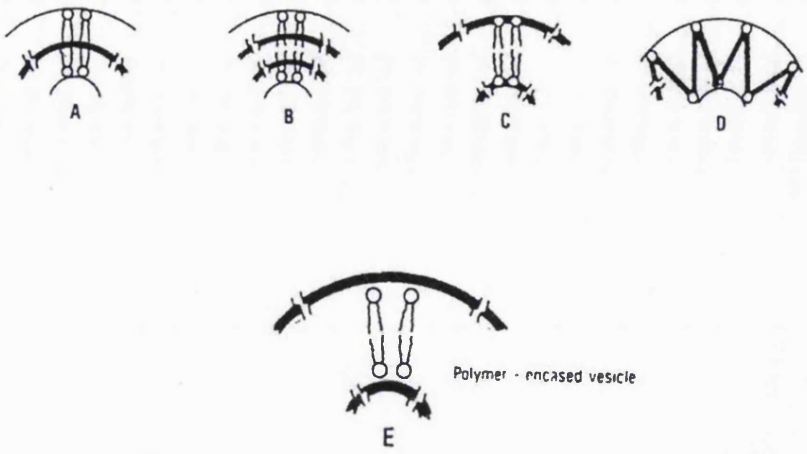

Figure 1.14: Diagrammatic representation of possible polymerisations in a typical bi-layered liposome (reproduced from Regen *et al.* 1984). Polymeric backbone may run through the centre of the phospholipid bi-layer (A), through the phospholipid chains themselves (B), through the polar head groups (C), across a bi-layer from one monolayer to the other (D) or concentrically outside of the bi-layer externally and internally without joining to the lipid or head group at all (E)

Several groups have synthesised polymerisable phospholipids for the construction of liposomes. Polymerisable lipids such as diacetylenic, methacrylate and conjugated diene phospholipids have been investigated and the resultant polymerised liposomes have been shown to possess increased stability than their non-polymerised counterparts (table 1.5). Reviews on the synthesis of various polymerisable phospholipids have been published by several authors (O'Brien *et al.*, 1985; Hayward *et al.*, 1985; Regen, 1987; and Freeman and Chapman, 1988).

There are two main methods for the preparation of polymerised vesicles; the first being the assembly of polymerisable phospholipid monomers into liposomes before polymerisation (known as polymerised liposomes) using UV irradiation or radical initiators (e.g. using azobisisobutyronitrile) and the second being the preparation of polymerised phospholipid chains first followed by subsequent assembly into liposomes (known as polymeric liposomes). Polymerised liposomes, unlike polymeric liposomes, have the advantage of being able to 'cross-link' with more adjacent phospholipid molecules (and therefore form higher molecular weight polymers), which has been shown to be associated with an increase in liposomal stability (reviewed by Regen, 1987). However, a disadvantage of polymerised liposomes is that polymerised phospholipids cannot be purified, as they are already part of the liposomal structure

unlike the polymerised phospholipids in polymeric vesicles, which can be purified before liposome assembly. A second disadvantage in polymerisation of the assembled liposome is that the process (UV irradiation or radical initiation) may be damaging to liposomally entrapped material. A further obstacle includes the reproducibility of bi-layer polymerisation.

An important consideration in contemplating the use of polymerised liposomes for delivery of drugs/vaccines to humans is that the phospholipid polymers must be biodegradable (studies reviewed by Bailey and Zhou, 1991). Other concerns are thrombogenicity of polymerised liposomes and harmful effects to human cells by free radicals (used in the polymerisation of liposomes) not removed or quenched before formulation administration (reviewed by Freeman and Chapman, 1988).

A study published by Okada and co-workers (Okada and Langer, 1995) demonstrated that polymerisation of 1,2-di (2,4-octadecadienoyl) phosphatidylcholine (DODPC), by radical initiation, did not result in leakage of encapsulated material (sucrose or BSA) and suggested that the structure of liposomes remained unchanged by the polymerisation process. Following the incubation of polymerised liposomes in gastrointestinal fluids *in vitro* approximately only 25 % of encapsulated material (sucrose or BSA) was released over two days and a higher stability was confirmed compared to regular liposomes (PC/Cholesterol). Chen and co-workers (Chen *et al.* (1996b) showed that following oral delivery to female BALB/c mice, 2.7 % of the dose of polymerised liposomes (composed of DODPC) was taken up from the gastrointestinal tract, the majority of which was thought to be *via* the Peyer's patches and was detected in the spleen, liver, kidney, heart, lung, blood, Peyer's patches and other tissues. The authors also showed that polymerised liposomes were degraded inside macrophages *in vitro* (using macrophages cultured from murine reticulum sarcoma cells) to release encapsulated calcein. The detection of polymerised liposomes in widespread tissues following oral delivery may possibly be due to the migration of macrophage-captured antigen from the intestinal lumen and from the basolateral side of M cells of the Peyer's patches to the systemic circulation. When polymerised DODPC liposomes were coated with UEA-1 (Chen *et al.*, 1996a), the coated liposomes were taken up from the gastrointestinal tract and into the spleen, liver, kidney, heart, lung, blood, Peyer's patches and other tissues to a higher extent (10.5 %) than non-coated polymerised liposomes (~3.2 %) following oral delivery to female BALB/c mice.

Table 1.5: Summary of polymerised liposomal stability studies

Polymerisable moiety	Polymerisation method	Main points	Reference
Conjugated diacetylene	UV irradiation (254 nm)	Polymerised liposomes do not precipitate in 50 $\%^{v}/_{v}$ ethanol and are stable when prepared for scanning electron microscopy unlike non-polymerised liposomes	Hub *et al.* (1980)
	UV irradiation (254 nm)	Polymerised liposomes are less permeable to glycerol than non-polymerised liposomes	Leaver *et al.* (1983)
	UV irradiation (254 nm)	BSA release at pH 2 and glucose-6-phosphate release in human saliva is lower for polymerised liposomes than non-polymerised liposomes Polymerised liposomes are not resistant to bile or pancreatin	Alonso-Romanowski *et al.* (2003)
Methacrylate	UV irradiation (254 nm)	Polymerised liposomes release less entrapped sucrose in water, salt soln., 20 $\%^{v}/_{v}$ ethanol, under sonication and when heated to 50 °C than non-polymerised liposomes	Regen *et al.* (1982)
	UV irradiation (254 nm)	Sonication displaces both hydrophilic and hydrophobic markers in non-polymerised liposomes but not in polymerised liposomes Solubilisation by ethanol is resisted by polymerised vesicles Hydrophilic markers are released from all liposomes on addition of sodium dodecyl sulphate, Brij 56 or deoxycholate but hydrophobic markers are not released from polymerised liposomes. Triton X-100 and Triton X-45 extract hydrophobic markers from all liposomes	Juliano *et al.* (1984)
	UV irradiation (254 nm)	Polymerisation is not of phospholipids but of an internal and external mono-layer. Polymerised vesicles released less sucrose after 24 h dialysis with water or 1 h dialysis with 23 $\%^{v}/_{v}$ ethanol	Regen *et al.* (1984)
	UV irradiation (254 nm)	Polymerised liposomes size distribution remains constant and entrapped sucrose is not released during freeze-drying. Sodium dodecyl sulphate addition does not affect polymerised liposomes but triggers the release of sucrose from non-polymerised liposomes	Regen *et al.* (1983)

Table 1.5 (cntd): Summary of polymerised liposomal stability studies

Polymerisable moiety	Polymerisation method	Main points	Reference
Conjugated diene	Free radical initiation using AIBN or AAPD UV irradiation (254 nm)	UV polymerised liposomes were solubilised in organic solvents whereas radical initiated polymerised liposomes were resistant Dual polymerisation using AAPD and AIBN resulted in the least leakage of carboxyfluorescein	Ohno *et al.* (1987)
	Free radical initiation using AIBN or AAPD UV irradiation (254 nm)	Partial polymerisation of liposomes using AIBN resulted in a higher leakage of carboxyfluorescein than liposomes subsequently 'fully' polymerised with AAPD or UV light	Takeoka *et al.* (1990)
	Free radical initiation using AIBN and AAPD	Polymerised liposomes released sucrose and BSA slower than regular liposomes in simulated intestinal fluid (sodium taurocholate and phospholipse A_2) and pH2	Okada *et al.* (1995)
	$Na_2S_2O_5$ and $K_2S_2O_8$ radical initiation	Non-polymerised liposomes entrapping BSA display kinetics resembling breakdown of liposomes in the small intestine *in vivo* whereas polymerised liposomes seem to stay intact	Chen *et al.* (1996)
	UV light and water-soluble photo initiator	Polymerised DODPC and DODPC/cholesterol liposomes encapsulating rhodamine-dextran (3 kDa, 10 kDa, 70 kDa) were no more stable than non-polymerised liposomes in simulated intestinal fluids. Size of polymerised vesicles was maintained unlike non-polymerised liposomes.	Jang *et al.* (2002)

Table 1.5 (cntd): Summary of polymerised liposomal stability studies

Polymerisable moiety	Polymerisation method	Main points	Reference
Other	Free radical initiation using AAPH[†]	The cationic polymerised liposome decreased rat hepatocyte and Hep G_2 cell toxicity compared to non-polymerised liposomes, DOTAP[‡] and lipofectamine Polymerised liposomes are more stable in serum and polymerised liposomes with cholesterol exhibit comparable transfection of plasmid DNA encoding luciferase in Hep G_2 and Alexander cells as DOTAP-cholesterol and lipofectamine using pGL_3-control vector	Wu *et al.* (2001)

[†] AAPH: 2,2'-azobis(2-methylpropionamidine) dihydrochloride
‡ *N*-(2,3-(Dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammonium chloride
* AIBN: azobis(isobutyronitrile) (water insoluble)
** AAPD: azobis(2-amidinopropane) dihydrochloride (water soluble)

1.6.1 Principles of photo-polymerisation

Light (electromagnetic radiation) exhibits both wave-like and particle-like properties. The electric and magnetic fields oscillate in perpendicular fields to each other and to the direction of propagation. Light (except for that emitted from a laser source) is emitted as a result of electron transitions within the individual atoms of the source. At the beginning of the 20th century Planck had explained some of his work with his assumption that radiation could behave as quanta of energy (photons) with defined and indivisible energy (directly proportional to the frequency of the wave and indirectly proportional to the wavelength of the wave). Einstein extended this concept further by showing that these photons once emitted continued to exist as individual 'packets' of energy. Einstein proposed (in his theory of the photoelectric effect) that when one photon and one electron collide that only two effects may occur: that the energy be reflected with no energy loss or that complete transfer to the electron occurs. By increasing the intensity of the light, the number of photons are increased and hence the chance of photon-electron collisions is increased.

Molecules contain energy in rotational, vibrational and electronic forms (Wayne and Wayne, 1996). Electronic energy changes occur when an electron moves between the different orbitals available to it. The type of reaction is dependent on the amount of energy absorbed, which is dependent on the wavelength of the light. Low energy infrared light mostly results in rotational and vibrational effects whereas ultraviolet light possesses a larger amount of energy that may result in electron excitation. The electron can be moved to either molecular bonding, non-bonding or anti-bonding orbitals. The route that an 'excited' electron takes is dependent critically on the absorbed energy, and these could include dissociation, reaction with another molecule, isomerisation *etc.*

Bearing the above in mind it is important to identify the exact reaction desired and then to identify the wavelength of light required to achieve this. It is important to use monochromatic light (or to filter out non-required waves) so that unwanted reactions do not occur and to use a high intensity light source to increase the rate of the reaction.

1.6.2 DODPC as a phospholipid for polymerised liposomes

DODPC is a phosphatidylcholine derivative possessing a conjugated diene group in each hydrophobic tail (figure 1.15). The diene groups in DODPC align parallel to each other (Binder and Kohlstrunk, 1999) even though they are in the same positions in the acyl chains, and the tilting of one of the acyl chains (chain number 2) when phospholipids are arranged into bi-layer membranes should misalign them. Ohno and co-workers (Ohno *et al.*, 1987) however, discussed that the diene groups in each acyl chain of the phospholipid were not aligned and therefore intramolecular polymerisation of the phospholipid (between the two acyl chains of one molecule) was not possible when the phospholipids are in a bi-layer membrane. Gaub and colleagues (Gaub *et al.*, 1984) state that molecules containing 4 diene groups (like DODPC) will form polymers with 4 neighbouring phospholipids resulting in a 2-D network as long as intermolecular bonding does not occur. Thus, the exact positioning of the diene bonds in DODPC in respect to each other remains unclear but will have an implication for the formation of both intramolecular and intermolecular phospholipid bonds.

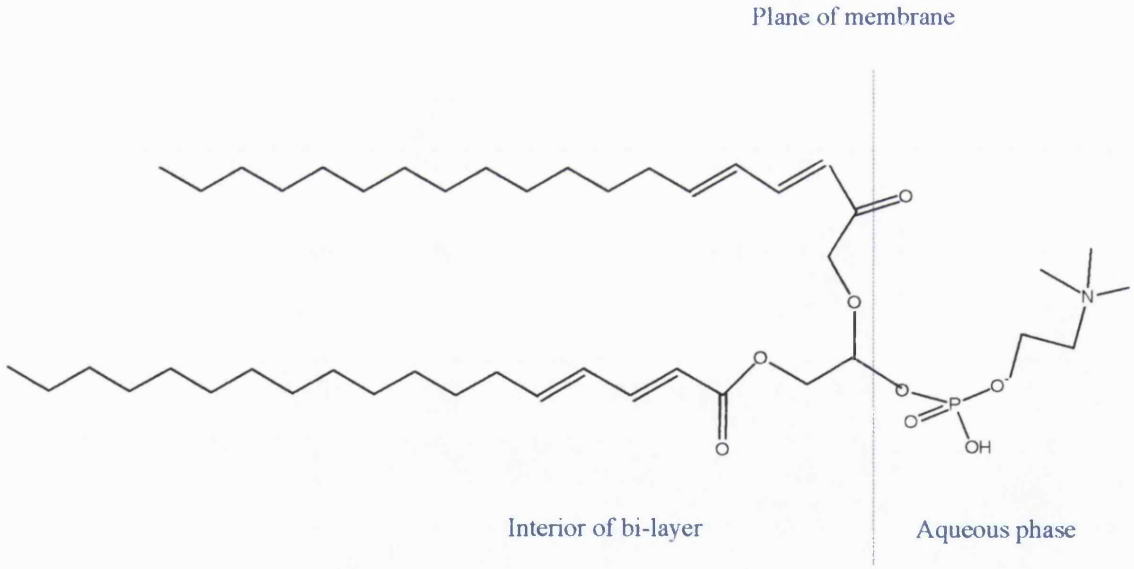

Figure 1.15: Chemical structure of DODPC (adapted from Binder and Kohlstrunk (1999); Ohno *et al.* (1987)). Irradiating DODPC with light at 254 nm will promote a photocyclisation reaction. This is a form of a photoaddition reaction where two σ-bonds are created from two π-bonds (from conjugated dienes) and should result in a more robust liposomal bi-layer with a 2-D polymerisation effect.

The polymerisation of DODPC SUVs using UV light or radical initiation was investigated by Ohno *et al.* (1987). They showed that polymeristion using UV light resulted in complete polymerisation after 3 h whereas the use of the radical initiators AIBN (water insoluble) or AAPD (water soluble) resulted in under 60 % polymerisation after 3 h and 12 h. UV polymerisation was carried out at 20 °C and radical polymerisation at 60 °C and the extent of polymerisation due to temperature alone (60 °C) was only 5-10 % after 12 h. The addition of AAPD to liposomes previously polymerised with AIBN led to a further increase in the extent of polymerisation suggesting that the two initiators polymerise diene groups which are in different areas of the bi-layer. As the authors proposed that the diene groups in each chain were not aligned (even though both are at the same position in the acyl chains) due to acyl chain tilting, it therefore followed that the diene group in acyl chain 2 may be more subjected to the aqueous phase and so prone to the effects of AAPD and the diene group in acyl chain 1 more prone to AIBN as it is located deeper into the hydrophobic region. Hence, the use of both initiators (AAPD and AIBN) was shown to lead to a higher extent of polymerisation.

To elucidate if there was a difference in polymerised liposomes prepared using either UV polymerisation or radical polymerisation, Ohno and co-workers (Ohno *et al.*, 1987) conducted stability studies investigating liposomal solubility in organic solvents such as methanol, ethanol and chloroform. UV Polymerised DODPC liposomes were found to be soluble in organic solvents and possessed an average molecular weight of 5,200 indicating that polymerisation resulted only in oligomers (not polymers) of DODPC. Radical polymerised liposomes (using AIBN, AAPD or both) however, were not soluble in the same solvents and it was suggested that they possessed a higher molecular weight than UV polymerised liposomes (i.e. an indication of polymer formation). The authors also investigated the release of carboxyfluorescein from UV and radical polymerised DODPC liposomes at pH 8.6 at 50 °C. Radical polymerised vesicles (using AIBN, AAPD or both) retained more carboxyfluorescein than UV polymerised liposomes at all time points and the concurrent use of both AIBN and AAPD resulted in the highest retention for encapsulated carboxyfluorescein compared to all other formulations. Light scattering was used to determine whether liposomes remained intact following the addition of Triton X-100 to the formulations. UV polymerised liposomes and monomeric liposomes both displayed a decrease in light scattering whereas the radical polymerised liposomes were resistant to Triton X-100. Thus,

though the extent of diene bond conversion was faster for UV polymerised liposomes than radical initiation, there is a clear difference in stability of the resultant polymerised liposome. Radical initiated polymerisation probably leads to longer polymer chains with a greater degree of cross-linking than UV polymerisation. The light scattering results were confirmed by studies by Okada and co-worker (Okada *et al.*, 1995) where it was shown that unlike liposomes polymerised by either radical alone, DODPC liposomes polymerised using both AAPD and AIBN displayed no light scattering change on Triton X-100 addition implying that such liposomes are more stable.

This effect of 'partial' polymerisation when using either AIBN or AAPD (but not both together) was used for the preparation of part-polymerised liposomes, which were loaded before completion of the polymerisation (Takeoka *et al.*, 1990). Initially the DODPC liposomes were polymerised using AIBN and then incubated with carboxyfluorescein at 60 °C for 100 h to allow for internal capture. The liposomes were then polymerised further by UV exposure or using AAPD. The further polymerisation was shown to decrease carboxyfluorescein leakage at 50 °C compared to partially polymerised liposomes and the decreased exposure of the encapsulate to polymerisation was proposed to cause less damage to it.

1.6.3 Polymerised liposomes used in vaccines

Though many studies have been reported proposing polymerised liposomes as more robust carriers for protein antigens, especially for the oral delivery of vaccines, few immunisation studies to date have been conducted (table 1.6). Thus, the potential of polymerised liposomes in vaccine delivery remains to be extensively investigated especially with respect to mucosal delivery.

Table 1.6: Summary of vaccine studies to date using polymerised liposomes

Lipid	Protein/Route/Animal	Outcome	Reference
Glycerophospholipid derivative with a dithiolane ring of lipoic acid	Lysozyme/Intraperitoneal/Mice	Polymerised liposomes elicit a higher anti-lysozyme IgG than conventional liposomes	Jeong *et al.* (2002)
DODPC	Diphtheria Toxoid/Oral/Mice	Polymerised formulation with encapsulated antigen evokes a higher IgG response than polymerised formulation mixed with free antigen and polymerised liposomes	Chen and Langer (1998)

1.7 Aims

The overall aim of this thesis was to design and characterise a polymerised liposomal formulation suitable for the intranasal delivery of a model protein antigen and to subsequently evaluate the existence of adjuvanticity by determining the immune response *in vivo*. An important consideration in the formulation of particulate vaccines for delivery to mucosal tissues is the effect of particle size on adjuvanticity and in this regard it was decided to investigate submicron particles with two particle size distributions. Another key factor affecting the immune response elicited is the way in which the protein antigen is associated to the particulate carrier and so it was planned to study the difference in adjuvanticity when proteins were surface adsorbed as opposed to being incorporated into the carrier.

In order to evaluate the effectiveness of polymerised liposomal vaccines after intranasal delivery, it was proposed to compare the immune responses observed to responses seen after administration by the more conventional intramuscular route. It was also hypothesised that the incorporation of adjuvants such as poloxamers and chitosan into polymerised liposomal formulations would further increase both systemic and mucosal immune responses after intranasal administration. A further goal of the project was to evaluate if the incorporation of specific adjuvants (liposomes, polymerised liposomes, poloxamers or chitosan) activated the cell-mediated branch of immunity, which is necessary to provide protection against intracellular pathogens such as HIV, tuberculosis and malaria.

2.0 Preparation of polymerised liposomes for protein antigen delivery

2.1 Introduction

Liposomal formulation of antigenic proteins has been shown to achieve a superior immune response than when the protein is presented alone in solution (first reported by Allison and Gregoriadis, 1974). Chen and Langer (1998) further enhanced the adjuvant properties of liposomes with their experiments using polymerised liposomes to deliver diphtheria toxoid orally (see section 1.6.3).

Important considerations in the preparation of a liposomal carrier include vesicle size and size distribution, surface charge, lamellarity, membrane stability, internal aqueous volume, drug loading and localisation. Once desirable loading has been achieved, the drug must be effectively retained with the vesicle throughout storage and until the end target is reached following administration to the body.

In the case where protein antigens are to be encapsulated/adsorbed to liposomes, the integrity of the epitopes (discrete immunologically active sites on the antigen recognised by either secreted antibody or antigen-specific membrane receptors on B and T cells) need to be preserved throughout the entire formulation process. The epitopes may be particularly vulnerable to the polymerisation stage when preparing polymerised liposomes and it was our aim to address this issue by post-production addition of the antigen using a modified DRV technique (Kirby and Gregoriadis, 1984) and FDEL method (Yachi *et al.*, 1996; Kikuchi *et al.*, 1999) (section 1.5.5).

The aim of this study was to prepare polymerised vesicles of a size appropriate to mucosal immunisation according to the findings of Tomizawa and co-workers (Tomizawa *et al.*, 1993) and Eldridge and co-workers (Eldridge *et al.*, 1990) and simultaneously optimise the encapsulation efficiency of protein antigens by DRV/FDEL method while preserving the biological activity i.e. antigenicity throughout.

2.2 Materials

2.2.1 Liposome preparation

Soya phospatidylcholine (SPC) (mwt ~ 786) and soya phosphatidylglycerol (SPG) (mwt ~ 797) were a gift from Lipoid GmbH (Ludwigshafen, Germany) (Lipoid S 100 and Lipoid S PG respectively). The polymerisable phospholipid 1,2-bis[(2E,4E)-octadecadienoyl]-*sn*-glycero-3-phosphocholin (DODPC) was manufactured by NOF corporation (Tokyo, Japan) and was a gift from DOR BioPharma, Inc. (Lake Forest, USA).

The LiposoFast 50 Extruder was purchased from Avestin Inc. (Ottawa, Canada), Isopore™ membrane filters for extrusion and woven mesh spacers (pre-filters used to support the membrane) were purchased from Millipore (Watford, UK) and Cyclopore™ Track Etched membranes from Whatman International Ltd (Maidstone, Kent).

The Edwards Micro-Modulyo and Edwards E2M5 high vacuum pump, purchased from Fred Baker Scientific (Runcorn, U.K.) was used for lyophilisation and glass freeze-drying vials (20 mL) and 20 mm stoppers were obtained from Fisher Scientific (Loughborough, U.K.). D(+)Trehalose (α-D-Glucopyranosyl α-D-glucopyranoside) dihydrate (from *Saccharomyces cerevisiae*) was used as a cryoprotectant during lyophilisation and was purchased from Sigma-Aldrich Co. (Poole, England).

2.2.2 Model protein antigens

Bovine serum albumin (BSA) was purchased from Sigma-Aldrich Company Ltd (Poole, UK). Tetanus toxoid (TT) was a gift from Aventis Pasteur S.A. (Marcy L'Étolie, France).

2.2.3 Radioiodination of proteins

^{125}I (as the sodium salt) and PD-10 Desalting column (containing Sephadex™ G-25 medium) were purchased from Amersham Biosciences (Little Chalfont, UK). IODO-BEADS® and Slide-A-Lyzer® dialysis cassettes were aquired from Perbio Science UK

Ltd. (Tattenhall, UK). Gamma-emitted radiation was detected using the Perkin Elmer Cobra II Auto-Gamma Counting System (model 5002, type I1) (Boston, U.S.A.).

2.2.4 Solutions

Phosphate-buffered saline (PBS) (pH 7.4) (quantities less than 1000 mL) was freshly prepared by dissolving one PBS tablet (Sigma-Aldrich Co. (Poole, England)) into 200 mL deionised water. Quantities greater than 1000 mL were freshly prepared as *per* the British Pharmacopoeia formula (2002) by dissolving 2.38 g of disodium hydrogen orthophosphate, 0.19 g of potassium dihydrogen orthophosphate and 8.0 g of sodium chloride in sufficient water to produce 1000 mL of buffer.

Sodium phosphate buffer (SPB) (0.1 M, pH 6.6) was freshly prepared by dissolving 0.52 g disodium hydrogen orthophosphate and 0.16 g of sodium dihydrogen orthophosphate in 100 mL of deionised water (Gomori, G., 1955).

Water used in all experiments was deionised and was further purified by passing through an Elgastat Option 3 Water purification unit (ELGA, High Wycombe, England). The pH varied from 5.1-5.6.

2.3 Methods

2.3.1 Procedure for liposome preparation

Various techniques have been used for the preparation of liposomes (section 1.5.2). Procedures such as and related to the classical thin film method are in essence composed of three main stages:

- Phospholipid solvation and subsequent drying onto a surface
- Phospholipid hydration to produce liposomes
- Modification of liposome lamellarity and size distribution

2.3.1.1 Procedure for the preparation of liposomes by the 'hand-shaking' thin film method

In early studies using SPC as the liposome component, the conventional 'hand-shaking' thin film method for the production of multi-lamellar liposomes was employed. Briefly, 20-100 mg total phospholipids (SPC containing 10 $\%^{m}/_{m}$ SPG where added) was dissolved in chloroform. Methanol was added to this solution so that the final ratio of chloroform: methanol was 2:1 by volume, resulting in an end concentration of 4 mg/ mL (mg phospholipid/ mg solvent). This mixture was 'swirled' by hand to ensure all phospholipid had dissolved and the solvent then removed using a rotary evaporator (using a Büchi waterbath B-480 and Rotavapor R-114 (Flawil, Switzerland) attached to a KNF Neuberger laboport vacuum pump (Freiburg-Munzingen, Germany)). The lipidic mixture was attached to the rotary evaporator using a round-bottomed flask (1mL solvent to 50 mL flask volume) and dried at 50 °C (using a water bath) for 30 min. The flask was further dried by attachment to the manifold of an Edwards Micro-Modulyo under vacuum drawn by an Edwards E2M5 high vacuum pump for 1 h. The next step aimed to rehydrate the finely dispersed phospholipid to form multi-lamellar liposomes. Water was added to the flask so that the end concentration of phospholipid in water was 5-10 mg/mL and the flask 'hand-shaken' for 1 min before revolving for 30 min in a 50 °C water bath using the rotary evaporator apparatus to allow further 'peeling' of phospholipids from the walls of the glass vessel to form liposomes. The flask was then vortexed for 1 min and left for 2 h at room temperature to ensure full 'swelling' of liposomes. The multi-lamellar liposomes thus formed were modified using a LiposoFast 50 extruder with filter membranes (of 0.1 μm, 0.2 μm, 0.6 μm, and 1.0 μm) and freeze-thawed (section 2.3.2) until the required size was obtained.

2.3.1.2 Procedure for the preparation of liposomes by a freeze-dried phospholipid hydration method

In the studies when DODPC was used as the liposome component, a different method for liposome preparation needed to be employed. This is because DODPC did not form a uniform and thin phospholipid film in a round-bottomed flask and so was not in the finely dispersed manner required for efficient phospholipid hydration and subsequent bi-layer formation. Therefore, the solvent containing the phospholipid was lyophilised and the dry phospholipid hydrated with water to form liposomes. Briefly, DODPC (20

mg-600 mg) was added to pre-warmed (50 °C) *t*-butanol (4 mg phospholipid/mL) and stirred until dissolved using a magnetic stirrer bead and the IKA magnetic stirrer RO 15 power® (Staufen, Germany) (360 rpm). Aliquots of 5 mL (containing 20 mg of DODPC) were added to 20 mL clear freeze-drying vials and sealed using 20 mm freeze-drying vial stoppers and frozen in liquid nitrogen (<195.79 °C, 1 min). The stoppers were then removed and the vials covered with Parafilm® M film, perforated with 12 needle width holes and lyophilised for 48 h using the Edwards Micro-Modulyo under vacuum drawn by an Edwards E2M5 high vacuum pump. The resulting lyophilised product was then hydrated with water to 10 mg/mL (mg phospholipid/ mL water) and stirred at 360 rpm for 2 h at room temperature using a magnetic stirrer plate and bead as above. The liposomes thus formed were modified using a LiposoFast 50 extruder with filter membranes (with pore diameters of 0.1 μm, 0.2 μm, 0.6 μm, and 1.0 μm) and freeze-thawed (section 2.3.2) until the required mean size and size distribution was obtained and polymerised using UV light (section 3.4).

In the final stage, liposomes were lyophilised by first freezing in liquid nitrogen (<195.79 °C, 1 min) and then attaching to the manifold of an Edwards Micro-Modulyo drier, under vacuum drawn by an Edwards E2M5 high vacuum pump for 48 h. The lyophilised product was stored in a desiccator at 0 % relative humidity (using silica gel) at 2-8 °C until required. The lyophilised vesicles were rehydrated in one of two ways; either by instant rehydration at room temperature and then rocked at 20 rpm for 1 h using the Stuart Scientific Platform Rocker STR6 (Redhill, U.K.) or rehydrated in a controlled manner (Gregoriadis *et al.*, 1999). The latter method involved the incremental addition of water; 100 μL twice and 0.8 mL the third time with 30 min incubations (37°C, 100 rpm) between each hydration (using the Sanyo Gallenkamp IOX400.XX2.C incubator (Loughborough, U.K.)).

2.3.2 Procedure for the preparation of liposomes with a well-defined particle size distribution

2.3.2.1 Extrusion through polycarbonate filter membranes in order to define upper limit for liposome diameter

The size distribution of liposomes can be 'capped' by passing the vesicle suspension through an extruder (figure 2.1) containing double-stacked polycarbonate filter membranes (with pore diameters ranging from 0.1-1 μm), which thus reduces the diameter of liposomes at the larger end of the size distribution.

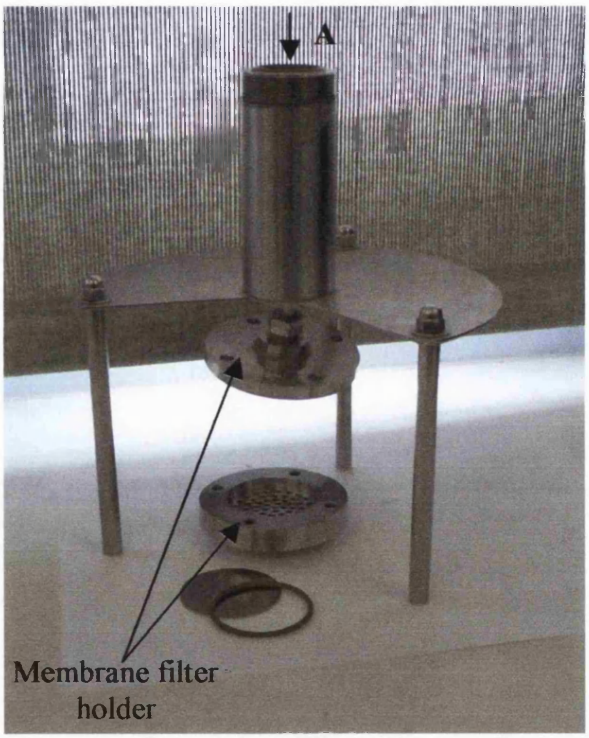

Figure 2.1 Photograph of the LiposoFast 50 extruder used for the preparation of liposomes with a well-defined particle size distribution. The liposome suspension was introduced into the extruder at point A, which was subsequently sealed by connecting to the outlet of a nitrogen cylinder. Polycarbonate filter membranes (and steel drain disc supports) had previously been placed into the base of the filter holder and this secured (using pictured O-ring) to the top cap of the filter holder using screws. A maximum pressure of 8 bars was used to collect extruded liposomes from the outlet on the underside of the base of the filter holder.

The liposomes were driven through the membranes under force created by compressed oxygen-free nitrogen gas (BOC Gases (Guildford, U.K.)). Liposomes larger than the

membrane pore diameter are ruptured as they pass through and then reseal or form smaller liposomes from fragments. After repeated passage through the extruder, larger multi-lamellar liposomes (as well as larger uni-lamellar liposomes) generally become oligo- /uni-lamellar liposomes with a diameter about the same as the filter membrane pore diameter. Sample loss during extrusion is minimised by prior passage of water through the extruder to wet surfaces and membranes. Also, where possible, large sample volumes are used to minimise the effect of the loss of phospholipid due to membrane and surface retention on the overall sample concentration. The volume of sample before and after extrusion is measured using a measuring cylinder to ensure sample loss has not occurred.

2.3.2.2 Freeze-thawing in order to increase liposomal diameter and convert liposomal structure from multi-lamellar to oligo- /uni-lamellar

The liposomal mean size, size distribution and lamellarity can be altered due to cycles of freezing and thawing. The formation of ice crystals during freezing leads to liposome rupture followed by resealing during the thawing stage. This was achieved by immersing the container containing the liposomes into liquid nitrogen (3 min) followed by thawing of liposomes by container immersion into a 50 °C water bath (5 min).

2.3.3 Procedure for the radiolabelling of model proteins with ^{125}I and purification of labelled protein from free isotope using column chromatography and dialysis

Radiolabelling of proteins provides a rapid, easy and precise method for their quantification. Soluble proteins are easily iodinated using ^{125}I which attaches to external tyrosine residues on the protein (and also to histidine and tryptophan in smaller quantities) by chemical oxidation using a reagent such as IODO-BEADS® iodinating reagent. The reagent is the sodium salt of *N*-chloro-benzenesulfonamide immobilised on to non-porous, polystyrene beads.

The procedure used to radiolabel BSA and TT was as follows:

1/ The protein to be labelled (50 µg protein/bead) was dissolved in 0.4 mL (SPB) (0.1 M, pH 6.6)

2/ IODO-BEADS® were removed from stock and agitated with 0.5 mL SPB in a small glass tube. Excess SPB was removed and the bead dried by rolling on filter paper. This step removed any loose reagent or particles from the bead. The bead was then transferred to a fresh glass tube.

3/ Behind lead shielding, 18.5 MBq of $Na^{125}I$ was added to the tube and made up to 0.1 mL with SPB. The vial was gently swirled and the bead and reagent were left to react for 5 min.

4/ BSA or TT in solution (or alternative protein to be labelled) was added to the glass reaction tube, swirled and permitted to react with the isotope and reagent for 15 min.

5/ The solution was transferred to a new glass vial. The bead was washed with 0.3 mL of fresh SPB to recover any remaining protein and this washing was also transferred.

The radio-labelled protein was then separated from free radionuclide using column chromatography and dialysis:

6/ The protein/radionuclide solution was made up to 2.5 mL with SPB and free $Na^{125}I$ was separated from iodinated protein by gel filtration using a PD-10 Desalting column (equilibrated with 25 mL of SPB). PD-10 Desalting columns contain Sephadex™ G-25 Medium and can separate substances with a molecular weight > 5,000 from lower molecular weight substances (< 1,000). Discarding the first eluent, 1 mL fractions were collected thereafter. The radioactivity of the fractions was determined using the Perkin Elmer Cobra II Auto-Gamma Counting System and the fractions corresponding to the iodinated protein were pooled. Figure 2.2 is a chromatogram showing the separation of $Na^{125}I$ from BSA as detected by the elution of radioactivity in each fraction. The first peak (peak A) corresponds to the labelled protein whereas the second peak (peak B) corresponds to free nuclide.

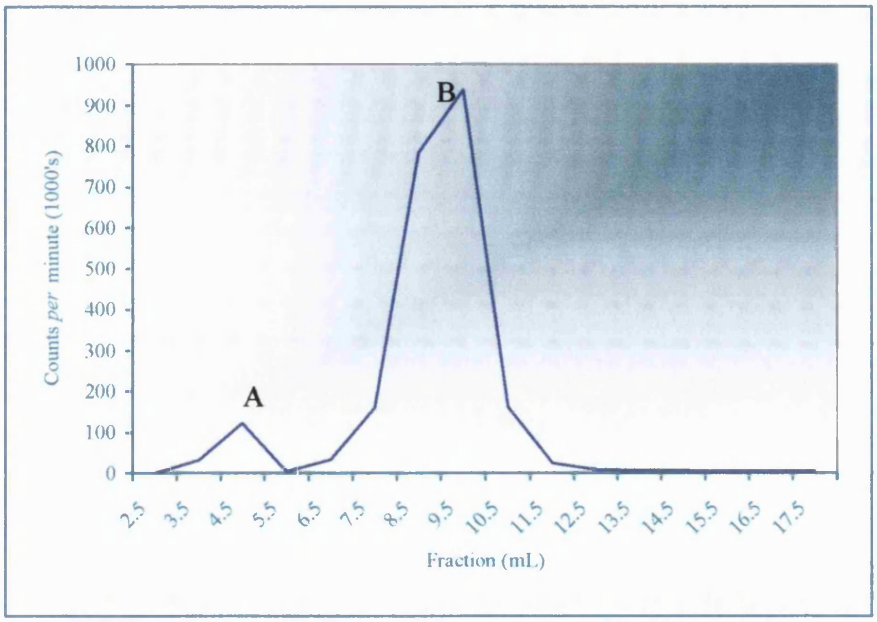

Figure 2.2 Chromatogram showing the detection of radioactivity in elution fractions following passage of non-purified iodinated BSA through gel filtration using a PD-10 desalting column. Peak A is typical of high molecular weight components such as proteins and peak B of low molecular weight components such as salts.

7/ Further purification of the pooled fractions containing radio-labelled protein was achieved by dialysing the sample (using 1 L of dialysate for each mL of sample) against PBS three times (twice, two hourly and the third time overnight). This was performed using a Slide-A-Lyzer® dialysis cassette (molecular weight cut off: 10,000, 3-15 mL capacity, 2-8 °C) and buoys being revolved in dialysate using a magnetic stirrer bead and Stuart Scientific magnetic stirrer SM1 (Redhill, U.K.). Dialysis cassettes were prepared for use by prior hydration of the membrane in PBS (30 s) before sample addition using a hypodermic needle and syringe. The sample was injected slowly through a syringe port (in the cassette) and after dialysis was removed through a second port.

The determination of the percentage of radioactivity associated to the protein in the purified sample was performed as follows:

8/ A small quantity (5 μL) of iodinated sample was added to 100 μL of BSA (10 $\%^{m}/_{v}$) (to bulk the total protein content so that following precipitation and sample centrifugation there would be a larger pellet which if disturbed decreases the loss of the radio-labelled protein from the pellet to the supernatant).

9/ Following the addition of the 'cold' protein the mixture was vortexed for 1 min. before the addition of 1.25 mL trichloroacetic acid (TCA) (20% $^{m}/_{v}$) to precipitate all protein out of solution. The mixture was left to incubated at 2-8 °C for 1 h. The precipitate was then pelleted by centrifugation (3000 g, 10 min) using a Micromax bench centrifuge (International Equipment Company, Needham Heights, U.S.A.). The radioactivity of the supernatant and the resuspended pellet (to the same volume in water) was then calculated (equation 2.1).

$$\text{Purity (\%)} = \frac{\text{Radioactivity pellet}}{\text{Radioactivity (pellet +supernatant)}} \times 100$$

Equation 2.1 Equation for the calculation of the purity of iodinated protein with regard to the presence of contaminating free radionuclide

The purity for I^{125}-BSA and I^{125}-TT was calculated to be 93.4 % and 87.6 % respectively. The purity can be increased if desired by repeating the dialysis procedure.

2.3.4 Procedure for liposome polymerisation

The polymerisation of liposomes can be effected using the Rayonet photochemical mini-reactor (RMR-600), RMR-2537 Å lamps and quartz vessels (figure 2.3) (all purchased from Southern New England Ultraviolet Co Inc. (Branford, USA)). RMR-2537 Å lamps (8) were used as they predominantly emit UV light only at 253.7 nm (see figure 2.4 for the spectral energy distribution) which was the wavelength of light required for the photo-polymerisation. Quartz vessels of 5 mL and 20 mL volume were used depending on sample volume, as glass vessels are not suitable for use with wavelengths below 360 nm due to light absorption by the glass. An aluminium 'scaffold' was constructed (inset figure 2.3) to hold four quartz vessels (containing the sample) within the photoreactor so that maximal direct exposure of sample vessels to

bulbs was possible. The mini-reactor was adapted for use in the U.K. using a BLOCK AIM 1.6/0.8 autotransformer (RS Components Ltd. (Corby, U.K.)). The photoreactor was switched on 3 min prior to sample introduction into the chamber to allow the intensity of the UV light emitted by the lamps to become stabilised.

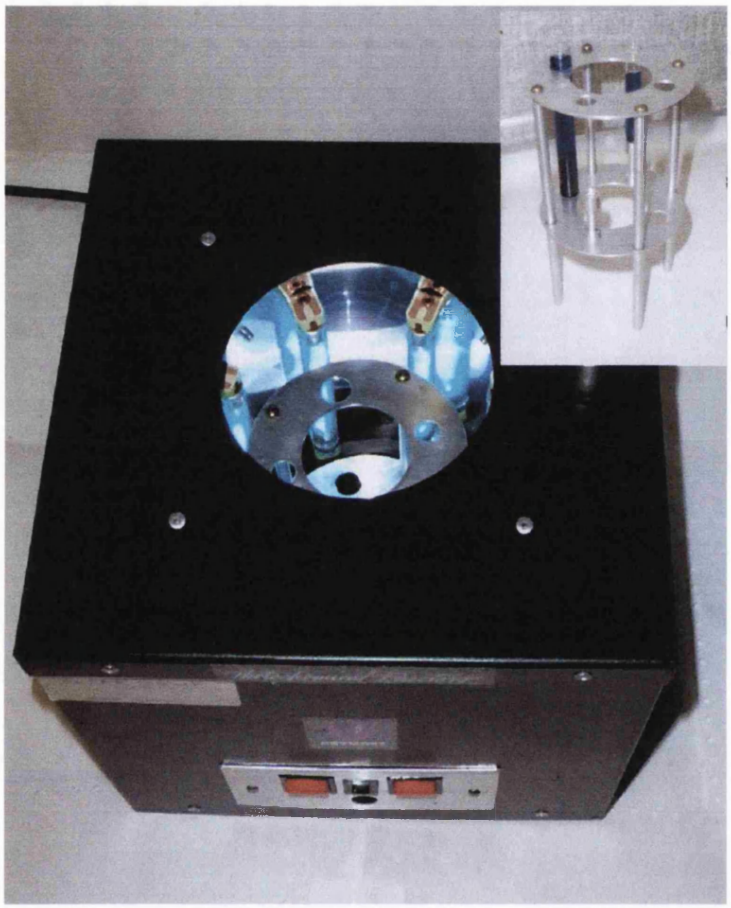

Figure 2.3 Photograph of Rayonet photochemical mini-reactor (RMR-600) housing eight RMR-2537 Å lamps used for the photo-polymerisation of DODPC liposomes. An aluminium 'scaffold' (inset picture) was constructed to hold four quartz test tubes containing liposome samples inside the photoreactor.

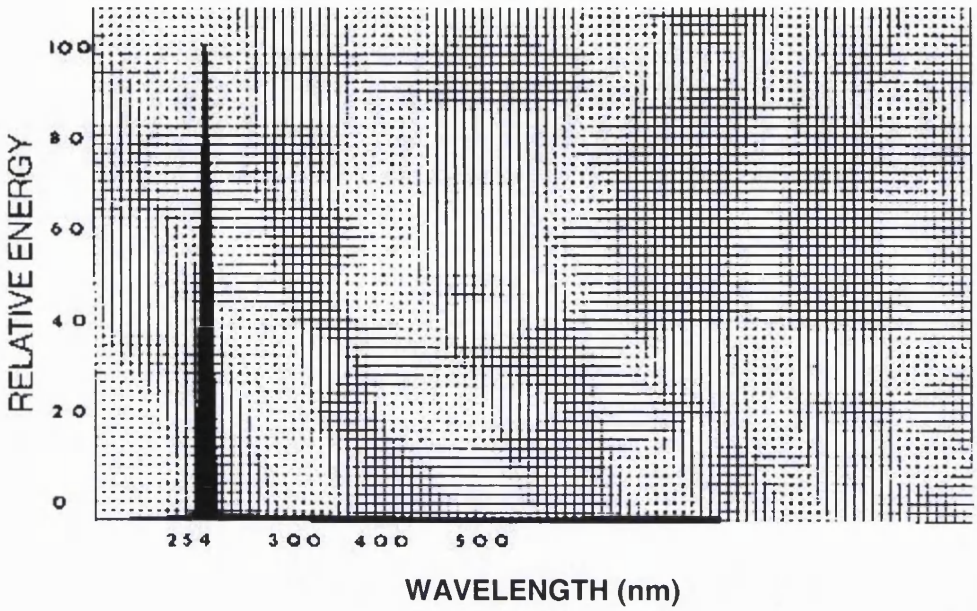

Figure 2.4: The representative spectral energy distribution for the RMR-2537 Å lamps (courtesy of Southern New England Ultraviolet Co Inc.)

2.3.5 Procedure for separation of liposome-associated protein from free protein

In the course of the studies it was necessary to separate liposomes and associated protein from non-associated 'free' protein. This step is essential for the quantification of liposomally associated protein and for the *in vitro* study of the release of protein from the liposomal formulation into release media over time. Such characterisation of the formulations is required prior to their *in vivo* administration to aid in the understanding of the observed results.

Typically, formulations are considered for administration once the free protein has been removed from the preparation so that the dose administered contains only formulation-associated protein. The protein dose in the formulations administered in the course of the *in vivo* studies presented in this thesis included both liposome-associated and free protein and so the separation of associated and free protein prior to dosing was not necessary.

The separation of free protein from liposome-associated protein was achieved by sedimentation of liposomes (with associated proteins) using centrifugation to form a re-dispersible 'pellet', leaving free protein in the supernatant. The Sorvall® Combi-Plus

ultracentrifuge and Sorvall® A-1256 rotor pre-cooled to 4 °C (Kendro Laboratory Products Ltd., Bishop's Stortford, U.K.) using 10 mL polycarbonate oak ridge bottles with ultraspeed sealing assembly (O-ring, plug and cap) (Kendro Laboratory Products Ltd., Bishop's Stortford, U.K.) were used for centrifugation of all formulations.

The structure of liposomes must not be disrupted during centrifugation as any rupture of the vesicles may cause undue release of associated protein into the supernatant. This must be balanced with the concern that if too little centrifugal force is used then liposomes of a much smaller size may not be sedimented and remain in the supernatant. Optimal centrifugal forces and time periods were investigated for all formulations and it was found that factors such as phospholipid composition, mean diameter of liposomes, and whether liposomes were polymerised or not, affected the extent of sedimentation. The following point-checks were used to ascertain that centrifugation did not result in detrimental effects on liposomes:

1 Liposome sediment easily resuspended after centrifugation (in fresh media) by centrifuge tube inversion and vortexing (1 min) without evidence of large aggregates.
2 Mean liposomal hydrodynamic diameter and sample size distribution as determined using PCS (section 2.3.6.1) remained unaffected after centrifugation and resuspension of sediment.
3 Liposomes remained visually intact (using transmission electron microscopy (section 2.3.7)) after centrifugation and resuspension of sediment.
4 The supernatant of centrifuged samples contained only a few liposomes (determined using PCS (section 2.3.6.1) and by visualisation using transmission electron microscopy (section 2.3.7).

Separation of free protein from liposomes was conducted using a centrifugational force of 150,000g for 40 min at 4 °C as this was found not to adversely affect liposomes yet sediment the majority of vesicles after investigation using the above check-points (results not shown).

2.3.6 Procedure for the determination of particle size and size distribution

2.3.6.1 Procedure for the determination of the size of submicron liposomes using photon correlation spectroscopy (PCS)

The random movement of particles in suspension (Brownian motion) is related indirectly to their size (larger particles move slower than smaller particles). This relationship between particle size and velocity is used in PCS, also known as Dynamic Light Scattering (DLS) or Quasi-Elastic Light Scattering (QELS), to determine the hydrodynamic diameter of particles.

The Malvern ZetaMaster 2000 using a 2mW laser diode (670 nm) was used for the determination of the hydrodynamic diameter of particles. In brief, a laser (Light Amplification by Stimulated Emission of Radiation) beam illuminates the sample and the intensity fluctuation of the scattered light is recorded at a fixed angle. This intensity and rate of fluctuation occur due to the Brownian motion of the particles (which is inversely related to particle size) as they enter into and out of the incident beam. The translational diffusion coefficient of the particles is thus calculated and from this the equivalent diffusional spherical hydrodynamic diameter of the particles using Stokes-Einstein equation (equation 2.2). The hydrodynamic diameter differs from the true spherical particle diameter in that it includes the electrical double layer (see section 2.3.8).

$$d = \frac{kT}{3\pi\eta D}$$

d = Hydrodynamic diameter
k = Boltzmann's constant
T = Absolute temperature
η = Viscosity of medium
D = Diffusion co-efficient of a spherical particle

Equation 2.2: Stokes-Einstein equation relating the hydrodynamic diameter of a spherical particle to it's diffusion coefficient

An indication of the breadth of the particle size distribution (the polydispersity index) is evaluated and ranges from 0 (completely monodisperse) to 1 (polydisperse). The manufacturer divides this scale for particle size distribution classification as 0-0.5 being monodisperse, 0.05-0.08 being nearly monodisperse, 0.08-0.7 as possessing mid-range polydispersity and above 0.7 as being very polydisperse. The number of particles entering the beam is also measured and is reported as KCPS (counts (in thousands) *per* second). If sample dilutions prior to analysis are kept consistent, it is possible to use the KCPS figure to give a qualitative indication of the number of particles present in the samples. All sample diameters reported in this thesis were measured in deionised water (pH 5.1-5.5) using the automatic mode for analysis.

2.3.6.2 Procedure for the determination of the size of micron-range liposomes using laser light diffraction

Incident light (e.g. from a laser source) on a particle will be both absorbed and refracted. To allow for the absorbing component, the refractive index for materials is divided into real (refractive properties) and imaginary (absorbing properties) components. In the case of particles suspended in a medium, the extent and intensity of the scattered (or diffracted) incident light is dependent on optical properties of the particle and the suspending medium (e.g. absolute refractive indices of both) and also on particle size. In general, large particles will diffract incident light at small angles and small particles diffract incident light at large angles. For particles below 25-50 μm in diameter, the Mie theory must be used to calculate the light diffracting pattern from such particles. The use of the Mie theory requires the knowledge of parameters such as particle refractive index (real and imaginary components), suspending medium refractive index and the wavelength of the incident light source. Further to this, the light diffracting pattern of the sample is analysed and compared against the trial distributions already constructed, and the trial distribution attenuated until it matches the sample's distribution from which the range of particle diameters is then known.

The Malvern Mastersizer S fitted with a 300 mm lens, 14.3 mm beam length and 15 mL sample chamber with a magnetically stirred cell and a helium-neon laser (632.8 nm, 2mW) was used to determine the size of the particles with diameters between 0.5-900 μm. The Mastersizer software version 2.18 was used for analysis of collected data. The real component of the refractive index for the particles was set as 1.5960 (characteristic

of emulsions), the imaginary component of the particles as 0.001 and the refractive index of the water (as the suspending medium) as 1.33. These optical characteristics are represented as a preset Malvern code in the machine (3PDD).

The size of the particles is quoted as the volume mean diameter, which represents the median value for particle diameter (50 % of the volume of liquid is made up of particles with lower diameters than the median value and 50 % of the volume of liquid is made up of particles with higher diameters than the median value). The distribution of particle diameters around this median value is stated as the span value (equation 2.3).

$$\text{Span} = \frac{\text{diameter}(90^{th}\ \text{percentile}) - \text{diameter}\ (10^{th}\ \text{percentile})}{\text{diameter}\ (50^{th}\ \text{percentile})}$$

Equation 2.3: Calculation of the breadth of the particle size distribution (span) to determine the spread of particle diameters around the median value

Broad particle size distributions will display a large span value (i.e. a polydisperse system) and a monodisperse distribution will display a low span value.

2.3.7 Procedure for the imaging of liposomes using transmission electron microscopy (TEM)

Liposome samples were imaged using TEM immediately after preparation. A drop of the liposome suspension was placed on a 3.05 mm 400 mesh carbon-coated copper grid (purchased from TAAB (Aldermaston, U.K.)) and the surplus drawn away using filter paper. The samples were then stained with a drop of either phospho-tungstic acid (1 $\%^{m}/_{v}$ in water) or uranyl acetate (1 $\%^{m}/_{v}$ in water), the excess drained with filter paper and the stain allowed to dry onto the samples at room temperature for 2 min prior to imaging using the Philips CM120 BioTwin microscope. An electron gun at the top of the microscope emit electrons which are accelerated across a potential difference before collision with the sample in the specimen chamber. The electrons are deflected by areas of the sample with a higher density or thickness and so such electrons are not transmitted through the sample. The prior addition of the water-soluble and electron dense negative stain (phospho-tungstic acid or uranyl acetate), which dries onto water accessible surfaces, increases the contrast of the image as electrons are deflected off the

dye. Thus, the light areas in transmission electron micrographs represent the structure of the specimen (i.e. the phospholipid bi-layers in the case of liposomes) and the dark areas the aqueous portions.

2.3.8 Procedure for zeta potential determination using laser Doppler velocimetry

Colloidal systems such as liposome may be unstable, leading to flocculation or coagulation. The DVLO theory proposes that if repulsive forces between particles (due to electric double layer repulsive forces) are sufficiently greater than attractive forces (due to van der Waals forces), then the colloidal system will be stable. Thus, increasing electrostatic repulsion between particles by either ionisation of surface groups on the particles or adsorption of ions or charged species onto the surface will increase colloidal stability. A second mechanism to increase stability within a system is through the addition of polymers to the particle surface (either by adsorption or conjugation) to prevent particles from contacting through steric hindrance or by steric repulsion by decreasing the effect of van der Waals attractive forces.

The occurrence of the surface charge on colloidal particles (such as liposomes) will lead to the attraction of counter-ions from the bulk media, which will associate with the colloid in two layers (figure 2.5). The inner layer (Stern layer) consists of adsorbed or 'fixed' counter-ions which would move with the particle if it were to be placed into an electric field. The outer layer or 'diffuse' layer contains ions that are less attached to the particle. The interface between the two layers is known as the Stern plane and associated with this layer is a further 'quantity' of solvent containing ions. The edge of this solvating layer (within the diffuse layer) is known as the plane of hydrodynamic shear (or the slipping plane), which represents the boundary of relative movement between the vesicle and the bulk solution. It is the potential difference between this layer and the bulk medium that is known as the zeta or electrokinetic potential. The interaction of particles is dependent on the magnitude of this zeta potential (and not the surface charge of the particle) and so is used as an indicator for colloidal stability.

Zeta potential was determined using the Malvern ZetaMaster 2000 (Malvern, U.K.) using a 5mW He-Ne laser at 632.8 nm using a field strength of 30 Vcm^{-1} to measure the electrophoretic mobility of the particles (the velocity of the particle moving through a medium under the influence of unit electric field) which is linked to zeta potential by

the Henry equation (equation 2.4). Factors that influence the zeta potential include particle surface charge, the dielectric constant and viscosity of the dispersant and ionic strength (in terms of ion concentration and ion valency) and pH of the medium.

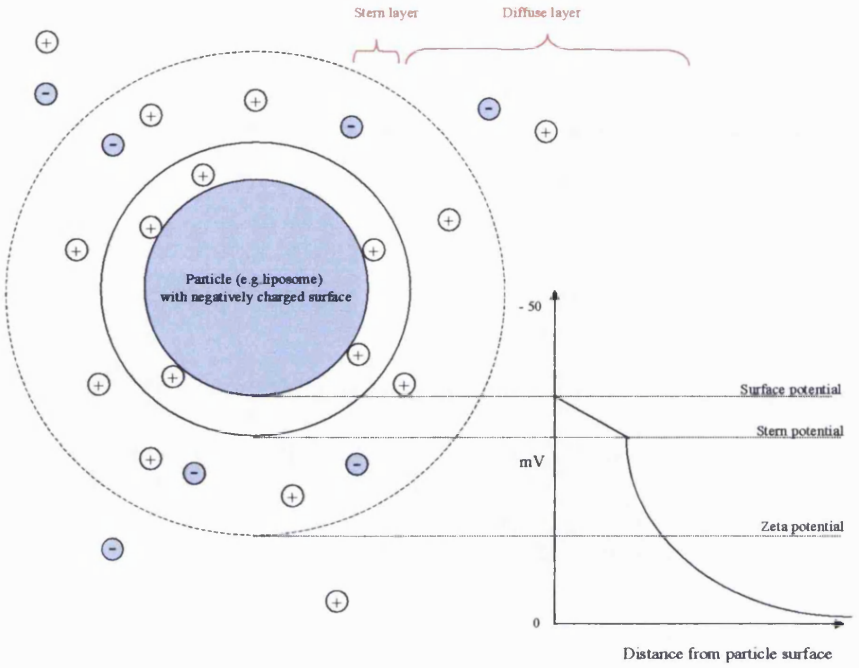

Figure 2.5 Diagrammatic representation of the electronic potential variance through the electric double layer surrounding a particle in an aqueous environment (adapted from Malvern ZetaMaster training manual)

$$U_E = \frac{2\varepsilon\zeta f(Ka)}{3\eta}$$

U_E = Electrophoretic mobility

ε = Dielectric constant of medium

ζ = Zeta potential

***f*(Ka) = Henry's function of reciprocal Debye length† (k) multiplied by particle radius (a). In polar media (dielectric constant greater than 20) this is 1.5 using Smoluchowski's approximation (particles larger than 0.2 μm dispersed in a medium containing more than 0.001 M salt)**

η = Viscosity of medium

†The Debye length is the thickness of the double layer of the particle and is indirectly related to ionic concentration of the medium (mol/L).

Equation 2.4: Henry equation for calculation of zeta potential from the determination of particle electrophoretic mobility

The experimental set up for the measurement of particle electrophoretic mobility involves placing the sample in a cell across which an electric field is applied using electrodes, thus causing the movement of charged particles. Undergoing such movement, the particles cross two overlapping laser beams, which results in a change in the frequency of scattered light due to the Doppler effect. The two overlapping beams are originally emitted from one source and are subsequently split using a beam splitter. One of the beams is then reflected using a modulator, which shifts the frequency of this reflected beam, by small alternating amounts. Where the two laser beams overlap in a specific area in the sample cell, Young's interference fringes with known interval widths are produced. The drift velocity of the particles under a known electric field across these fringes can thus be ascertained by detection of the fluctuation in the intensity of scattered light (due to Doppler effect). Heterodyne detection (subtracting the scattered light from non-scattered light to determine change in frequency) is used to increase the measurement resolution. The purpose of one of the beams being reflected by the modulator is so that the Young's interference fringes subsequently formed also possess changing frequency so that non-charged particles (i.e. not under the influence of the electric field and hence not moving under the influence the field through the interference pattern) can also be detected. In the Malvern ZetaMaster 2000, the lasers are set to cross at the stationary layer in the sample cell where it has been shown that the electrophoretic mobility of the particle measured is free of electro-osmotic effects (the movement of the dispersion medium moving under the influence of it's component ions and thus affecting particle flow to differing extents at different parts of the sample cell).

In all studies reported in this thesis, the medium used was deionised water and for comparison studies water was ensured to be of the same pH for all studies (pH 5.1-5.5). The zeta potential standard DTS5050 (Malvern, U.K.), which contains latex particles with a zeta potential of –50 mV (± 5 mV) was used regularly to ensure the instrument was working accurately.

2.3.9 Procedure for the measurement of liposomal surface hydrophobicity using Rose Bengal

Differences in the surface hydrophobicity between non-polymerised and polymerised liposomes were determined by using the hydrophobic dye Rose Bengal (4,5,6,7-Tetrachloro-2',4'5'7'tetraiodofluorescein) (Müller (1991), Lukowski *et al.*, 1992). The

method is based on the dye's hydrophobic characteristics due to which it partitions itself between the liposome's bi-layer (hydrophobic) and the aqueous dispersion medium (hydrophilic) depending on its competing hydrophilic/hydrophobic affinity for either.

In brief, 10 mg of liposomal phospholipids (1mL of 10 mg/mL solution) were added to 3 mL of Rose Bengal in solution (20 μg/mL Rose Bengal in water) and the resulting mixture allowed to interact at room temperature for 3 h under mild agitation (20 rpm) using the Stuart Scientific Platform Rocker STR6 (Redhill, U.K.). Non-adsorbed Rose Bengal was removed by centrifugation (100,000 g for 40 min). The measurement of the supernatant at 564 nm visible light against a Rose Bengal-water blank (peak at 549 nm) is noted for both polymerised and non-polymerised liposomes. The partitioning quotient is calculated as the fraction of liposome-adsorbed Rose Bengal to a Rose Bengal-water blank (equation 2.5) and represents the separation of the Rose Bengal between the liposomes and surrounding aqueous media. A partitioning quotient of 0 would represent no association of Rose Bengal to liposomes (i.e. liposomes are less hydrophobic) and a partitioning quotient of 1 would represent complete association of Rose Bengal to the liposomes (i.e. liposomes are more hydrophobic). This method assumes that all samples used will possess the same surface area (i.e. when comparing one liposomal formulation to another liposomal formulation, both samples must have the same number of particles of the same diameter (and hence same surface area)) so that the Rose Bengal partitioning is between the same volume of medium and surface area of particle. For this purpose, liposomes with a narrow size distribution were prepared and polymerised liposomes were produced from the non-polymerised batch (so as to eliminate variation in particle size distributions between polymerised and non-polymerised liposomes).

$$\textbf{Partitioning quotient} = \frac{\textbf{(Rose Bengal-water blank)}_{UV\,\lambda 549} - \textbf{(supernatant)}_{UV\,\lambda 564}}{\textbf{(Rose Bengal-water blank)}_{UV\,\lambda 549}}$$

Equation 2.5 Calculation of the partitioning quotient for the comparison of the surface hydrophobicities of liposomal formulations using Rose Bengal

2.3.10 Procedure for measuring retention of protein to liposomal formulations *in vitro* after incubation in release medium

The retention of protein (BSA or TT) to liposomal formulations after incubation in release medium at various time-points will give an indication of the depth of protein encapsulation (within, for instance a multi-lamellar liposomal formulation), the robustness of liposomal bi-layers in aqueous media (and hence delay in the release of encapsulated protein into the surrounding medium) or the strength of protein association to liposomal bi-layers due to hydrophobic interactions. The *in vitro* protein release profile can give an indication of the *in vivo* protein release from liposomal formulations in biological fluids. Thus, such characterisation would help to ascertain the potential of liposomal formulations in delivering the protein by controlled or instant release.

In vitro release profiles were performed by suspending 20 mg of phospholipids in liposomal formulations (containing free and liposome-associated protein) into 5 mL PBS (pH 7.4) in 10 mL polycarbonate oak ridge bottles with ultraspeed sealing assembly (O-ring, plug and cap) (Kendro Laboratory Products Ltd. (Bishop's Stortford, U.K.)). Prior to adding the protein to the liposome samples, the protein was mixed with a small quantity of radio-labelled protein which acts as a tracer. Samples were incubated at 37 °C (100 rpm) in a Sanyo Gallenkamp IOX400.XX2.C incubator (Loughborough, U.K.) and removed at various time-points. The released protein (in suspending medium) was separated from liposome-associated protein by centrifugation (150,000g, 40 min, 4 °C) (see section 2.3.5) and quantified by measuring the emission of gamma radiation from the pellet and supernatant. The retention of protein to liposomal formulations was expressed as a percentage of the original total protein (free and liposome-associated). Hence, the retention of protein to liposomal formulations at various time-points can be followed.

2.3.11 Statistical analyses

Results are expressed as the arithmetic mean of n observations ± the standard deviation (SD) or standard error (SE) (also known as standard deviation of the mean). The SD represents the variability of individual data points of a sample or population in connection to a central value (usually the mean) and the SE represents the variability of

a set of sample means derived from the population. SD and SE were calculated using Microsoft Excel.

Significance analysis between two groups was carried out using the Student's t-test. A paired-sampled Student's t-test was used to analyse the difference in means within a group before and after an intervention or procedure and two-sampled t-test when the means of two unconnected groups were being analysed. When examining the latter it was important to ascertain whether or not the variances between the two groups were equal or different, for which the F-test was used. The use of the t-test required assuming that the data was randomly collected and the data was from a normally distributed population. The analysis of the Student's t-test and the F-test for variances were calculated using Microsoft Excel.

Significance analyses between multiple groups were not carried out using the Student's t-test due to the increased chance of the occurrence of a type I error. This would be the error of detecting that a significant difference between two groups existed, when one did not (i.e. rejecting the null hypothesis when it should have been accepted). Conversely, the error of accepting the null hypothesis when it is false (i.e. not accepting the alternative hypothesis and thus missing a difference) is a type II error. Where multiple statistical analyses (multiple comparisons) were to be made, the one-factor between subjects (one-way) analysis of variance (ANOVA) was used to calculate significant differences. ANOVA calculates the variability within a group against the variability between groups to indicate evidence of equality of means (or lack of such) between the groups. The use of ANOVA is valid when the data is assumed to be randomly collected and from a normally distributed population. The homogeneity of variances (F-) test (calculated by the Levene test using SPSS) was employed to assess the similarity or inequality of variances between groups, by comparing the largest variance to the smallest variance (as multiple groups were analysed). ANOVA and *a posteriori* (post hoc) testing were performed using SPSS. *A posteriori* testing (Tukey's a (Tukey's honestly significant difference (HSD)) test, Dunnett's T3 test, Tukey-Kramer's test and Games-Howell's test) was performed according to suitability, to identify where the exact statistical significant difference lay. Tukey's HSD test was performed where groups possessed equal variances and each group consisted of an equal number of data points. Where variances were equal but group sizes differed, the Tukey-Kramer test

was performed. Dunnett's T3 and Games-Howell were used when variances were unequal and consisted of equal or unequal group sizes respectively.

Probability (p) is the likelihood of the occurrence of an event (probability of the occurrence of an error) and where significant differences in the text are stated this is statistically significant ($p<0.05$) unless otherwise stated.

2.4 Results and discussion

2.4.1 Determination of the number of extrusion cycles required to prepare a liposome formulation with a narrow size distribution

The number of extrusions required to prepare liposomes with a mean diameter similar to that of the pore diameters of the filter membranes was investigated (table 2.1). Multi-lamellar SPC/SPG liposomes were constructed by the thin film method (see section 2.3.1.1) and the mean hydrodynamic diameter of the particles determined using PCS (section 2.3.6.1) before and after increasing numbers of extrusions through double-stacked polycarbonate filter membranes with pore diameters of 0.1 μm (section 2.3.2.1).

Table 2.1: The effect of the number of extrusions through double-stacked polycarbonate filter membranes (with pore diameters of 0.1 μm) on mean SPC/SPG liposomal hydrodynamic diameter (n = 3)

N°. extrusions	Mean hydrodynamic diameter (nm) (± SD)	Polydispersity index (± SD)	KCPS (± SD)
0	302.7 (20.5)	0.745 (0.081)	96.1 (0.7)
5	185.7 (6.5)	0.661 (0.18)	266.7 (0.9)
10	168.8 (4.3)	0.512 (0.127)	299.6 (7.9)
15	162 (2.8)	0.371 (0.09)	292.4 (25.1)
20	155.7 (0.4)	0.318 (0.012)	297.9 (3)

Mean hydrodynamic diameter of liposomes was greatly reduced after 5 extrusion cycles from 302.7 ± 20.5 nm to 185.7 ± 6.5 nm (mean size ± SD). The decrease in mean

hydrodynamic diameters of liposomes beyond 5 extrusions was less marked than after the initial 5 extrusions, though the polydispersity index of the samples continued to decrease with further extrusion passages. Mean hydrodynamic diameter even after 20 extrusions (155.7 nm) remained higher than the pore diameters of the filter membrane (0.1 μm). This was reasoned to be due to the repulsion between bi-layers (due to the negative charge acquired as a result of the incorporation of the negatively charged phospholipid SPG) increasing liposomal diameter following extrusion.

It is notable that there was an increase in the count rate (KCPS) post-extrusion. This was reasoned to be because many larger multi-lamellar vesicles were broken to create a higher number of smaller vesicles. The count rate does not increase beyond 10 extrusions indicating that larger vesicles were no longer being converted into smaller ones. Any further polydispersity change was probably due to removal/change of the few large vesicles that remained, yet were skewing the analysis. The Student's paired sample t-test indicated that there was no significant difference ($p<0.05$) in the change in mean hydrodynamic diameter of liposomes beyond 10 extrusions. Hence, for the preparation of liposomes with a narrow size distribution using extrusion, 10 extrusion cycles are sufficient.

Figure 2.6 shows a transmission electron micrograph (using 1 $\%^{m}/_{v}$ phospho-tungstic acid in deionised water as a negative stain) (section 2.3.7) of SPC liposomes produced after 10 extrusions. The liposomes can be seen to be multi-lamellar and intact and by cross reference with the scale bar, are of a diameter that corresponds to the mean hydrodynamic diameter as determined using PCS (168.8 ± 4.3 nm).

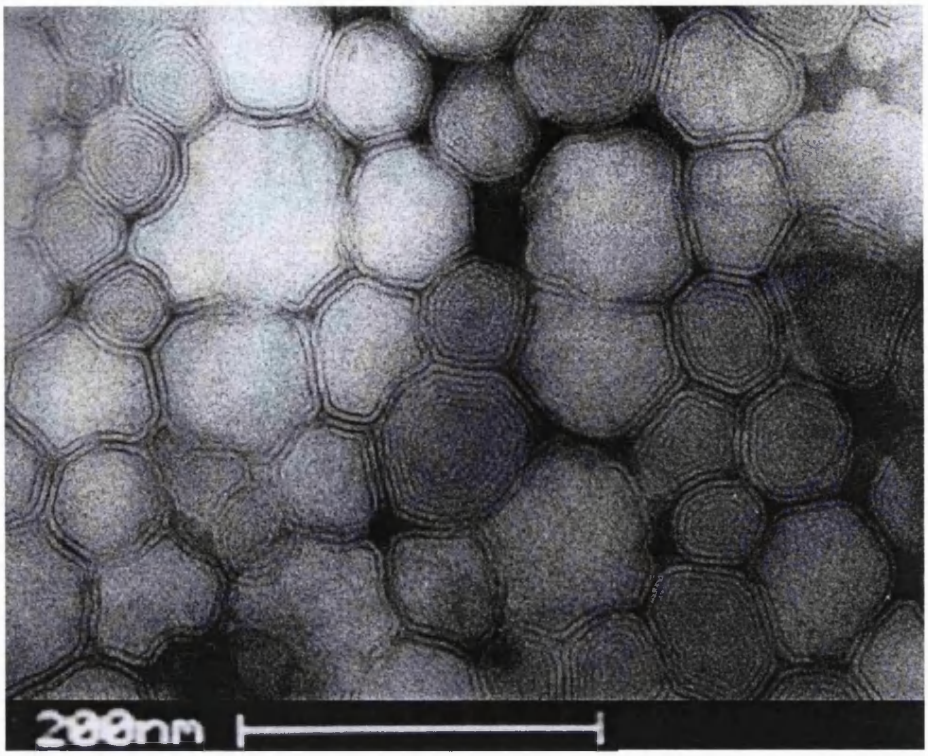

Figure 2.6 Transmission electron micrograph of SPC liposomes stained with 1 $\%^{m}/_{v}$ phospho-tungstic acid in deionised water. Liposomes were prepared using the 'hand-shaking' thin film method and a narrow size distribution obtained after 10 extrusions through double-stacked filter membranes (with pore diameters of 0.1 μm)

2.4.2 Employment of combined freeze-thaw and extrusion cycles to prepare a liposomal formulation with a well-defined particle size distribution

For nasal immunisation, it is important to prepare liposomes of a size that will lead to optimal uptake by the NALT (section 1.4.6.3). Studies by Ghirardelli and colleagues (Ghirardelli *et al.*, 1999) on excised nasal mucosa of male New Zealand rabbits, showed that following application of insulin coated fluorescent polystyrene nanoparticles (0.5 μm), nanoparticles were largely found bound to non-ciliated microvillar cells overlying lymphoid aggregates whereas none were found bound to ciliated cells. The authors concluded that these non-ciliated microvillar cells (possibly M cells) were the transporting cells for particulate matter in the nasal tissues.

An extensive investigation on the effect of particle size on uptake by the nasal microvillar cells (e.g. nasal M cells) has still not been undertaken in animal or human

subjects. However, such studies investigating the effect of particle size on particle uptake by M cells located in the GALT (see section 1.4.6.4) may give an indication for the particle size preferentially taken up by nasal M cells. On reviewing the findings of the studies conducted on the gastro-intestinal and nasal mucosal tissues (section 1.4.6.3 and section 1.4.6.4 respectively), it was hypothesised that in order to optimise the systemic and mucosal immune response to a nasally administered vaccine, it would be necessary to prepare liposomes with a diameter between 0.3-1 μm. To achieve this, filter membranes with pore diameters of 0.6 μm and 1 μm were used in the extruder to prepare liposomes with a higher mean hydrodynamic diameter than the liposomes prepared in section 2.4.1.

The use of polycarbonate filter membranes with pore diameters of 0.6 μm in the extruder and freeze-thawing was employed for the conversion of multi-lamellar SPC liposomes (produced by the thin film method (see section 2.3.1.1)) with a broad size distribution to produce a liposome population with a narrower size distribution. Particle size analysis using PCS (section 2.3.6.1) following 10 extrusions revealed that the mean liposomal hydrodynamic diameter was far below (237.3 ± 6.9 nm) (mean ± SD) the intended diameter of 0.6 μm (table 2.2). This was attributed to the formation of many smaller vesicles that resulted in the vesicle size distribution being weighted towards smaller diameter values.

The freeze-thaw procedure (Castile *et al.*, 1999) was used in order to increase the diameter of liposomes following the extrusion stage. The authors reviewed that the mechanism by which the diameter of liposomes increased was due to the fragmentation of bi-layers by ice crystal (formed during the freezing stage) and the production of a new population of liposomes (fragments forming new liposomes or fusing with existing liposomes) during the thawing stage (section 1.5.4). This was followed by a second cycle of extrusions to re-size the upper end of the distribution (table 2.2).

Table 2.2 The effect of the freeze-thaw (F-T) and extrusion procedure using double-stacked filter membranes with pore diameters of 0.6 μm on the mean hydrodynamic diameter of SPC liposomes (n = 3)

Stage	Mean hydrodynamic diameter (nm) (± SD)	Polydispersity index (± SD)	KCPS (± SD)
Pre-extrusion	467.9 (21)	1 (0)	33.7 (2.5)
10 extrusions	237.3 (6.9)	0.234 (0.075)	48.6 (2.1)
1 F-T	238.1 (2.9)	0.253 (0.049)	192.6 (4.6)
5 F-T's	400.7 (7)	0.455 (0.06)	132.2 (2.3)
10 F-T's	470.7 (6.3)	0.283 (0.109)	127.5 (1)
10 extrusions	360.4 (8.1)	0.166 (0.097)	165 (1.2)

Multi-lamellar SPC liposomes (prepared by the thin film method (see section 2.3.1.1)) were also subjected to the freeze-thaw and extrusion procedure using extruder filter membranes with pore diameters of 1 μm, to prepare liposomes with a higher mean hydrodynamic diameter than when using filter membranes with pore diameters of 0.6 μm (table 2.3). Particle size analysis using PCS (section 2.3.6.1) following 10 extrusions revealed that the mean liposomal hydrodynamic diameter was far below (297.0 ± 4.1 nm) (mean ± SD) the intended diameter of 1 μm (table 2.2) and so the freeze-thaw procedure was once again used for the preparation of liposomes with a higher mean diameter.

Table 2.3 The effect of the freeze-thaw (F-T) and extrusion procedure using double-stacked filter membranes with pore diameters of 1 μm on the mean hydrodynamic diameter of SPC liposomes (n = 3)

Stage	Mean hydrodynamic diameter (nm) (± SD)	Polydispersity index (± SD)	KCPS (± SD)
Pre-extrusion	486.8 (61.2)	1.0 (0)	44.7 (6.4)
10 extrusions	297.0 (4.1)	0.225 (0.193)	41.4 (0.1)
10 F-T's	415.5 (8.6)	0.268 (0.078)	156.7 (11.8)
10 extrusions	400.0 (12.7)	0.287 (0.079)	146.9 (2.4)

Table 2.2 and 2.3 show that the mean hydrodynamic diameter (of vesicles extruded through filter membranes with pore diameters of 0.6 μm and 1 μm respectively) increased following the freeze-thaw procedure. The second cycle of extrusions aimed to decrease the size of any 'overly' large vesicles produced during the freeze-thaw stage. As a result, there was a decrease in the polydispersity index from 0.283 to 0.166 for vesicles extruded through filter membranes with pore diameters of 0.6 μm. The same was not observed for the liposomes extruded through filter membranes with pore diameters of 1 μm (polydispersity index was 0.268 after the freeze-thaw stage and 0.287 after the second extrusion cycle). This may be due to the particle size of liposomes produced after the freeze-thaw stage remaining below the diameter of the pores (1 μm) in the filter membrane.

The count rate (KCPS) also increased following the freeze-thaw procedure for liposomes prepared using filters with both pore diameters of 0.6 μm (from 48.6 KCPS to 127.5 KCPS) and 1 μm (from 41.4 KCPS to 156.7 KCPS). This may be due to the conversion of multi-lamellar vesicles to uni- /oligo-lamellar vesicles (Castille *et al.*, 1999), which may be beneficial in those cases where a high lamellarity is not a requirement. This may be where the drug/antigen is to be surface adsorbed or where the encapsulate is hydrophilic (and thus does not require a high lipid:aqueous liposomal volume for sufficient entrapment) or when many bi-layers are not required for liposomal stability (as the stability of the single bi-layer may be sufficient). In such cases, more efficient use of phospholipid will be made as a higher number of liposomes will be produced.

Figures 2.7 and 2.8 represent the volume distributions (measured using PCS) for the vesicles prepared by extrusion through filter membranes with pore diameters of 0.6 μm and 1 μm respectively. The volume distribution depicts the particle size distribution in terms of particle volume (i.e. the relative amounts of particles by the volume they enclose). Thus, 10 liposomes with a mean diameter of 500 nm would have a far higher volume than 10 liposomes with a mean diameter of 50 nm. Conventionally, it is the intensity distribution that is portrayed for particle distribution analyses, which shows the relative intensity of scattered light from particles of different sizes. However, as particle diameters increase (from 50 nm to 500 nm) the intensity of scattered light at 90° decreases (i.e. one liposome of diameter 50 nm scatters far more light at 90° than one liposome of diameter 500 nm). Data from the volume distribution was therefore

deemed far more applicable for the representation of the liposomal size distribution as liposomes with a broader particle size range were being investigated.

Figures 2.7 and 2.8 show that post-swelling, most vesicles possessed hydrodynamic diameters above 1 μm. After the first extrusion cycle (10 extrusions) the particle size distribution contained many smaller liposomes that were transformed to larger ones after the freeze-thaw cycles (10 freeze-thaws). The second extrusion cycle (a further 10 extrusions) remodelled the upper end of the particle size distribution (which was also affected by the freeze-thaw cycle) and resulted in a liposomal population with a narrower particle size distribution. Following the second extrusion cycle, for liposomes produced by extrusion through filter membranes with pore diameters of 0.6 μm the volume distribution indicated that the majority of the liposomal volume was derived from liposomes with a mean hydrodynamic diameter that was smaller than for those liposomes produced by extrusion through filter membranes with pore diameters of 1 μm.

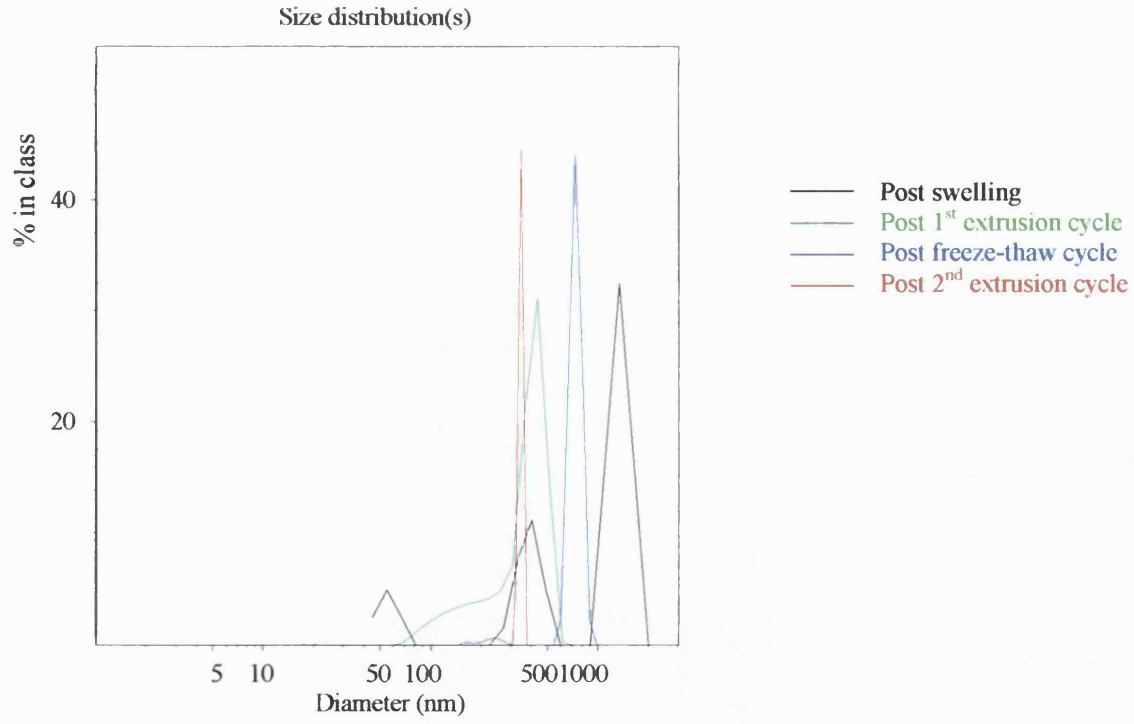

Figure 2.7 Effect of extrusion (through filter membranes with pore diameters of 0.6 μm) and freeze-thawing on the volume distribution of SPC vesicles

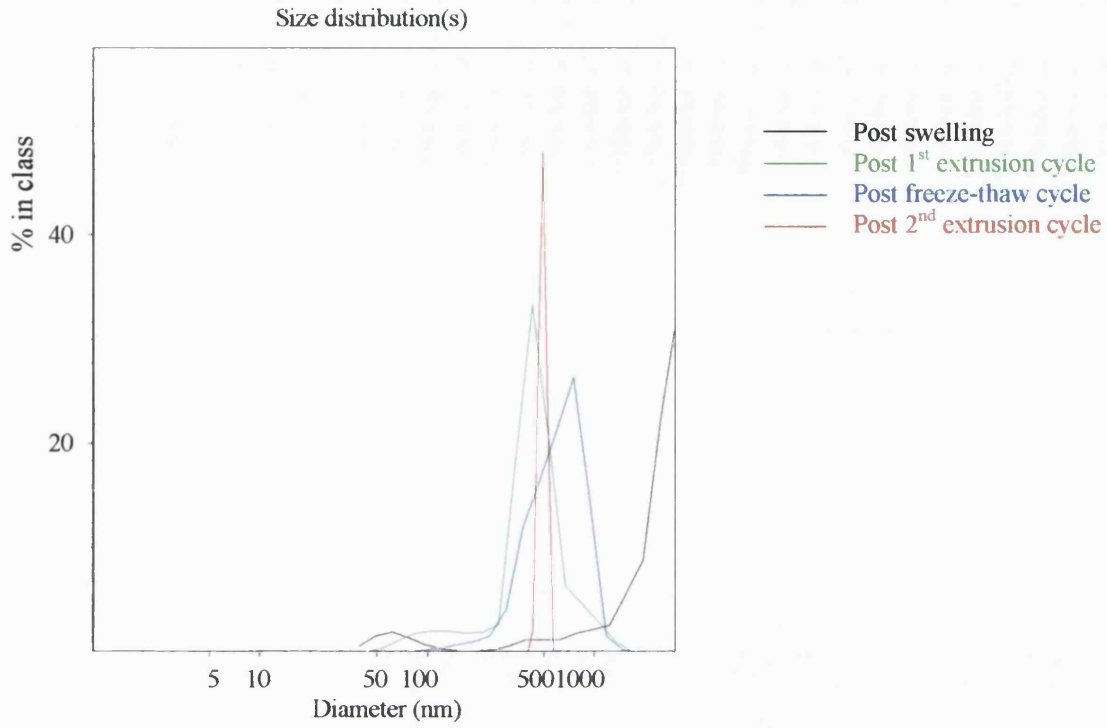

Figure 2.8 Effect of extrusion (through filter membranes with pore diameters of 1 μm) and freeze-thawing on the volume distribution of SPC vesicles

2.4.3 Effect of lyophilisation and centrifugation on liposomal size distribution

Ideally, the size and integrity of liposomes should be maintained throughout processes such as lyophilisation (section 2.3.1) and centrifugation (section 2.3.5). SPC vesicles were prepared by the thin film method (section 2.3.1.1) and a well-defined particle size distribution obtained by extrusion ten times through double-stacked filter membranes with pore diameters of 0.1 μm (see section 2.3.2.1 and section 2.4.1).

Table 2.4 shows the effect of lyophilisation and centrifugation (after rehydration of the freeze-dried product to 10 mg phospholipid/mL water) on the mean hydrodynamic diameter of liposomes and polydispersity index. SPC liposomes were lyophilised by freezing in liquid nitrogen (<-195.79 °C, 1 min) followed by drying for 48 h (using the Edwards Micro-Modulyo drier under vacuum drawn by an Edwards E2M5 high vacuum pump) using 125 mM trehalose as a cryoprotectant and centrifuged at 150,000 g for 40 min at 4 °C.

Table 2.4: The effect of lyophilisation and centrifugation on mean hydrodynamic diameter and polydispersity index of SPC liposomes (n=3)

Stage	Mean hydrodynamic diameter (nm) (± SD)	Polydispersity index (± SD)	KCPS (± SD)
Pre-lyophilisation	117.1 (0.8)	0.097 (0.008)	355.3 (4.5)
Post-rehydration/ pre-centrifugation	107.3 (0.7)	0.136 (0.013)	338.7 (0.8)
Post-centrifugation	109.4 (0.9)	0.112 (0.006)	225.2 (16.9)

Table 2.4 shows that the count rate decreases post-centrifugation from 338.7 KCPS (post-rehydration/pre-centrifugation) to 225.2 KCPS. This possibly indicates a lower number of liposomes in the end sample (post-centrifugation) and may be due to the loss of smaller vesicles during centrifugation that were not sedimented and remained in the supernatant. The two-sampled Student's paired t-test showed that there was a significant difference ($p<0.05$) in the mean hydrodynamic diameter of liposomes after lyophilisation but that there was no significant difference ($p<0.05$) in mean hydrodynamic diameter of liposomes following centrifugation. The change in mean hydrodynamic diameter and particle size distribution of liposomes during lyophilisation was low and was deemed acceptable for the purposes of the further experiments to be conducted.

2.4.4 Calculation of the phospholipid yield for preparation of liposomes following freeze-thaw extrusions, centrifugation and freeze-drying

The quantification of phospholipid loss during the preparation of the liposomes is important as it indicates the efficiency of the production method. When using expensive phospholipids such as DODPC, it is essential to minimise such raw material losses, especially if such methods were to be scaled up.

In the phospholipid yield studies conducted, the measurement by weight, before and after production of liposomes was used to quantify the loss of phospholipid. DODPC liposomes were prepared using the freeze-dried phospholipid hydration method (section 2.3.1.2) and modified by freeze-thaw extrusions (10 extrusions through double-stacked filter membranes with pore diameters of 1 μm, 10 freeze-thaw cycles then 10 further

extrusions) (see section 2.3.2.1, section 2.3.2.2 and section 2.4.2). The liposomes were then lyophilised by freezing in liquid nitrogen (-195.79 °C, 1 min) followed by drying for 48 h (using the Edwards Micro-Modulyo drier under vacuum drawn by an Edwards E2M5 high vacuum pump) (see section 2.3.1). The phospholipid yield for DODPC vesicles was calculated to be 85.5 $\%^{m}/_{m}$ when initial quantity of DODPC was 100 mg. Loss during the first lyophilisation stage was calculated to be 5 $\%^{m}/_{m}$ for a 40 mg sample. The remainder of the phospholipid loss could be assumed to be in the filter of the extruder, lost into the vacuum of the lyophiliser or incomplete recovery from glassware used. However, it was previously reported (Berger *et al.*, 2001) that following extrusion of SPC and SPG through the same extruder as used in the studies in this thesis, through a filter membrane with pore diameters of 0.05 μm (much smaller pore diameter than used in the studies presented here), that the phospholipid recovery was 100 %, though the authors used pressures much higher (54 bar) than the pressure used in the investigations reported in this thesis (8 bar). As the quantity of phospholipid used *per* batch is increased, it would be logical to assume that the percentage yield would increase towards an upper limit.

Using the weight measurement for yield quantification can only be used when there is only one liposome component (i.e. for the yield quantification of one phospholipid only). When samples consisting of mixed compositions of materials are produced then it becomes essential to quantify each material individually (as one particular material may be retained preferentially on the extruder filter due to electrostatic charges *per* say) by techniques such as high performance liquid chromatography.

2.4.5 Employment of the DRV method and modified DRV method (using cryoprotection) as a tool to enhance protein association to liposomes compared to conventional methods

The stage at which the protein is allowed to interact with the phospholipids/preformed liposomes influence the extent and depth of encapsulation or the degree of surface adsorption of the protein and hence may affect release/dissociation properties of the protein from the formulated liposomes. Processing stresses during liposome preparation can also affect protein conformation and so may decrease protein immunogenicity.

As it was planned to polymerise phospholipids once liposomes had been formed, it was considered desirable to add the protein after this stage so as to protect the epitope(s) in the protein from any detrimental effects that would reduce protein immunogenicity. This would entail adding the protein at one of two stages after polymerisation; either pre-lyophilisation or post-lyophilisation. It was believed that the addition of protein before lyophilisation would result in a higher degree of encapsulation (see section 1.5.5) (Gregoriadis *et al.*, 1999) than post-lyophilisation addition of protein (as a part of the rehydration medium), which would probably result in a high level of surface adsorption of protein and a lower degree of entrapped protein. The former method would entail protein addition to liposomes during formulation production (under manufacturer control) and the latter at the bed-side (by trained personnel or by the patient) when the stored product is re-hydrated.

SPC/SPG liposomes were prepared using the thin film method (section 2.3.1.1) and extruded through double-stacked filter membranes with pore diameters of 0.1 μm (ten times) to produce liposomes with a narrow size distribution (section 2.3.2.1 and section 2.4.1). Liposomes were lyophilised (section 2.3.1) by freezing in liquid nitrogen (< -195.79 °C, 1 min) followed by drying for 48 h (using an Edwards Micro-Modulyo under vacuum drawn by an Edwards E2M5 vacuum pump) using 125 mM trehalose (where used) as a cryoprotectant.

The cryoprotective action of sugars such as sucrose and trehalose has been reported for the preservation of liposomal size on freezing (Vemuri *et al.*, 1991; Ausborn *et al.*, 1992). A major disadvantage of using a cryoprotectant in the DRV method was thought to be a diminished efficiency of protein association to liposomes as the method relies on the rupture and fusion of vesicle bi-layers during lyophilisation for protein incorporation. Thus, it was first necessary to ascertain if the lyophilisation of liposomes without cryoprotection resulted in the preservation of vesicle size and size distribution (table 2.5).

Table 2.5 The effect of the DRV method and the modified DRV method with cryoprotection on mean diameter of SPC/SPG liposomes (n=3)

Method	Stage	Mean volume diameter[1] (μm) [Span]	Mean hydrodynamic diameter[2] (nm) (± SD) [polydispersity index]
DRV	Pre-lyophilisation	-	119.0 (3.8) [0.33]
	Post-lyophilisation	24.62 [4.846]	-
DRV with cryoprotection	Pre-lyophilisation	-	130.2 (1.2) [0.118]
	Post-lyophilisation	-	119.4 (4.5) [0.164]

[1]Mean volume diameter as measured using laser light diffraction (section 2.3.6.2)

[2]Mean hydrodynamic diameter as measured using photon correlation spectroscopy (section 2.3.6.1)

When DRVs were prepared without cryoprotection, the mean diameter of the liposomes increased from the nanometre range (119.0 ± 3.8 nm) to the micrometer range (24.62 μm). This may be due to rupture and re-fusion of the vesicles during lyophilisation without cryoprotection. When the DRV method was modified to include trehalose (125 mM) as a cryoprotectant, the change in mean hydrodynamic diameter of liposomes after lyophilisation was marginal (from 130.2 ± 1.2 nm before lyophilisation to 119.4 ± 4.5 nm following lyophilisation), though the means were determined to be significantly different ($p<0.05$) (paired two-sampled Student's t-test). However, the change in mean hydrodynamic diameter of liposomes and the increase in polydispersity index was small and so, for the purpose of the further studies to be conducted, this level of cryoprotection was deemed to be sufficient.

Once it was ascertained that the modified DRV method with cryoprotection could be used without adversely affecting mean liposomal size and size distribution, the effect on protein association to liposomes was examined. The loading efficiency of DRVs was compared to that of vesicles prepared by the classical thin film method where the protein was added as part of the hydration solution to the thin phospholipid film (concentrated protein solution added first, followed by bulk water) or protein added post-hydration but before extrusion (table 2.6). Theoretical loadings of 1 $\%^{m}/_{m}$ and

$2\%^{m}/_{m}$ were investigated for protein association to liposomes using the DRV method (the theoretical loading represents the percentage of protein to phospholipid in the formulation). Trehalose (125 mM) was used as a cryoprotectant for all preparations. At this stage, the addition of protein post-lyophilisation was not investigated.

Bovine serum albumin was used as a model protein and was purchased pre-conjugated to fluorescein isothiocyanate (FITC-BSA). Liposome-associated protein was separated from free protein by centrifugation (150,000 g, 40 min, 4 °C) (section 2.3.5) and subsequently released from sedimented vesicles into free solution using Triton-X100 (5 $\%^{v}/_{v}$ final concentration). The concentration of FITC-BSA was quantified against a calibration curve (known FITC-BSA concentration in solution) after adjusting against a control (empty liposomes and Triton-X100) by using fluorescence spectroscopy (excitation λ = 485 nm, emission λ = 535 nm) using the Wallac 1420 Victor^2 (PerkinElmer® Analytical Instruments (Seer Green, UK)).

Table 2.6: The effect of the protein addition stage on the loading efficiency of BSA into SPC/SPG liposomes (n=3)

Method	Theoretical loading ($\%^{m}/_{m}$)	Actual loading ($\%^{m}/_{m}$) (± SD)	Loading efficiency ($\%^{m}/_{m}$) (± SD)	Association (mg protein/ mg lipid)	Association (mg protein/ μmol lipid)
Thin film method	2	0.104 (0.005)	5.195 (0.268)	0.001039	26.64
Post-swelling addition	2	0.144 (0.002)	7.18 (0.087)	0.001436	36.82
DRV with cryoprotection	2	0.156 (0.006)	7.789 (0.305)	0.001558	39.94
DRV with cryoprotection	1	0.126 (0.01)	12.64 (0.954)	0.001264	32.41

Key: Theoretical loading represents the percentage of overall (free and associated) protein to phospholipid in the formulation
Actual Loading represents the percentage of associated protein to phospholipid in the formulation
Loading efficiency represents the percentage of protein associated to liposomes in respect to the total protein added to the formulation

Loading efficiency of BSA for liposomes prepared using the thin film rehydration method was significantly lower ($p<0.05$) than by the other three methods. There was no significant difference ($p<0.05$) in the association of BSA to liposomes irrespective of whether the BSA was added post swelling or by the DRV method. However, it must be noted that at this stage, the exact localisation of the BSA within the liposome (e.g. encapsulated or surface adsorbed) had not been evaluated. There was a significant increase ($p<0.05$) in the BSA association to liposomes when a theoretical loading of 1 $\%^{m}/_{v}$ was used compared to either the post-swelling addition of BSA or DRV method with 2 $\%^{m}/_{v}$. Significance between groups was analysed using a one-way ANOVA and Tukey's HSD test.

The lower BSA association to liposomes when using the classical thin film method may be due to loss of BSA on the surface of the round bottomed flask. It is of interest to note that the post swelling and DRV method have similar loadings even though with the post-swelling method there was greater opportunity for loss of BSA on filters and surfaces during the extrusion stage.

Therefore, it can be seen that associating protein to non-polymerised vesicles using the DRV method with cryoprotection is a good alternative to conventional techniques such as the classical thin film method.

2.4.6 Effect of the length of exposure of non-polymerised DODPC liposomes to UV light on the extent of polymerisation and mean hydrodynamic diameter of liposomes

DODPC liposomes were prepared using the freeze-dried phospholipid hydration method (section 2.3.1.2) and modified by freeze-thaw extrusions (10 extrusions through double-stacked filter membranes with pore diameters of 1 μm, 10 freeze-thaw cycles, 10 further extrusions) (see section 2.3.2.1, section 2.3.2.2 and section 2.4.2). The liposomes were subjected to UV light (254 nm) for varying time periods and UV spectroscopy was used to observe the decrease in UV absorption at 254 nm, which corresponds to the decrease in the presence of diene bonds due to polymerisation (table 2.7). The UV absorption (254 nm) for non-polymerised liposomes was used as a reference (i.e. total number of diene bonds in non-polymerised liposomes) to which the UV absorption (254 nm) for

polymerised liposomes (at various time-points) was compared against to calculate the progress in diene bond conversion (progress of polymerisation) as a percentage.

Table 2.7 Progress of polymerisation of DODPC vesicles (modified with freeze-thaw cycles and extruded through double-stacked filter membranes with pore diameters of 1 μm) following exposure to UV light for various time periods

Time (hr,min)	UV absorption (254 nm)	Diene bonds remaining (%)	Mean extent of polymerisation (%)
0	0.801	100	0
0,15	0.623	77.8	22.2
1,15	0.170	21.2	78.8
3,15	0.088	11.0	88.0

The mean extent of polymerisation was found to be significantly different ($p<0.05$) at each increase in time-point investigated (paired two-sampled Student's t-test (equal variances)). The initial rate (in first 1 h 15 min) of polymerisation was higher than at later stages of the experiment (22.2 % in the first 15 min, a further 56.6 % in the next 1 h and only a further 9.2 % in the next 2 h before termination of the experiment. The results observed here are comparable to those reported by Ohno and co-workers (Ohno *et al.*, 1987) who showed that polymerisation of DODPC liposomes using a UV light source was almost 100 % after 3 h. In our studies, polymerisation conversion after 3 h 15 min was 88 %.

The occurrence of vesicle aggregation/precipitation during UV polymerisation was examined by determining mean hydrodynamic diameter of liposomes (using PCS) at each time-point (table 2.8). The mean hydrodynamic diameter of liposomes was not significantly different (paired two-sampled Student's t-test ($p < 0.05$)) after polymerisation for 3.25 h to the mean liposomal size of non-polymerised liposomes. Vesicles integrity following polymerisation was confirmed using TEM (section 2.3.7) (figure 2.9).

Table 2.8 Mean hydrodynamic diameter of liposomes at various time-points during UV polymerisation (n = 3)

Time (h, min)	Mean hydrodynamic diameter (nm) (± SD)	Polydispersity index (± SD)	KCPS (± SD)
0	285.3 (21.5)	0.287 (0.399)	43.8 (1.7)
0, 15	256.7 (5.2)	0.118 (0.058)	75.0 (0.2)
1, 15	277.0 (9.6)	0.339 (0.412)	29.0 (1.5)
3, 15	248.6 (5.7)	0.113 (0.133)	56.6 (1.0)

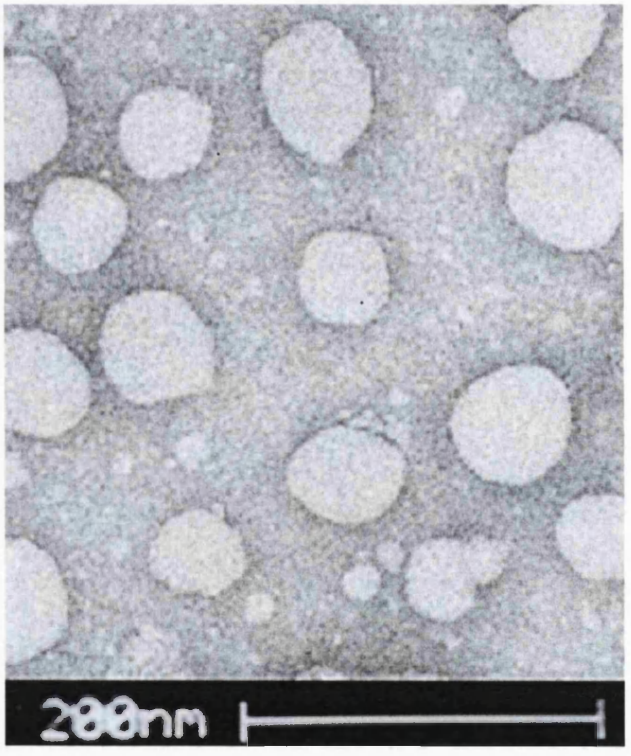

Figure 2.9 Transmission electron micrograph of polymerised DODPC liposomes stained with 1 $\%^{m}/_{v}$ uranyl acetate in deionised water. Liposomes were prepared using the freeze-dried phospholipid hydration method and modified by freeze-thaw extrusions (10 extrusions through double-stacked filter membranes with pore diameters of 1 μm, 10 freeze-thaw cycles, 10 further extrusions) before polymerisation for 3 h 15 min.

2.4.7 Effect of temperature during liposomal rehydration (post-lyophilisation) on protein association to liposomes

Gregoriadis (1999) reported that, when using the DRV method, a higher protein loading/association could be achieved by rehydrating the liposomes (post-lyophilisation) at a temperature exceeding the transition temperature of the liposomal membrane. This was reasoned to be due to the liposomal bi-layer being in a more 'fluid' and more permeable nature at a temperature above it's transition temperature (see section 1.5.1.1). This was also reported by Yachi and co-workers (Yachi *et al.*, 1996) on their experiments on the rehydration of FDELs (section 1.5.5).

DODPC phospholipids have been reported (Koynova and Caffrey, 1998) to have a transition temperature of 18.7 ± 2.8 °C (mean ± SD). The polymerisation of the phospholipids would be expected to affect this value, either by increasing the transition temperature to a higher figure or by suppressing it altogether (Gaub *et al.*, 1984). Also, the change in transition temperature may be affected by the extent of phospholipid polymerisation and thus beyond a certain level/extent of polymerisation, liposomes may no longer undergo transition from the solid gel state to a fluid crystalline state. Therefore, the use of high temperatures (than estimated Tc value) in the rehydration of lyophilised polymerised liposomes may not lead to a higher protein association.

It was decided to investigate any possible temperature effect on the loading/association of two model proteins, BSA and TT (figure 2.10) to polymerised DODPC liposomes. Polymerised liposomes were prepared using the freeze-dried phospholipid hydration method (section 2.3.1.2) and modified by freeze-thaw extrusions (10 extrusions through double-stacked filter membranes with pore diameters of 1 μm, 10 freeze-thaw cycles, 10 further extrusions) (section 2.3.2.1 and section 2.3.2.2 and section 2.4.2). The liposomes were polymerised for 3 h 15 min (section 2.3.4) prior to the addition of the protein (mixed with a small quantity of radio-labelled protein) (see section 2.3.3). The liposome-protein samples were lyophilised by freezing in liquid nitrogen (-195.79 °C, 1 min) followed by drying for 48 h (using the Edwards Micro-Modulyo drier under vacuum drawn by an Edwards E2M5 high vacuum pump) using 125 mM trehalose as a cryoprotectant (section 2.3.1.2). After rehydration of the lyophilised samples by either incremental or instant hydration, liposome-associated protein was separated from free protein by centrifugation (150,000 g, 40 min, 4 °C) (section 2.3.5). Instant hydration of

the lyophilised product was performed at room temperature and the sample rocked at 20 rpm for 1 h using the Stuart Scientific Platform Rocker STR6 (Redhill, U.K.). Incremental hydration was performed by rehydration in a controlled manner (Gregoriadis *et al.*, 1999) by addition of small quantities of water (preheated to 70 °C, 37 °C or at room temperature) at a time (100 µl twice and 0.8 mL the third time with 30 min incubations between each addition). During the incubations, the samples were kept at temperatures corresponding to the temperature of the hydration water i.e. at 70 °C in a water bath, at 37 °C by keeping the samples in a Sanyo Gallenkamp IOX400.XX2.C incubator (Loughborough, U.K.) or at room temperature. These temperatures were chosen as it was thought that at 70 °C the liposomes would be at a temperature much higher than the liposomal T_c, at 37 °C they would be at a temperature corresponding to just above any possible T_c and also similar to a temperature the liposome would experience in a biological environment and at room temperature to simulate simple conditions that could be used easily (for interests of method scale-up). Non-associated free protein was separated from liposome-associated protein by centrifugation (150,000 g, 40 min, 4 °C) (section 2.3.5) and the quantity of protein associated to liposomes determined indirectly by measuring the emission of gamma radiation from radio-labelled proteins present in the supernatant. For the purposes of these studies, both BSA and TT were added to polymerised liposomes at a theoretical loading of 1 $\%^{m}/_{m}$ (mg protein/mg phospholipid).

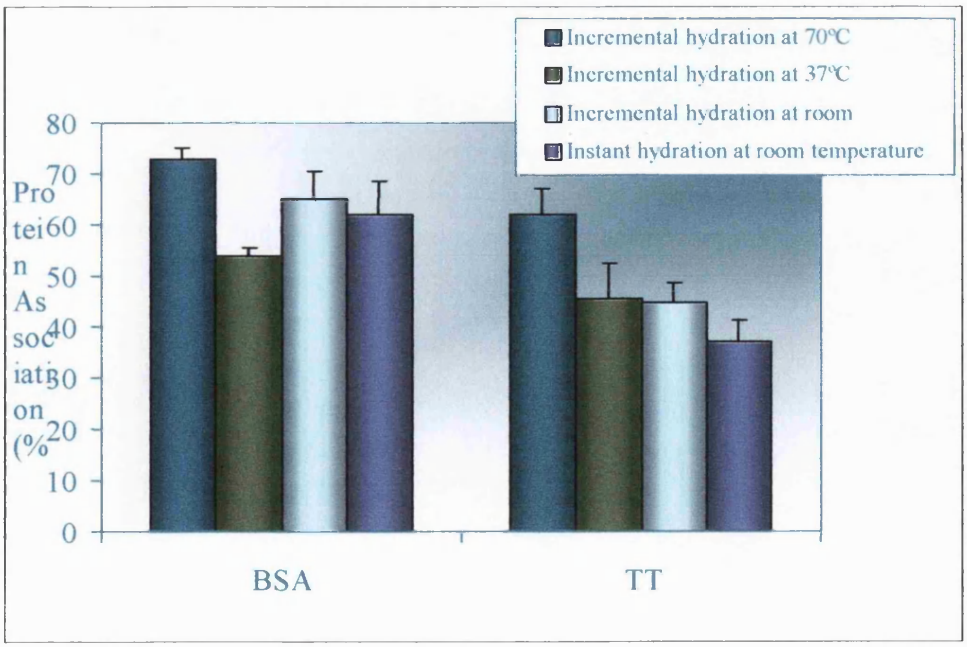

Figure 2.10 The effect of hydration temperature on protein association for polymerised DODPC vesicles (n = 3). Results are means (± SD)

The mean percentage of protein associated to liposomes was highest when the rehydration temperature was 70 °C but this was significantly higher ($p<0.05$) only when the protein used was TT. Neither instant hydration nor incremental hydration resulted in a significantly higher ($p<0.05$) protein association for either BSA or TT at any temperature (excluding TT association to liposomes when hydrated at 70 °C). The mean TT association to liposomes was less than that for BSA at all comparative points.

We concluded from our results that though our finding were in agreement with those of Gregoriadis and co-workers (Gregoriadis *et al.*, 1999) and Yachii and co-workers (Yachii *et al.*, 1996), in that the mean BSA and TT association to liposomes was higher when using a high rehydration temperature (70 °C) and incubation period, that it would be better to use a lower hydration temperature than 70 °C so as to avoid any possible detrimental heating effects on the protein and to simplify the formulation procedure. Thus, it was decided to rehydrate lyophilised liposomes at room temperature. The use of incremental rehydration resulted in a higher (though not significantly higher ($p<0.05$)) mean BSA and TT association to liposomes than instant hydration of the same formulations at the same temperature which is in agreement with the findings of Gregoriadis and co-workers (Gregoriadis *et al.*, 1999) and so it was decided to use incremental hydration when proteins were to be associated to liposomes using the DRV method. The difference in protein release in simulated media from vesicles prepared by hydration at different temperatures or hydrated incrementally or instantly was not investigated. Significance between groups was analysed using a one-way ANOVA and Tukey's HSD test.

2.4.8 Evaluation of the effect of various theoretical loadings on the extent of protein association to liposomes using the DRV method with cryoprotection

Loading efficiency can be improved by increasing the amount of phospholipid used whilst keeping the protein content the same (theoretical loading decrease). Polymerised DODPC liposomes were prepared using the freeze-dried phospholipid hydration method (section 2.3.1.2) and particle size modified by freeze-thaw extrusions (10 extrusions through double-stacked filter membranes with pore diameters of 1 μm, 10 freeze-thaw cycles, 10 further extrusions) (section 2.3.2.1 and section 2.3.2.2 and section 2.4.2). The liposomes were polymerised for 3 h 15 min (section 2.3.4) prior to the addition of TT (mixed with a small quantity of radio-labelled TT) (see section 2.3.3). The

TT (mixed with a small quantity of radio-labelled TT) (see section 2.3.3). The liposome-TT samples were lyophilised by freezing in liquid nitrogen (-195.79 °C, 1 min) followed by drying for 48 h (using an Edwards Micro-Modulyo drier under vacuum drawn by an Edwards E2M5 high vacuum pump) using 125 mM trehalose as a cryoprotectant (section 2.3.1.2). The samples were rehydrated by incremental hydration (100 µl twice and 0.8 mL the third time), allowing the samples to be rocked at 20 rpm for 30 min between each incremental addition using the Stuart Scientific Platform Rocker STR6 (Redhill, U.K.). Free protein was separated from liposomally-associated protein by centrifugation (150,000 g, 40 min, 4 °C) (section 2.3.5) and the quantity of TT associated to liposomes determined by measuring the emission of gamma radiation from the radio-labelled protein.

Figure 2.11 shows the effect of a decrease in protein to phospholipid theoretical loading on final protein association. The mean TT loading efficiency to polymerised DRVs increased as theoretical loading was decreased, though this was not significantly different (one-way ANOVA ($p< 0.05$)). This may be due to the presence of a higher quantity of phospholipid as theoretical loading is decreased, to which protein can be associated (by encapsulation and adsorption).

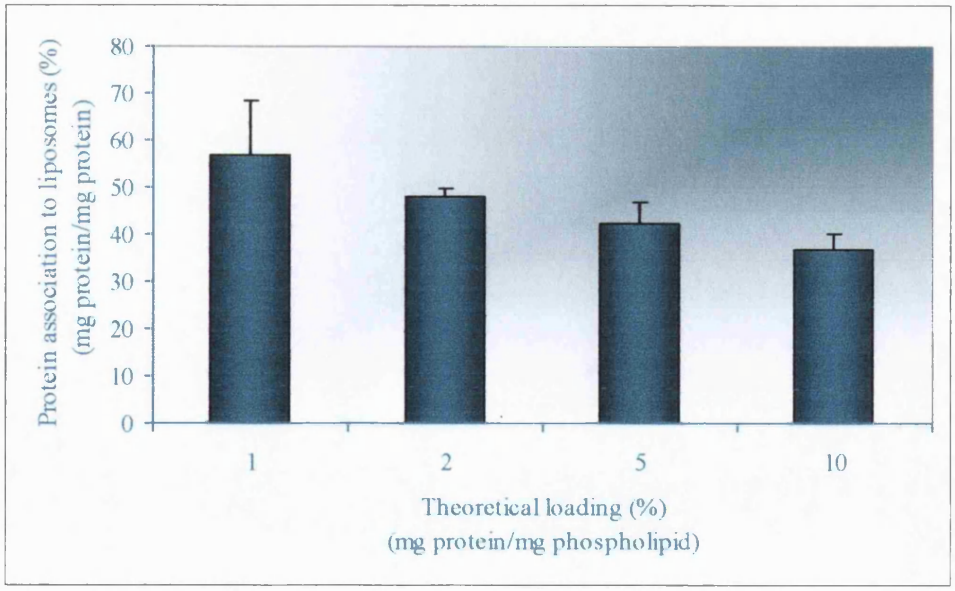

Figure 2.11 Effect of protein:phospholipid theoretical loading on percentage protein association to liposomes (n=3). Results are mean protein association (± SD)

The difference in protein association between protein associated to non-polymerised liposomes using the DRV method with cryoprotection (NP DRVs) and protein associated to polymerised liposomes using the DRV method with cryoprotection (P DRVs) was investigated to evaluate the impact of vesicle polymerisation on protein association (table 2.9).

The use of the DRV method for the association/encapsulation of proteins into liposomes is discussed in section 1.5.5. The use of the DRV method to create protein-vesicle association may result in the internalisation of protein as well as in surface adsorption. The protein association of P DRVs was compared to that of polymerised vesicles where the protein was added at least 1 h after rehydration of the polymerised liposomes (post-lyophilisation) (table 2.9). This time delay will have allowed sufficient time for vesicle re-formation leaving only external membrane surfaces for protein adsorption. Such vesicles will be termed polymerised surface adsorbed vesicles (P SAVs) (see section 1.5.5). Figure 1.13 represents the protein association stages in both DRV and SAV methods.

Non-polymerised and polymerised DODPC liposomes were prepared using the freeze-dried phospholipid hydration method (section 2.3.1.2) and particle size modified by freeze-thaw extrusions (10 extrusions through double-stacked filter membranes with pore diameters of 1 μm, 10 freeze-thaw cycles, 10 further extrusions) (section 2.3.2.1 and section 2.3.2.2 and section 2.4.2). For the production of polymerised liposomes the vesicles were subjected to UV light (254 nm) for 3 h 15 min (section 2.3.4). The liposome samples were lyophilised by freezing in liquid nitrogen (-195.79 °C, 1 min) followed by drying for 48 h (using an Edwards Micro-Modulyo under vacuum drawn by an Edwards E2M5 high vacuum pump) using trehalose (125 mM) as a cryoprotectant (section 2.3.1.2). For the preparation of DRVs, the protein was added just prior to the freezing stage of the lyophilisation procedure (mixed with a small quantity of radio-labelled protein which was used as a tracer) (see section 2.3.3). In the case of DRVs the lyophilised samples were rehydrated by incremental hydration (with water at room temperature) (100 μl twice and 0.8 mL the third time) and the samples rocked at room temperature at 20 rpm for 30 min between each incremental addition, using the Stuart Scientific Platform Rocker STR6 (Redhill, U.K.). For the preparation of SAVs, the lyophilised liposome sample was 'instantly' hydrated (section 2.3.1.2) at room temperature and the sample rocked at 20 rpm for 1 h using the Stuart Scientific Platform

Rocker STR6. Liposome-associated protein was separated from non-associated protein by centrifugation (150,000 g, 40 min, 4 °C) (section 2.3.5) and the quantity of protein associated to liposomes determined indirectly by measuring the emission of gamma radiation from non-associated radio-labelled protein in the supernatant. A theoretical loading of 5 $\%^{m}/_{m}$ (mg initial BSA added/ mg phospholipid) was used for all formulations.

Table 2.9 The effect of polymerisation on BSA association to DODPC vesicles (using the DRV method) and the comparison of this association to vesicles with surface adsorbed protein only (n=9)

Vesicle type	Protein association ($\%^{m}/_{m}$) (± SD)
NP DRV	64.6 (7.5)
P DRV	89.0 (1.6)
P SAV	84.7 (5.1)

The interaction of BSA to polymerised vesicles (both P DRVs and P SAVs) was significantly higher ($p<0.05$) than to non-polymerised vesicles (one way ANOVA, Dunnett's T3 test). To investigate this difference in protein association to polymerised and non-polymerised liposomes, the surface charge/characteristics of liposomes was investigated by determining the liposomal zeta potential using laser Doppler velocimetry (section 2.3.8) and surface hydrophobicity analysis using the Rose Bengal method (section 2.3.9). Table 2.10 shows that the zeta potential for non-polymerised liposomes (-31.0 ± 0.9 mV) is more negative than for polymerised liposomes (-22.0 ± 0.7 mV) (mean ± SD) and that the non-polymerised liposomes also display a lower surface hydrophobicity than polymerised liposomes. The difference in zeta potential measurement and surface hydrophobicity (as determined using Rose Bengal) between polymerised and non-polymerised vesicles was significantly different (p <0.01) (two-sample Student's t-test with equal variances).

Table 2.10 Difference in surface characteristics between polymerised and non-polymerised DODPC vesicles (prepared using filter membranes with pore diameters of 0.1 μm)

Vesicle Type	Partitioning quotient (± SD) (n=3)	Zeta potential (mV) (± SD) (n=5)
Non-polymerised	0.849 (0.0006)	-31.0 (0.9)
Polymerised	0.893 (0.013)	-22.0 (0.7)

Key: A partitioning quotient of 0 represents no association of Rose Bengal to liposomes (i.e. liposomes are less hydrophobic) and a partitioning quotient of 1 would represent complete association of Rose Bengal to the liposomes (i.e. liposomes are more hydrophobic).

The polymerised liposomes attracted the hydrophobic dye Rose Bengal to a higher extent than non-polymerised liposomes. This could be both visually seen (in the supernatant of samples once free and associated Rose Bengal was separated using centrifugation) and by UV analysis of the supernatants at 564 nm. Analysis of the zeta potential of both polymerised and non-polymerised liposomes further confirmed the greater hydrophobicity of polymerised liposomes as they were determined to be less negatively charged than their non-polymerised counterparts.

BSA (67 kDa) has an isoelectric point of pH 4.7. The isoelectric point (pI) of a protein is that pH at which the protein has no net charge (positive charges equal negative charges). At a high pH (above the protein isoelectric point), there will usually be a net negative charge and at a low pH (below the protein isoelectric point) there will usually be a net positive charge. Thus, at pH 7.4, BSA is above it's pI at pH 7.4 and should possess a net negative protein charge. Hence, it may be that the net negatively charged BSA would associate more to the less negatively charged polymerised liposomes than the more negatively charged non-polymerised liposomes (as determined by analysing liposomal zeta potential using laser Doppler velocimetry (table 2.10)).

The difference in mean protein association between the polymerised DRVs and polymerised SAVs (table 2.9) was not significantly different (p = 0.1) (one way ANOVA, Dunnett's T3 test). This would imply that there is little or no protein internalisation during the DRV process for polymerised vesicles. However, overall protein association is not a complete indicator for protein association as it has not been

demonstrated that DRVs contain as much surface adsorbed protein as SAVs and that only any amount above this is related to encapsulation (i.e. overall protein association to DRVs and SAVs may be the same though the extent of encapsulation and surface adsorption may vary between formulations). To investigate this matter further, the release profile of protein from DRVs and SAVs in PBS should provide further understanding on the nature of protein association as it would be expected that superficially encapsulated protein would be released from liposomes faster than protein encapsulated deeper within the liposome (section 2.4.10).

2.4.10 Determination of BSA retention to liposomal formulations at various time-points in PBS using an *in vitro* release study

The initial association to and subsequent retention of BSA by polymerised and non-polymerised liposomal formulations (which contained both liposomally associated and free BSA) was investigated following incubation (37 °C, 100 rpm) in PBS (pH 7.4) (see section 2.3.10) (figure 2.12). P DRVs, P SAVs and NP DRVs were prepared using the procedure as outlined in section 2.4.9 using BSA as a model protein (mixed with radio-labelled BSA as a tracer for quantification).

Figure 2.8 shows that the mean percentage of BSA associated initially (0 h) with polymerised DRVs and SAVs was 89.1 ± 2.4 $\%^{m}/_{m}$ and 85.6 ± 0.4 $\%^{m}/_{m}$ respectively (mean (mg protein associated to liposomes/total protein in formulation) ± SD), which were shown to be not significantly different ($p<0.05$) to each other. The mean percentage of BSA associated with non-polymerised DRVs was much lower (56 ± 5.0 $\%^{m}/_{m}$) and was significantly different ($p<0.05$) to the mean association to P DRVs and P SAVs. Significance was analysed using a one-way ANOVA and Dunnett's T3 test.

After 24 h of incubation of liposomal formulations in the release medium, the polymerised SAVs showed a much higher release of associated protein (77.7 ± 9.2 $\%^{m}/_{m}$ of protein associated) than non-polymerised DRVs (64.1 ± 2.6 $\%^{m}/_{m}$) resulting in their mean remaining liposomally associated BSA percentages not being significantly different (19.1 ± 8.0 $\%^{m}/_{m}$ and 20.2 ± 3.1 $\%^{m}/_{m}$ respectively) ($p<0.05$). Polymerised DRVs however, released only 47.6 ± 7.1 $\%^{m}/_{m}$ of their associated protein leaving a mean 46.8 ± 7.5 $\%^{m}/_{m}$ association which is significantly higher ($p<0.05$) than the mean

percentage remaining associated BSA to either P SAVs or NP DRVs. Release rates thereafter for protein from both non-polymerised DRVs and polymerised SAVs followed a similar pattern. The polymerised SAVs were no more effective at BSA retention post 24 h than non-polymerised DRVs ($p<0.05$).

Following 2 weeks (336 h) of incubation of liposomal formulations in release media, the mean percentage of BSA associated to P DRVs remained significantly higher ($p<0.05$) (23.6 ± 7.7 $\%^{m}/_{m}$) than the mean remaining BSA association with P SAVs or NP DRVs from which virtually all protein had been released. Significance was analysed using a one-way ANOVA and Tukey's HSD test.

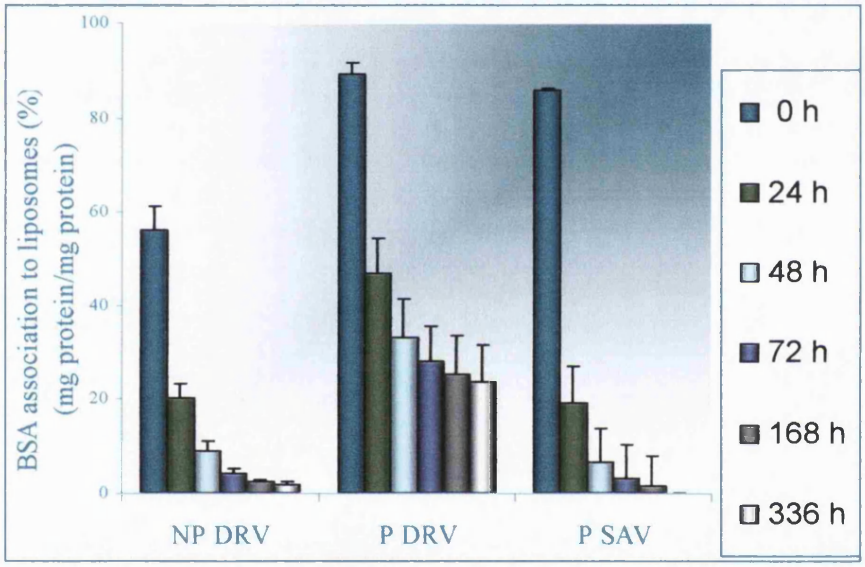

Figure 2.12: The BSA retention profile (expressed as the percentage of BSA associated to liposomes as opposed to free in solution) for various liposomal formulations (NP DRVs, P DRVs and P SAVs) in PBS pH 7.4, 37 °C, 100 rpm (n = 3). Results are means (± SD)

This difference in release pattern of liposomally associated BSA from DRVs and SAVs showed that there was a difference in the way that BSA was associated to the liposomes between the two formulations. In the case of polymerised liposomes, this may possibly be due to stronger BSA-phospholipid membrane interactions due to changes in liposome surface hydrophobicity and zeta potential, resulting in hydrophobic and electrostatic differences as well as a degree of internal BSA capture by the polymerised DRV. In conclusion, even though the percentage retention is approximately the same

between polymerised formulations (P DRVs and P SAVs) it is clear that the nature of the protein association to liposomes differs.

2.5 Conclusions

The adjuvant nature of liposomes was first reported by Allison and Gregoriadis (1974) who showed that intramuscular, intravenous and subcutaneous administration of liposomally encapsulated diphtheria toxoid raised higher serum antibody titres than when free diphtheria toxoid was administered alone. Many studies on the use of liposomes as vaccine adjuvants have been conducted since these early findings and are reviewed by Gregoriadis *et al.* (1988); Gregoriadis (1990); Childers and Michalek (1994) and Rogers and Anderson (1998).

Polymerised liposomes have been projected as more robust carriers for proteins than conventional non-polymerised liposomes (table 1.5). To date, numerous articles have been published regarding the synthesis of polymerisable lipids for the preparation of polymerised liposomes (O'Brien *et al.*, 1985; Hayward *et al.*, 1985; Regen, 1987 and Freeman and Chapman, 1988) though only a few authors have reported their use as vaccine adjuvants (Chen and Langer (1998) and Jeong *et al.*, 2002).

The effect of particle size on the uptake of particulate formulations by mucosal tissues following intranasal and oral administration was discussed in section 1.4.6.3 and 1.4.6.4 respectively and it was concluded that for nasal administration, a high systemic and mucosal immune response against a protein antigen would probably be achieved by administering the protein in liposomes with diameters of between 0.3-1 μm. With this in mind, liposomes with two particle size distributions were prepared using a freeze-thaw and extrusions procedure as used by Castile and co-workers (Castile *et al.*, 1999). The vesicles were shown to remain intact throughout processing stages such as centrifugation and lyophilisation (using trehalose as a cryoprotectant). The polymerisation of DODPC liposomes using UV light (254 nm) did not result in a significant ($p<0.05$) difference in vesicle size and had progressed to 88.0 % completion after 3 h 15 min, which was in agreement to the observations of Ohno and co-workers (Ohno *et al.*, 1987).

We believe that this is the first demonstration of the use of the DRV method to incorporate/associate protein to pre-polymerised liposomes. This brings new promise to protein/sensitive molecule formulation when using polymerised liposomes as an adjuvant/carrier, as exposure to the detrimental effects of free-radical or UV light (used in polymerisation) is avoided by utilising the DRV method. The association of BSA to non-polymerised SPC/SPG liposomes using the DRV method with cryoprotection (7.8 ± 0.3 $\%^{m}/_{m}$ (mg protein associated/mg initial protein added)) was shown to be significantly higher ($p<0.05$) than when the classical thin film hydration method was used (5.2 ± 0.3 $\%^{m}/_{m}$ (mg protein associated/mg initial protein added)). The use of trehalose as a cryoprotectant was shown to be essential in preserving the particle size of non-polymerised liposomes (table 2.5) during the lyophilisation stage of the DRV method.

The effect of the temperature during rehydration of lyophilised liposomes (after using the DRV method) was investigated and it was shown that high temperatures (70 °C) resulted in higher BSA (not significant higher ($p<0.05$)) and TT (significantly higher ($p<0.05$) association to polymerised liposomes though it was thought that a more ambient temperature (room temperature) could be used to simplify the formulation procedure. It was also shown that incremental rehydration resulted in a higher (though not significantly higher ($p<0.05$)) BSA and TT association to polymerised liposomes than when lyophilised liposomes were rehydrated in one step (instant hydration). On examining the effect of the theoretical loading of protein (amount of protein added to formulation as a percentage of the amount of phospholipid), it was shown that as theoretical loading was increased from 1 $\%^{m}/_{m}$ to 10 $\%^{m}/_{m}$ (mg protein/mg phospholipid) that the protein association to liposomes decreased from 56.8 ± 11.5 $\%^{m}/_{m}$ (mg protein association/mg initial protein added) to 36.6 ± 3.4 $\%^{m}/_{m}$ (mg protein association/mg initial protein added) though this was not significantly different ($p<0.05$).

Studies conducted on the effect of polymerisation of liposomes on protein association to vesicles showed that BSA associated to a significantly higher ($p<0.05$) extent to polymerised liposomes prepared using the DRV method (89.0 ± 1.6 $\%^{m}/_{m}$ (mg liposome associated protein/mg initial protein added)) or prepared by surface adsorption only (84.7 ± 5.1 $\%^{m}/_{m}$ (mg liposome associated protein/mg initial protein added)) in comparison to non-polymerised liposomes prepared using the DRV method (64.6 ± 7.5

$\%^{m}/_{m}$ (mg liposome associated protein/mg initial protein added)). The difference in the association of protein to polymerised and non-polymerised liposomal formulations was thought to be due to differing surface characteristics between formulations (table 2.10). Non-polymerised liposomal formulations were shown to possess a lower zeta potential (-31.0 ± 0.9 mV) and Rose Bengal partitioning quotient (0.849) than polymerised liposomal formulations (-22.0 ± 0.7 mV and 0.893 respectively). On investigating the release of BSA from polymerised and non-polymerised liposomal formulations, it was observed that P SAVs released more associated protein in the first 24 h than P DRVs resulting in the mean remaining protein association for P SAVs reducing to similar levels as NP DRVs. P DRVs however, retained a significantly higher ($p<0.05$) BSA association (46.8 ± 7.5 $\%^{m}/_{m}$ (mg remaining liposome-associated protein/mg total protein in formulation)) compared to P SAVs and NP DRVs (19.1 ± 8.0 $\%^{m}/_{m}$ and 20.2 ± 3.1 $\%^{m}/_{m}$ respectively (mg remaining liposome-associated protein/mg total protein in formulation), after 24 h. After 2 weeks, P DRVs retain a significantly higher ($p<0.05$) BSA association to vesicles (23.6 ± 7.7 $\%^{m}/_{m}$ (mg remaining liposome-associated protein/mg total protein in formulation)) compared to P SAVs and NP DRVs from which virtually all protein has been released. Thus, it can be concluded that there is a stronger BSA-liposome interaction when BSA is associated to polymerised liposomes using the DRV method with cryoprotection than when BSA was adsorbed to the surface of polymerised liposomes only. Both DRVs and SAVs may have their own benefits as carriers depending on the type of controlled release desired.

3.0 Comparison of immune responses to intramuscularly and intranasally delivered polymerised liposomal vaccines

3.1 Introduction

Intramuscular and deep subcutaneous administration routes are currently the most commonly utilised methods for the delivery of vaccines (British National Formulary, 2004). Reasons for such regimes have included the stimulation of high systemic responses to many infectious pathogens and the absence of economically viable effective alternatives. The general disadvantages associated with the invasive delivery of vaccines include pain at site of injection, need for trained medical personnel for administration and risk of transmission of blood borne diseases through needle-stick injuries and reuse of equipment. Manufacturing obstacles include the preparation of sterile vaccine solutions and cold-storage requirements for the traditional parenteral aluminium based adjuvant vaccines. The availability of non-invasive and self-administrable vaccines may increase the speed of mass vaccination campaigns and enhance patient compliance to immunisation regimes.

Mucosal immunisation offers many benefits over invasive methods. Practical advantages could include the self-administration of vaccines and from an immunological perspective there is the opportunity to elicit a mucosal response (section 1.4.6.7.2). Chen and Langer (1998) administered diphtheria toxoid encapsulated within polymerised DODPC vesicles *via* the oral route and showed that a systemic serum IgG response to the antigen was raised. However, the detection of any mucosal response and/or cell-mediated response was not reported. Other mucosal sites of administration using polymerised DODPC vesicles remain to be investigated.

The intranasal route benefits from the presence of fewer degradative enzymes than the gastrointestinal tract and is an easily 'accessible' mucosal site for drug and vaccine delivery purposes. Effective immune responses following intranasal vaccination have been shown by many authors (Almeida *et al.*, 1993; de Haan *et al.*, 1995a; Almeida and Alpar, 1996) with various carriers. Studies by de Haan and co-workers (de Haan *et al.*, 1995a) demonstrated the induction of both a serum IgG and a mucosal secretory IgA

response to a liposomal influenza virus subunit (aggregates of viral haemagglutinin and neuraminidase derived from H1N1, A/Chile/83 virus and H3N2) vaccine formulation which when administered nasally was superior to that elicited by nasal delivery of the free antigen alone.

The studies conducted in this chapter aim to investigate the difference *in vivo* not only of vesicle formulation parameters such as size of vesicles (discussed in section 1.4.6.3, section 1.4.6.4 and 1.4.6.5), theoretical loading (the ratio of initial protein added to phospholipid), polymerised versus non-polymerised vesicles (see section 1.6) and the effect of protein localisation in the liposome (surface adsorbed protein compared to internalised protein) but also to compare the difference in immune responses when the same formulation is presented to the body using a different route (i.e. intramuscular *versus* intranasal).

3.2 Materials

3.2.1 Animals used for immunisation studies

Female BALB/C mice (6-8 weeks old) were purchased from Harlan Olac (Blackthorn, U.K.) and were acclimatised for 1 week prior to use. The animals were allowed food and water *ad libitum* for the course of the experiments.

3.2.2 Materials for the procedure for detection of antibody production using an Enzyme Linked Immunosorbent Assay (ELISA)

Immulon® 2 HB flat bottom Microtiter® plates were purchased from Thermo Labsystems (Franklin, USA). The goat anti-mouse antibody (IgG, IgG_1, IgG_{2A}, IgA and IgM) horseradish peroxidase conjugates were obtained from Serotec Ltd. (Kidlington, UK).

Solutions required for the ELISA were prepared fresh and were of the following composition:

- PBS (pH 7.4): One PBS tablet (Sigma-Aldrich Co. (Poole, England)) dissolved in 200 mL deionised water.
- Wash buffer (PBS-T): 40 g sodium chloride, 1 g potassium chloride, 7.21 g Di-sodium hydrogen orthophosphate, 1 g potassium di-hydrogen orthophosphate, 2.5 mL Tween® 20, deionised water to 5 L.
- Block buffer: 4 $\%^{m}/_{v}$ BSA in PBS (pH 7.4).
- Citrate buffer (pH 4.0): 4.6 g citric acid, 9.78 g Di-sodium hydrogen orthophosphate, deionised water to 500 mL.
- Substrate solution: One tablet of 2,2'-Azino-bis(3-Ethylbenzthiazoline-6-sulfonic acid) tablets (diammonium salt) (ABTS) is dissolved in 15 mL of citrate buffer. Immediately prior to use 1.5 μL of hydrogen peroxide (30 $\%^{v}/_{v}$) was added.

3.2.3 Materials for the procedure for determination of cytokine production after stimulation of spleen cell suspension (of naïve and immunised mice) with soluble TT using an ELISA

RPMI 1640 medium (with L-glutamine), Foetal Bovine Serum (FBS) and L-glutamine were purchased from Invitrogen Ltd. (Paisley, UK). Penicillin-streptomycin solution Hybri-Max® was obtained from Sigma-Aldrich Company Ltd. (Poole, UK). Nunclon™ Surface tissue culture microwell 96F plates were purchased from Nunc™ A/S (Roskilde, Denmark).

Immulon® 2 HB flat bottom Microtiter® plates were purchased from Thermo Labsystems (Franklin, USA). The DuoSet® ELISA development kit for interleukin-2 (IL-2), IL-4, IL-6, IL-12, tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) and the substrate solution pack was obtained from R&D Systems, Inc. (Abingdon, UK).

Solutions required for the determination of cytokine production using an ELISA were prepared fresh and were of the following composition:

- PBS and wash buffer (pH 7.2-7.4):(section 3.2.2)
- Block buffer: 1 $\%^{m}/_{v}$ BSA, 5 $\%^{m}/_{v}$ sucrose in PBS

- Reagent diluent:
  IL-4, IL-6, IL-12 and TNF-α: 1 $\%^{m}/_{v}$ BSA in PBS (pH 7.2-7.4)
  IL-2 and IFN-γ: 0.1 $\%^{m}/_{v}$ BSA, 0.05 $\%^{v}/_{v}$ Tween® 20 in Tris-buffered saline (20 mM Trizma® base, 150 mM NaCl) (pH 7.2-7.4)
- Substrate solution: Color Reagent A and Color Reagent B (hydrogen peroxide and tetramethylbenzidine respectively) are mixed in equal volumes just prior to use.

3.2.4 Miscellaneous chemicals and reagents

Aluminium Hydroxide Gel adjuvant (Alhydrogel® 2 $\%^{m}/_{v}$) was purchased from Superfos Biosector (Frederikssund, Denmark). All chemicals and reagents not specified in the text were supplied by Sigma-Aldrich Company Ltd. (Poole, England).

3.3 Methods

3.3.1 Liposomal and non-liposomal formulations used for intramuscular and intranasal delivery of tetanus toxoid

Liposomes were formulated to contain 10 μg of TT in total (liposome associated and free) *per* priming dose and 5 μg TT (liposome associated and free) for booster doses using either the DRV method for protein association to liposomes (section 1.5.5) or by protein surface adsorption alone. Polymerised DRVs (P DRVs) were compared to their non-polymerised counterparts (NP DRVs) for assessment of the effect of polymerising the bi-layers on vaccine adjuvanticity.

Liposomal formulations contained DODPC as the sole constituent phospholipid and were prepared as described in detail in section 2.3.1. In brief, 600 mg of DODPC was added to 150 mL pre-warmed (50 °C) *t*-butanol (4 mg phospholipid/mL *t*-butanol) and stirred until dissolved. Aliquots of 5 mL (containing 20 mg of DODPC) were added to 20 mL clear freeze-drying vials and sealed using 20 mm freeze-drying stoppers and frozen in liquid nitrogen (<195.79 °C, 1 min). The stoppers were then removed and the vials covered with Parafilm® M film, perforated with 12 needle width holes and lyophilised for 48 h using the Edwards Micro-Modulyo under vacuum drawn by an

Edwards E2M5 high vacuum pump. The resulting phospholipid 'cake' was then rehydrated with water to ~10 mg/mL (mg phospholipid/mg water) and stirred at 360 rpm for 2 h at room temperature using a magnetic stirrer plate and bead. The particle size distribution and structure of the liposome suspension thus formed was modified (section 2.3.2) by using freeze-thawing and extrusions. Liposomes of two particle size distributions were prepared. Vesicles with a mean hydrodynamic diameter of approximately 100 nm were produced by serial extrusion through polycarbonate membranes with decreasing pore diameters (5 extrusions through membranes with a pore diameter of 1 μm, 0.6 μm and 0.2 μm and finally 10 extrusions through filter membranes with pore diameters of 0.1 μm) and will be termed as possessing a narrow particle size distribution throughout the remainder of the thesis. Vesicles with a higher mean hydrodynamic diameter and broader particle size distribution were prepared by 10 extrusions through membranes with a pore diameter of 1 μm only and will be termed as possessing a wider particle size distribution throughout the remainder of the thesis. Liposomes were then freeze-thawed 10 times before repeating the extrusion step as before to yield uni-/oligo-lamellar DODPC liposomes. In the case of polymerised DODPC liposomes, the samples were then placed into quartz vessels before subjecting to UV light (254 nm) using the Rayonet photochemical mini-reactor (RMR-600) for 3 h 15 min (section 2.3.4 and 2.4.6).

The vesicles were then lyophilised by freezing in liquid nitrogen (<195.79 °C, 1 min) and attaching to the manifold of an Edwards Micro-Modulyo drier, under vacuum drawn by an Edwards E2M5 high vacuum pump for 48 h. In the case of liposomes where TT was added to the formulation using the dehydration-rehydration vesicle method (DRV - section 1.5.5), the protein was added to the liposome formulation just prior to the lyophilisation stage. DRV formulations with TT theoretical loadings of 1 $\%^{m}/_{m}$ (mg TT/mg phospholipid) and 5 $\%^{m}/_{m}$ (mg TT/mg phospholipid) were produced. DRVs were then rehydrated by incremental addition of saline; 100 μl twice and 0.8 mL the third time with 30 min incubations (room temperature) between each hydration. In the case of liposomes where TT was added to the liposomes so that only surface adsorption occurred, the vesicles (SAVs - section 1.5.5) were first redispersed after lyophilisation by instant rehydration with saline at room temperature and then the resultant suspension rocked at 20 rpm for 1 h to permit complete liposome formation. SAV formulations with a TT theoretical loading of 5 $\%^{m}/_{m}$ (mg protein added/mg phospholipid) were produced.

TT adsorbed aluminium hydroxide gel adjuvant was used as a positive control. In brief, 200 μg of TT was added to 1 mg of $Al(OH)_3$ (Alhydrogel®) and vortexed together for 5 min in a total volume of 1 mL saline solution. The TT was allowed to adsorb to the aluminium hydroxide gel for a further 1 h at room temperature prior to administration. The intramuscular administration volume of 50 μL represents a dose of 10 μg TT (free and adsorbed) and 50 μg aluminium hydroxide gel. Free TT in saline was used an adjuvant free control for both intranasal and intramuscular administration. A summary of formulations for intramuscular administration is given in table 3.1 and for intranasal administration in table 3.2.

Table 3.1: Liposomal and non-liposomal formulations used for the intramuscular administration of tetanus toxoid

Key: P =polymerised, NP = non-polymerised, DRV = dehydration-rehydration vesicle, SAV = surface adsorbed vesicle, 5 % = theoretical loading (mg TT/ mg phospholipid)

Abbreviation of formulations	Description of formulations
P DRV 5 % 1μm	Polymerised Dehydration-Rehydration Vesicles containing associated and free TT (5 $\%^{m}/_{m}$ theoretical loading) produced using an extruder filter membrane with 1 μm pore diameter
P SAV 5 % 1μm	Polymerised Surface Adsorbed Vesicles containing surface associated and free TT (5 $\%^{m}/_{m}$ theoretical loading) produced using an extruder filter membrane with 1 μm pore diameter
NP DRV 5 % 1μm	Non-Polymerised Dehydration-Rehydration Vesicles containing associated and free TT (5 $\%^{m}/_{m}$ theoretical loading) produced using an extruder filter membrane with 1 μm pore diameter
$Al(OH)_3$/TT	10 μg TT and 50 μg $Al(OH)_3$ (20 $\%^{m}/_{m}$ theoretical loading (mg TT/ mg aluminium hydroxide))
Free TT	10 μg TT in solution

All formulations were delivered in saline in a volume of 50 μL to hind leg quadriceps

Table 3.2: Liposomal and non-liposomal formulations used for the intranasal administration of tetanus toxoid

Abbreviation of formulations	Description of formulation
P DRV 5 % 1μm	Polymerised Dehydration-Rehydration Vesicles containing associated and free TT (5 $\%^{m}/_{m}$ theoretical loading) produced using an extruder filter membrane with 1 μm pore diameter
P DRV 1 % 1μm	Polymerised Dehydration-Rehydration Vesicles containing associated and free TT (1 $\%^{m}/_{m}$ theoretical loading) produced using an extruder filter membrane with 1 μm pore diameter
P SAV 5 % 1μm	Polymerised Surface Adsorbed Vesicles containing surface associated and free TT (5 $\%^{m}/_{m}$ theoretical loading) produced using an extruder filter membrane with 1 μm pore diameter
NP DRV 5 % 1μm	Non-Polymerised Dehydration-Rehydration Vesicles containing associated and free TT (5 $\%^{m}/_{m}$ theoretical loading) produced using an extruder filter membrane with 1 μm pore diameter
P DRV 5 % 0.1μm	Polymerised Dehydration-Rehydration Vesicles containing associated and free TT (5 $\%^{m}/_{m}$ theoretical loading) produced using an extruder filter membrane down to a size of 0.1 μm pore diameter
P DRV 1 % 0.1μm	Polymerised Dehydration-Rehydration Vesicles containing associated and free TT (1 $\%^{m}/_{m}$ theoretical loading) produced using an extruder filter membrane down to a size of 0.1 μm pore diameter
Free TT	TT in solution

All formulations were delivered in saline in a volume of 20 μL

3.3.2 Immunisation protocol for the delivery of liposomal and non-liposomal TT formulations by the intramuscular route

Groups of five mice were immunised by intramuscular injection to alternate hind leg quadriceps using a 50 μL injection volume. The mice were primed with 10 μg of TT free in saline or formulated with aluminium hydroxide or liposomes and delivered in suspension in saline (as detailed in table 3.1) and boosted on day 35 with 5 μg of TT in the same formulation (by diluting priming formulations with an equal volume of saline). Tail bleeds were taken on days 7, 14 and 28 and sera prepared for TT-specific antibody analysis as described in section 3.3.4. At the end of the study, spleens were removed and processed as detailed in section 3.3.7 for the analysis of cytokine production.

3.3.3 Immunisation protocol for the delivery of liposomal and non-liposomal TT formulations by the intranasal route

Groups of five mice were placed into an inhalation anaesthesia chamber and anaesthetised using isoflurane (a volatile liquid anaesthetic) at a rate of 2-3 L *per* minute using oxygen as a carrier gas (1.5 L *per* minute). The mice were anaesthetised to prevent the gag reflex on intranasal delivery of formulations. TT (either free in saline or formulated with liposomes and delivered in suspension in saline) was administered in a 20 μL volume by drop-wise application to alternate nostrils using a 20 μL pipette. The mice were primed with 10 μg of free or formulated TT in saline (as detailed in table 3.2) and boosted on day 35 and 59 with 5 μg of TT in the same formulation (by diluting priming formulations with an equal quantity of saline). Tail bleeds were taken on days 7, 14, 28, 49 and 69 and sera prepared for TT-specific antibodies as described in section 3.3.5. Faecal pellets were collected on day 69 and processed as detailed in section 3.3.6. At the end of the study, spleens were removed and processed as detailed in section 3.3.7 for the analysis of cytokine production.

3.3.4 Procedure for collection and preparation of serum from mice for antibody analysis

Prior to collecting bleeds, mice were transferred to an incubator (37 °C) for 5 min to encourage vein dilatation and a razor blade used to 'nick' the tail vein. Approximately 100 μl of blood was collected (whilst keeping the mouse under a strong heat-emitting lamp to prevent the nick from clotting) using 100 μL heparin coated (1 $\%^{m}/_{v}$) capillary tubes and transferred to micro-centrifuge tubes. The blood was allowed to clot (either by storage overnight at 4 °C or 2 h at 37 °C) and serum removed by centrifugation at 21,000 g for 10 min and stored at –20 °C until required.

3.3.5 Procedure for collection and processing of faecal stools for secretory IgA analysis

Mice were transferred to a metabolic chamber and 10 faecal pellets were collected fresh from each group and kept on ice. Faecal pellets were suspended in PBS (10 μL PBS/mg

pellet) and crushed with a glass hand-held homogeniser. The suspension was then vortexed before being flash-frozen in liquid nitrogen (<195.79 °C, 1 min). The mixture was then allowed to thaw on ice and transferred to micro-centrifuge tubes and spun at 21,000 g for 15 minutes. The supernatant was collected and stored at –20 °C until required.

3.3.6 Procedure for determination of antibody titres using an Enzyme Linked Immunosorbent Assay (ELISA)

Serum TT-specific antibodies were detected using a modified microplate ELISA:

1. Immulon® plates were coated with 50 μl of TT (1 μg TT/50 μL PBS (pH 7.4)) *per* well and incubated overnight at 4 °C.
2. The solution was removed from the wells by inversion and the plates 'washed' three times with PBS-T and once in deionised water by immersion into the relative solution and subsequent agitation by tapping each side of the plate twice using the palm of the hand before removal of the solution by plate inversion. After the final wash, the plates were dried by inverting and blotting against clean paper towels and placed in a 37 °C incubator for 5 min.
3. Block buffer (100 μL) was added to each well and the plates were incubated for 1 h at 37 °C. The plates were then washed and dried as in step 2.
4. Serum samples diluted in PBS (initial dilution) were then placed into the first column of each plate typically as a 1 in 16 to a 1 in 640 dilution and then serially diluted (1 in 2) in PBS along each plate row so that the final volume in each well was 50 μL. The initial dilution was estimated so that within subsequent serial dilutions, the sample antibody concentrations would be reduced to the levels of the naïve standard serum obtained from non-immunised mice (used on each plate as a negative control). The plates are then incubated for 1 h at 37 °C. The plates were then washed and dried as in step 2.
5. Antibody conjugate (50 μL), appropriate to the antibody being investigated, was added to each well as *per* the following dilutions:

IgG: 1 in 1,000
IgM: 1 in 3,000

IgA: 1 in 2,000

IgG_1: 1 in 2,000

IgG_{2a}: 1 in 2,000

The plates were then incubated for 1 h at 37 °C and then washed and dried as in step 2.

6. Substrate solution (50 μL) was added to each well and colour development was allowed to occur for 30 min at 37 °C. The plates were then read at 405 nm and antibody titres calculated by comparison to the naïve standard serum on each plate.

3.3.7 Preparation of spleen cell suspensions from naïve and immunised mice and procedure for *in vitro* stimulation with soluble TT

At the end of the study (70 days post priming dose), all animals were killed by cervical dislocation and the spleens removed and placed into sterile PBS on ice. Pooled spleens were then gently pressed and ground through a fine wire strainer. RPMI 1640 was used as the working media and was supplemented with 20 mM L-glutamine, FBS (10 $\%^{v}/_{v}$), 10^5 U of penicillin/L and 100 mg of streptomycin/L. The spleen cells were added to 10 mL of this working media. The cell suspensions were allowed to settle for at least 10 min and they were then transferred (less the sedimented debris) to sterile Falcon tubes. These were spun at 200 g for 10 min and the pellet resuspended in 15 mL of fresh working media, centrifuged as before and the new pellet resuspended again in 15 mL of fresh working media. The cells (100 μL) were then seeded in triplicate into sterile 96 well tissue culture plates holding 100 μL volumes of working media containing either TT (200 μg/mL), concanavalin A (1 μg/mL) as a positive control or PBS as a negative control. The plates were covered and placed into a humidity controlled incubator (5 % CO_2) at 37 °C for 48 h. Supernatants were removed from each of the microcultures and stored at –70 °C until required.

3.3.8 Procedure for determination of cytokine production during co-culture of spleen cell suspension (of naïve and immunised mice) with soluble TT using an ELISA

The production of IL-2, IL-4, IL-6, IL-12, TNF-α and IFN-γ was measured using a modified procedure as directed by the Duoset® ELISA Development kit.

1. Immulon® 96 well flat bottom microtitre plates were coated with the capture antibody (diluted to the working concentration in PBS as directed by the manufacturer) and left overnight at room temperature.
2. The solution was removed from the wells by inversion and the plates 'washed' three times with PBS-T and once in deionised water by immersion into the relative solution and subsequent agitation by tapping each side of the plate twice using the palm of the hand before removal of the solution by plate inversion. After the final wash, the plates were dried by inverting and blotting against clean paper towels and placed in a 37 °C incubator for 5 min.
3. Block buffer (200 μL) was added to each well and plates were incubated at room temperature for 1 h. The plates were then washed and dried as in step 2.
4. Standards (100 μL) were placed in duplicate on each plate and serially diluted (1 in 2) using reagent diluent. Samples and blank reagent diluent (100 μL) were also plated. After 2 h incubation at room temperature, the plates are washed and dried as in step 2.
5. 100 μL of the appropriate detection antibody is added to each well and the plates are incubated again for 2 h at room temperature before being washed and dried as in step 2.
6. Streptavidin-HRP (100 μL of the working dilution) was added to each well and plates incubated for 20 min at room temperature. The washing and drying procedure in step 2 was repeated.
7. Substrate solution (100 μL) was added to each well and the plates incubated for 20 min at room temperature. To terminate the reaction, 50 μL of 2 N H_2SO_4 was added to each well and the plates read at 450 nm with wavelength correction set at 540 nm or 570 nm.

3.4 Results and discussion

3.4.1 Determination of the mean hydrodynamic diameter and size distribution of liposomal and non-liposomal formulations used for the intramuscular and intranasal delivery of tetanus toxoid

The mean hydrodynamic diameter for liposomal and non-liposomal formulations prepared for intramuscular and intranasal administration (section 3.3.1) was determined using PCS (section 2.3.6.1) (table 3.3).

Table 3.3: Determination of the mean hydrodynamic diameter and size distribution of liposomal and non-liposomal formulations used for the intramuscular and intranasal delivery of tetanus toxoid using PCS (n=3)

Abbreviation for formulation	Mean hydrodynamic diameter (nm) (± SD)	Polydispersity index (± SD)
P DRV 5 % 1 μm	236.3 (4.7)	0.386 (0.034)
P DRV 1 % 1 μm‡	229.6 (6.9)	0.379 (0.029)
P SAV 5 % 1 μm	238.1 (5.2)	0.394 (0.038)
NP DRV 5 % 1 μm	242.4 (4.6)	0.397 (0.009)
P DRV 5 % 0.1 μm‡	98.3 (0.4)	0.242 (0.005)
P DRV 1 % 0.1 μm‡	99.6 (0.4)	0.243 (0.008)
$Al(OH)_3$/TT†	723.9 (26.8)	0.176 (0.061)

Key (refer to table 3.1 and table 3.2 for further information): P =polymerised, NP = non-polymerised, DRV = dehydration-rehydration vesicle, SAV = surface adsorbed vesicle, 1% or 5 % = theoretical loading (mg TT/mg phospholipid), 1 μm = liposomes with a wide particle size distribution prepared using an extruder filter with 1 μm pore diameter, 0.1 μm = liposomes with a narrow particle size distribution prepared using an extruder filter with 0.1 μm final pore diameter

† intramuscular study only

‡ intranasal study only

Liposomes with a wider particle size distribution, prepared using a polycarbonate membrane with pore diameters of 1 μm during the extrusion stage were determined to have a significantly higher (one way ANOVA, Dunnett's T3 test) mean hydrodynamic diameter than liposomes with a more narrow size distribution which were extruded

serially through filter membranes with decreasing pore diameters (smallest pore diameter of 0.1 μm).

Liposomes prepared by using the 1 μm pore diameter filter membranes for extrusion had a broad particle size distribution (truncated at upper end at 1 μm due to extrusion) and thus the value of the mean hydrodynamic diameter is not an ideal method for particle size distribution characterisation.

The volume distribution plot of the liposomes is a better tool for understanding the distribution of particle size within the formulations when polydisperse systems are to be analysed. The volume distribution plot represents the distribution of the total volume of liposomes against liposome size. Examples of such distribution traces are shown in figures 2.3 and 2.4 and are different to the intensity distribution plots usually reported. The intensity distribution plot represents the distribution of the total intensity of scattered light against particle size and is not a useful method for looking at polydisperse particle size distributions as larger particles scatter less light than smaller particles at 90 ° (the angle of detection used in the ZetaMaster 2000). Thus, although the mean hydrodynamic diameter is much lower than the pore size of the filter membrane used in the case of the liposomes with a wide particle size distribution, the volume distribution (not shown) shows that the majority of the 'volume' created in the formulation is due to much larger than mean size liposomes. The lower than expected mean hydrodynamic diameter value suggests that a large number of smaller liposomes are produced and skew the result to the lower end. The analysis of the liposome with a narrow particle size distribution shows that the peak particle size in both volume distribution and intensity distribution plots is about 100 nm which is due to the absence of any larger particles that would otherwise skew the volume distribution plot.

Thus, though the use of two extrusion procedures (one using filter membranes with final pore diameters of 0.1 μm and the other using filter membranes with pore diameters of 1 μm) has produced particles with not very different mean hydrodynamic diameters (as determined using PCS), it is evident that this is not a true reflection of the true particle size distribution. The larger liposomes present in the polydisperse formulations may have a large impact on administration of the formulation for *in vivo* immunisation studies.

3.4.2 Determination of TT retention to liposomal formulations at various time-points in PBS using an *in vitro* release study

The initial association and subsequent retention of TT with polymerised and non-polymerised liposomal formulations (which contain both liposomally associated and free TT) was investigated (figure 3.1) following incubation (37 °C, 100 rpm) in PBS (pH 7.4) (see section 2.3.10). Polymerised and non-polymerised DODPC liposomes with a wide particle size distribution were prepared as detailed in section 3.3.1. A theoretical loading of 1 $\%^{m}/_{m}$ TT (mg initial TT added/ mg phospholipid) was used for all formulations mixed with radio-labelled TT as a tracer for quantification.

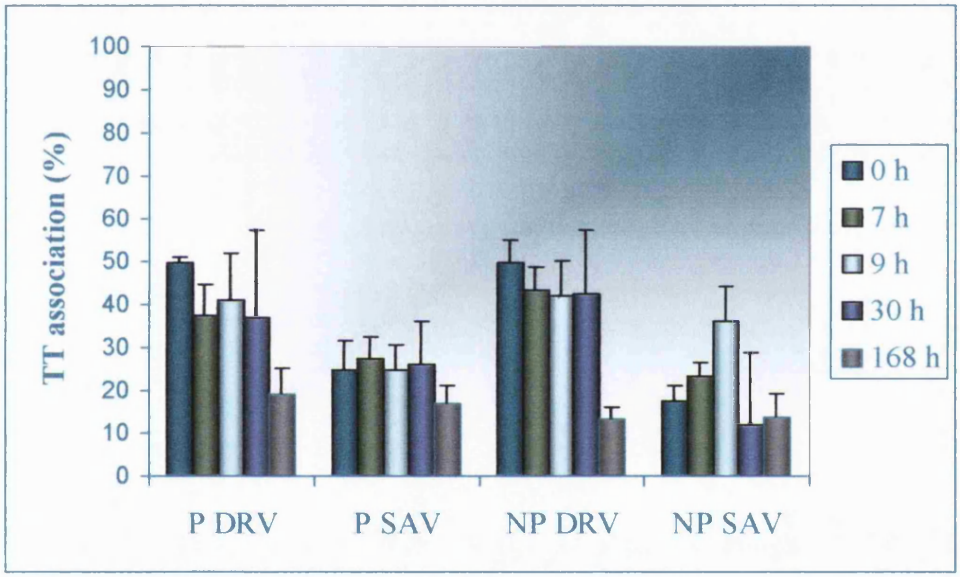

Figure 3.1: The TT retention profile (expressed as the percentage of TT associated to liposomes as opposed to free in solution) for various liposomal formulations (P DRVs, P SAVs, NP DRVs and NP SAVs) in PBS pH 7.4, 37 °C, 100 rpm (n = 3). Results are means (± SD)

Key (refer to table 3.1 and table 3.2 for further information): P =polymerised, NP = non-polymerised, DRV = dehydration-rehydration vesicle, SAV = surface adsorbed vesicle

The mean percentage of TT associated initially (0 h) with the P SAV formulation was only 24.7 ± 6.6 $\%^{m}/_{m}$ (mean (mg protein associated to liposome/ mg total protein in formulation) ± SD). This meant that ~75.3 $\%^{m}/_{m}$ of the total TT in the P SAV formulation was in the free form in solution. In comparison, the mean initial percentage of TT associated to P DRVs and NP DRVs was 49.7 ± 1.2 $\%^{m}/_{m}$ and 49.6 ± 5.3 $\%^{m}/_{m}$

respectively. Both DRV formulations (P DRVs and NP DRVs) were determined to have a significantly higher ($p<0.05$) initial TT association than either of the SAV preparations (P SAVs or NP SAVs). There was found to be no significant difference ($p<0.05$) in the initial percentage of protein associated to vesicles that were polymerised in comparison to non-polymerised vesicles (i.e. initial percentage of TT associated to P DRVs was not significantly different to the initial percentage of TT associated to NP DRVs and the initial percentage of TT associated to P SAVs was not significantly different to the initial percentage of TT associated to NP SAVs.

Following 7 h of incubation of the liposomes in the release medium, the mean percentage of TT associated with P DRVs (37.2 ± 7.5 $\%^{m}/_{m}$) was higher than the mean percentage of TT associated with P SAVs, though this no longer remained significantly different ($p<0.05$). This may be due to a greater initial release/desorption of TT from the liposomes for the former formulation resulting in a similar percentage mass of antigen remaining associated to liposomes for both P DRVs and P SAVs at the 7 h time point. However, the NP DRV formulation still had a significantly ($p<0.05$) higher percentage of TT associated (43.0 ± 5.4 $\%^{m}/_{m}$) to it than both P SAVs (27.1 ± 5.1 $\%^{m}/_{m}$) and NP SAVs (23.3 ± 3.2 $\%^{m}/_{m}$). The NP DRV formulation did not show the same initial TT release/desorption from liposomes as the P DRV formulation. This may possibly be due to a deeper TT incorporation inside the vesicles for NP DRVs (due to more protein entering the aqueous compartments of liposomes rather than being surface-associated) than P DRVs during the lyophilisation stage in formulation preparation.

At later time points, i.e. beyond 7 h (9h, 30 h and 168 h) there were no significant difference ($p<0.05$) in the percentage of TT remaining associated to vesicles between any of the formulations. It is notable that the percentage of TT associated to NP SAVs was increasing at initial time points (7 h, and 9 h) which may possibly be due to extra vesicular TT (free TT) still penetrating or associating (by adsorption) with the vesicles before it's subsequent release. Significance was analysed using a one-way ANOVA and Tukey's HSD test.

The trends seen with the initial (0 h) association of TT with polymerised and non-polymerised liposomal formulations (as opposed to free in solution) is in contrast to that observed when BSA was used as the model protein (section 2.4.10) for loading/release

studies from these liposomes. The initial percentage of BSA associated to liposomes was highest for the polymerised liposomes (89.1 ± 2.4 $\%^{m}/_{m}$ for P DRVs and 85.6 ± 0.4 $\%^{m}/_{m}$ for P SAVs) and much lower for NP DRVs (56 ± 5.0 $\%^{m}/_{m}$). In comparison, the initial percentage of TT associated to liposomal formulations was 49.7 ± 1.2 $\%^{m}/_{m}$ for P DRVs, 24.7 ± 6.6 $\%^{m}/_{m}$ for P SAVs and 49.6 ± 5.3 $\%^{m}/_{m}$ for NP DRVs (i.e. the percentage of liposomally associated TT was higher for liposomes prepared using the DRV method regardless of whether the phospholipid bi-layers were polymerised or not).

As the composition of the liposomal formulations and experimental conditions for the release study were the same for investigation of both BSA and TT, the differences seen in the initial associated percentages and subsequent release of proteins is most probably due to the nature of the proteins. In their experiments investigating the surface adsorption and subsequent release of TT and BSA to poly(lactide) microspheres, Alpar and Almeida (1994) attributed the higher BSA adsorption to microspheres (in comparison to TT adsorption) and differences in release profiles to factors such as variation between proteins in the degree of van der Waals interactions, hydrophobic interactions and hydrogen bond formation between the protein and the carrier surface. In addition the differences in molecular weight of BSA and TT will be an important consideration when comparing encapsulation/adsorption differences.

The experiments conducted in section 2.4.9 on BSA association to liposomes used a theoretical loading of 5 $\%^{m}/_{m}$ (mg protein/mg phospholipid) and in the experiments on TT association to liposomes, the theoretical loading was only 1 $\%^{m}/_{m}$ (mg protein/ mg/phospholipid). Even so, there was a higher BSA than TT association to polymerised liposomes when using the DRV method, which may be due to the higher molecular weight of TT. As a result, TT would be more difficult to entrap than BSA (a smaller protein) as the passage of larger molecules into the aqueous compartments of liposomes through bi-layers may be more difficult than the passage of smaller molecules and that fewer larger molecules could fit into a given space.

BSA (67 kDa) and TT (150 kDa) have isoelectric points of pH 4.7 and pH 6.2 respectively. The isoelectric point (pI) of a protein is that pH at which the protein has no net charge (positive charges equal negative charges). At a high pH (above the protein isoelectric point), there will be a net negative charge and at a low pH (below the

protein isoelectric point) there will be a net positive charge. Thus, in the case of BSA and TT, the former was further from it's pI at pH 7.4 and should be more negative in terms of net protein charge than the latter. Hence, it may be that the net negatively charged BSA would associate to a greater extent to the less negatively charged polymerised liposomes (-22.0 ± 0.7 mV) than the more negatively charged non-polymerised liposomes (-31.0 ± 0.9 mV) as determined using laser Doppler velocimetry (table 2.10). In the case of TT, which at pH 7.4 is only just above it's pI, the TT may possess only a small overall negative charge and so the surface charge of liposomes may not be as important a factor in protein association and hence in this case, this could possibly be why the protein associates to non-polymerised and polymerised liposomes in approximately equal amounts.

The retention of BSA to liposomal formulations after incubation in release media also differed to the release of TT under the same conditions. Whereas the retention of BSA to P DRVs (23.6 ± 7.7 $\%^{m}/_{m}$ of total BSA) remained significantly higher ($p<0.05$) compared to P SAVs and NP DRVs after 2 weeks, there was no significant differences ($p<0.05$) in the retention of TT to liposomal formulations after 7 h. Significance between groups was analysed using a one-way ANOVA and Tukey's HSD test.

Thus, the differences in the initial protein association to various liposomal formulations and subsequent retention of protein in release media for two different model proteins (BSA and TT) illustrates that the loading and subsequent release characteristics of any particular protein antigen from a liposomal formulation cannot be assumed to be applicable for other protein antigens. Factors such as the molecular weight, the hydrophobic composition and the isoelectric point of a protein antigen are three factors that may govern the characteristics of the resultant formulation. The polymerisation of liposome bi-layers may lead to a higher retention of protein by the liposome due to a decreased leakage of the encapsulated portion into the release media (table 1.5) due to more robust bi-layers or may also be due to differences in surface characteristics such as surface charge.

3.4.3 Systemic (humoral) immune response to liposomal and non-liposomal TT formulations following intramuscular administration

Serum IgM (see section 1.1.2) antibody production is generally an early indicator of the activation of an immune response following the administration of an immunogenic agent (Playfair and Chain, 2001). Figure 3.2 shows the TT-specific serum IgM antibody titres (determined using an ELISA) following the intramuscular administration of 10 μg of TT as a priming dose, in various liposomal and non-liposomal formulations. Mice were boosted intramuscularly on day 35 with 5 μg of TT in the same formulations (by diluting priming formulations with an equal volume of saline).

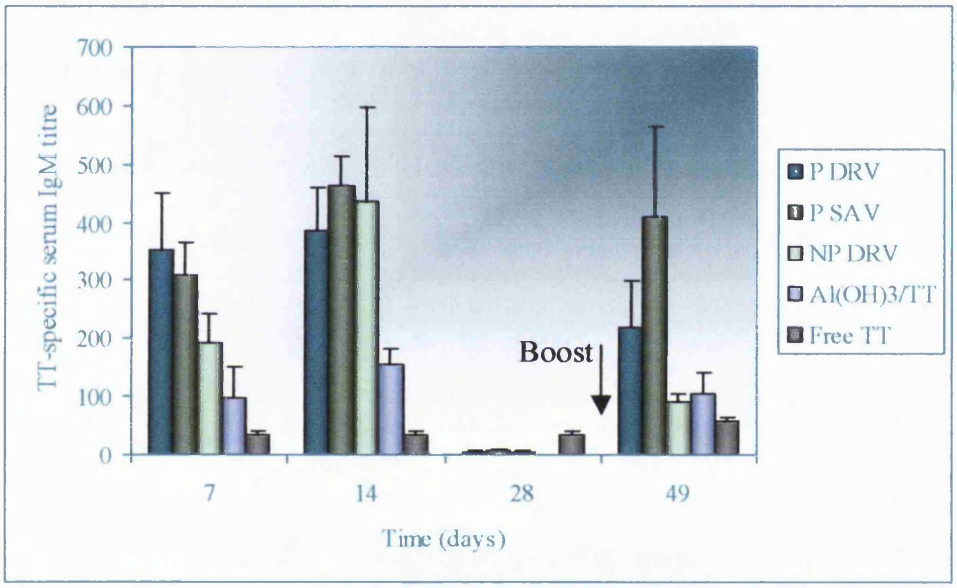

Figure 3.2: TT-specific serum IgM antibody titres raised in mice in response to intramuscular administration of liposomal and non-liposomal formulations of TT (n=5). Mice were primed with 10 μg of formulated TT and boosted on day 35 with 5 μg of formulated TT. Results are mean titres (± SE)

Key (refer to table 3.1 for further information): P =polymerised, NP = non-polymerised, DRV = dehydration-rehydration vesicle, SAV = surface adsorbed vesicle

Mean early TT-specific serum IgM antibody titres on day 7 were highest in animals given liposomal preparations (though results were not significantly different ($p<0.05$)) followed by $Al(OH)_3$/TT (96 ± 54) (mean titre ± SE) and then free TT (32 ± 9). Administration of polymerised liposomes (P DRVs and P SAVs) was shown to elicit higher mean TT-specific serum IgM response (352 ± 96 and 307 ± 57 respectively) than

non-polymerised liposomes (NP DRVs) (192 ± 48). All liposomal preparations (P DRVs, P SAVs and NP DRVs) continued to show higher mean TT-specific serum IgM responses on day 14 (384 ± 74, 461 ± 51 and 435 ±160 respectively) compared to conventional $Al(OH)_3$/TT (154 ± 26) and free TT (32 ± 9), though results were not significantly different ($p<0.05$) but by day 28, serum titres for all groups had dropped to base-line levels. Two weeks post boosting (boosted on day 35) mean TT-specific serum IgM titres had again risen for groups immunised with polymerised vesicles but only slightly for groups immunised with non-polymerised vesicles (90 ± 60), $Al(OH)_3$/TT (102 ± 38) or free TT (14 ± 6). Mice immunised with P SAVs displayed a mean TT-specific serum IgM titre about twice of that by P DRVs (410 ± 154 and 218 ± 80 respectively). This indicated that the TT-specific serum IgM response to polymerised liposomal formulations (and non-polymerised formulations on day 14 only) is higher though not significantly ($p<0.05$) than the antibody response induced by $Al(OH)_3$/TT or free TT. Though the TT-specific serum IgM antibody titres determined are of similar levels to or much lower than the TT-specific serum IgG titres (figures 3.3 and 3.4 respectively), IgM is more potent in antigen binding and complement activation than IgG and so the low titres of IgM antibody titres observed may be sufficient to provide protection from pathogens at early time points after infection. Kuby (1997) reported that 100-1000 times fewer antibody molecules of IgM than IgG are required to achieve the same level of anti-red blood cell agglutination. Significance between groups (for mean TT-specific serum IgM antibody titres) was analysed using a one-way ANOVA.

Serum IgG production in response to an antigenic stimulus is generally observed following the early IgM response (see section 1.1.2). Figure 3.3 shows the mean total TT-specific serum IgG antibody titres (determined using an ELISA) following the intramuscular administration of 10 μg TT as a priming dose in various liposomal and non-liposomal formulations. An early bleed (day 7) showed very low (base-line) levels of TT-specific serum IgG antibodies for all formulations. For animals which had received $Al(OH)_3$/TT, day 14 bleeds indicated a higher, though not significantly ($p<0.05$), mean primary response (264 ± 132) (mean titre ± SE) in comparison to animals which received liposomal formulations or free TT. By day 28, the mean TT-specific serum IgG titres for all liposomal formulations had declined to base-line levels (from low levels at day 14) whereas mean serum antibody titres had increased further for animals which had received either $Al(OH)_3$/TT (384 ± 180) or free TT (285 ± 100). The TT-specific serum IgG antibody response to all liposomal formulations is in

agreement with studies by Alpar and co-workers (Alpar *et al.*, 1992) who administered DSPC/cholesterol liposomes encapsulating 3 μg of TT intramuscularly to pigmented guinea pigs. The serum TT-specific IgG titres for DSPC/cholesterol liposomes 3 weeks after priming, were similar to levels elicited by free TT (6 μg) or non-immunised mice. After boosting (week 4) with the same formulations, TT-specific serum IgG antibodies detected at week 6 were higher for liposomal and free TT compared to non-immunised mice. This secondary immune response to TT administration seen by Alpar and colleagues (Alpar *et al.*, 1992) concurs with the results seen post-boosting in our studies (figure 3.4).

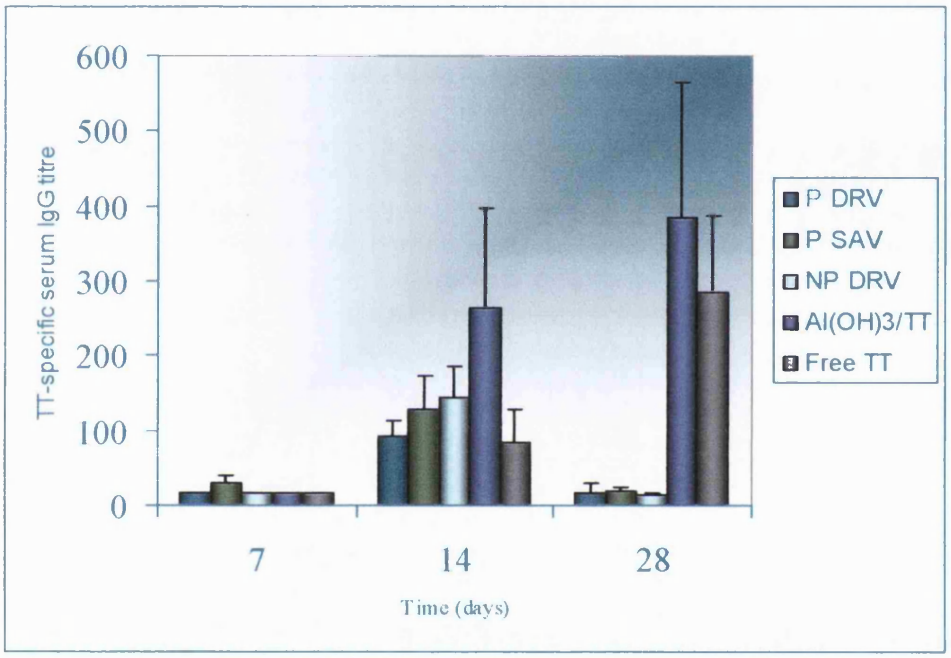

Figure 3.3: Primary TT-specific serum IgG antibody titres raised in mice in response to intramuscular administration of liposomal and non-liposomal formulations of TT (n=5). Mice were primed with 10 μg of formulated TT. Results are mean titres (± SE)

Key (refer to table 3.1 for further information): P =polymerised, NP = non-polymerised, DRV = dehydration-rehydration vesicle, SAV = surface adsorbed vesicle

Figure 3.4 shows the mean total TT-specific serum IgG antibody titres (determined using an ELISA) elicited on day 49 (secondary response) after priming with 10 μg of TT in various liposomal and non-liposomal formulations (figure 3.3) and boosting intramuscularly on day 35 with 5 μg of TT (by diluting priming formulations with an equal volume of saline).

Secondary responses (day 49) showed that animals immunised with $Al(OH)_3$//TT produced a significantly higher ($p<0.05$) mean TT-specific serum IgG antibody response in comparison to liposomal formulations or free TT whereas no significant differences ($p<0.05$) in mean antigen-specific serum IgG antibody levels were detected between the liposomal formulations and free TT. Administration of $Al(OH)_3$/TT evoked a mean TT-specific serum IgG titre (71,111± 14,222) approximately 5.1 times that of the mean antibody titre when animal were immunised with free TT (13,926 ± 5,116). Animals immunised with P SAVs and NP DRVs produced similar mean TT-specific serum IgG antibody titres to each other (26,859 ± 6,937 and 24,192 ± 2,859 respectively), which were both approximately 1.8 times greater than the antibody response to free TT. The administration of P DRVs (6,512 ± 1,844) however, resulted in a TT-specific serum IgG antibody response approximately only one half of that elicited following the delivery of free TT. Significance between groups (for mean TT-specific serum IgG antibody titres) was determined using a one-way ANOVA and Tukey's HSD test.

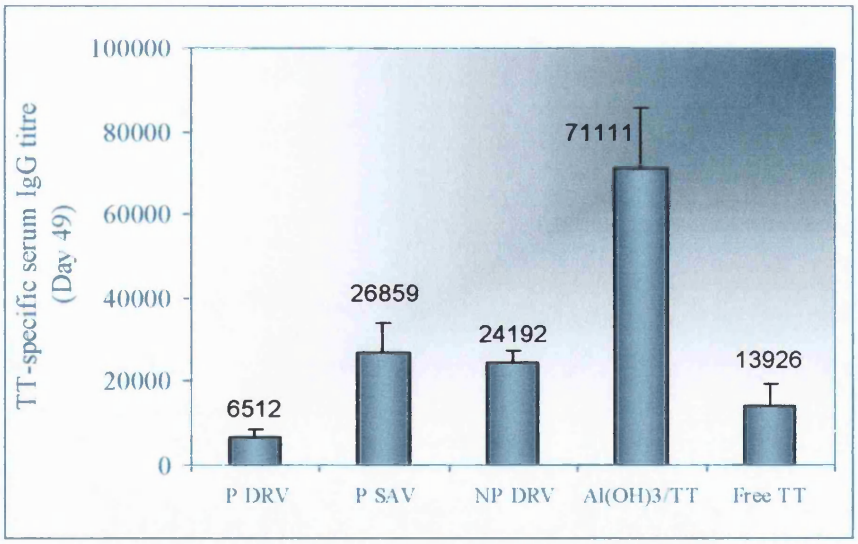

Figure 3.4: TT-specific serum IgG antibody titres raised in mice (Day 49) in response to intramuscular administration of liposomal and non-liposomal formulations of TT (n=5). Mice were primed with 10 μg of formulated TT and boosted on day 35 with 5 μg of formulated TT. Results are mean titres (± SE)

Key (refer to table 3.1 for further information): P =polymerised, NP = non-polymerised, DRV = dehydration-rehydration vesicle, SAV = surface adsorbed vesicle

The lower levels of TT-specific serum IgG antibodies observed after administration of P DRVs (in comparison to the administration of free TT) is in contrast to the adjuvant

properties of liposomes previously reported (Gregoriadis *et al.*, 1988; Childers and Michalek, 1994); Rogers and Anderson, 1998). However, studies by Davis and Gregoriadis (1987) using TT entrapped in liposomes composed of a variety of phospholipids with cholesterol, showed that after intramuscular priming and boosting (four weeks later) with the same formulations, antigen-specific serum IgG_1 and IgG_{2b} antibody responses were similar for liposomes composed of various phospholipids with transition temperatures between –32 to 41.5 °C but were significantly lower for liposomes prepared from DSPC (T_c = 54 °C) which showed low or no adjuvanticity. The authors concluded that phospholipids with a high transition temperature have a lower adjuvanticity for delivery of TT than phospholipids with lower transition temperatures.

It has been shown that liposomes with a high transition temperature are more stable in a biological environment than liposomes with a lower transition temperature (Aramaki *et al.*, 1993) and so may retain more of the encapsulated antigen for a longer time. This may be expected to increase adjuvanticity of liposomes due to the release of the antigen over a longer period of time after intramuscular administration and hence prolonging the depot action of delivered liposomes. However, the use of cholesterol in the formulation of DSPC liposomes by Davis and Gregoriadis (1987) may have resulted in an increased fluidity of liposomal membrane and hence a higher 'leakage' of entrapped TT than if DSPC alone had been used (Qi *et al.*, 1996). This is thought to be due to cholesterol disrupting the tight packing of bi-layers when they are below their transition temperature and in the case of the studies by Davis and Gregoriadis (1987), may have made the DSPC formulation less stable. The authors suggest in various publications (Davis and Gregoriadis, 1987; Gregoriadis *et al.*, 1988; Gregoriadis, 1990; Gregoriadis, 1994) reasons for their observations that liposomes with a higher transition temperatures reduced immune responses when using soluble antigens like TT. They reason that protein processing after presentation to macrophages is dependent upon whether or not the liposomes were above or below their gel-liquid crystalline transition temperature. Membrane soluble antigens would be directly transferred to the membranes of APCs during vesicle internalisation and associated with MHC molecules whereas soluble antigens (such as TT and diphtheria toxoid) require internal lysosomal catabolism before their surface exposure. This dependence on the characteristics of the antigen used may be why opposing observations on studies using antigens with differing properties have been reported in the literature (Gregoriadis, 1990) about the effect of

liposomal transition temperature on adjuvanticity. Thus, the association of TT to a more 'solid' phospholipid would hinder internal antigen processing and reduce adjuvant properties.

Results from our group have previously agreed with the link between transition temperature of liposomes and adjuvanticity. Alpar and co-workers (Alpar *et al.*, 1992) administered TT (3 μg) encapsulated in DSPC/cholesterol liposomes intramuscularly to pigmented guinea pigs and showed that TT-specific serum IgG titres were statistically the same ($p<0.05$) as when free TT (6 μg) was administered. Experiments comparing the administration of the same dose of TT in both liposomal and free form were not conducted.

The transition temperature for non-polymerised DODPC is 18.7 ± 2.8 °C (Koynova and Caffrey, 1998) and so bi-layers are 'fluid' at 37 °C but the transition temperature of polymerised DODPC liposomes (not determined) is expected to be much higher or absent (Gaub *et al.*, 1984) due to the possible restriction in bi-layer fluidity imposed by covalent bond formation between phospholipid molecules and so in effect the liposome may act as a more 'solid' vesicle (i.e. as one that would possess a much higher transition temperature). The desorption/release of TT from P SAVs is rapid (see figure 3.1) and so the polymerised phospholipid would not affect the processing of the released protein and so the antibody response in terms of TT-specific serum IgG is still higher than free TT alone. However, in the case of P DRVs where some of the TT may be incorporated within the aqueous portions of the liposome, the polymerised phospholipid would affect antigen processing by APCs.

Antibodies of the IgG isotype can be further divided into subclasses which possess differing immunological activities. The production of antibodies of the IgG_1 subclass are a good indicator of the activity of a T_h2 response whereas the presence of antibodies of the IgG_{2a} subclass are an indicator of the activity of a T_h1 response (cell-mediated immunity) (section 1.1.2). Alving and co-workers (Alving *et al.*, 1995) showed using immunogold electron microscopy that macrophage ingested liposomes can release their antigen from endosomal vacuoles into the cytoplasm. The antigen would thus be acting as an endogenous antigen and have the potential of being presented to T cells by association to MHC class I molecules, leading potentially to a predominantly T_h1 type response. If liposomes (and associated antigen) were only to be processed within

endocytic vacuoles (as an exogenous antigen) then T cell presentation would occur *via* the MHC class II pathway resulting in a T_h2 response. Factors such as the phospholipid transition temperature may affect the pathway (endosomal or cytosolic) *via* which the liposomes and associated protein will be processed.

Figures 3.5 and 3.6 show the mean TT-specific serum IgG_1 and IgG_{2a} antibody titres respectively, on day 49 (determined using an ELISA) following the intramuscular administration of 10 μg of TT as a priming dose on day 1 and boosting *via* the same route on day 35 with 5 μg of TT (by diluting priming formulations with an equal volume of saline) in various liposomal and non-liposomal formulations.

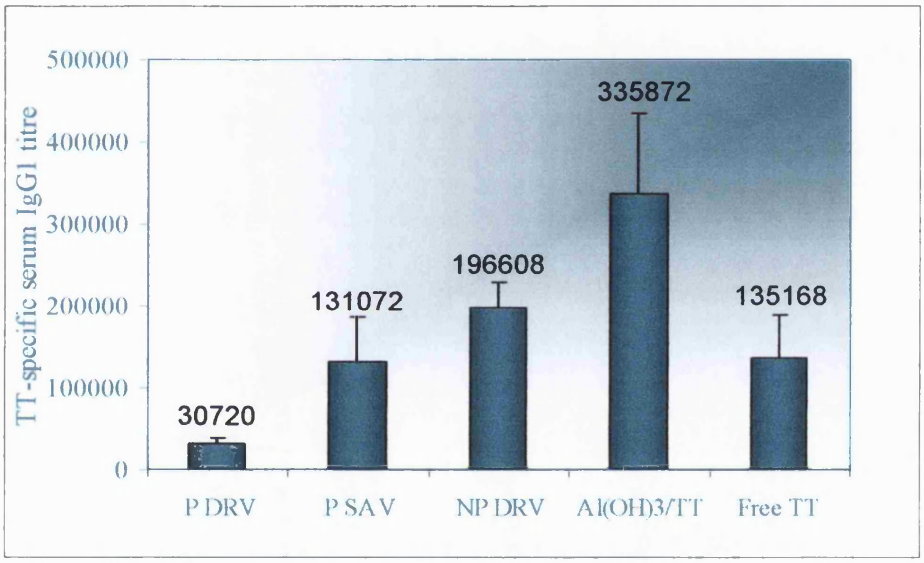

Figure 3.5: TT-specific serum IgG_1 titres (day 49) raised in mice in response to intramuscular administration of liposomal and non-liposomal formulations of TT (n=5). Mice were primed with 10 μg of formulated TT and boosted on day 35 with 5 μg of formulated TT. Results are mean titres (± SE)

Key (refer to table 3.1 for further information): P =polymerised, NP = non-polymerised, DRV = dehydration-rehydration vesicle, SAV = surface adsorbed vesicle

Animals immunised with $Al(OH)_3$/TT produced the highest (though not significantly higher ($p<0.05$)) mean TT-specific serum IgG_1 antibody titres (335,872 ± 97,275) followed by animals given TT with NP DRVs (196,608 ± 32,768). Immunising with P SAVs produced a similar TT-specific serum IgG_1 antibody response to that elicited when free TT was administered (131,072 ± 54,032 and 135,168 ± 53,248 respectively). Animals given P DRVs produced the lowest TT-specific serum IgG_1 antibody response

(30,720 ± 6,476), which was determined to be significantly lower (p<0.05) only in comparison to animals given NP DRVs (one-way ANOVA and Dunnett's T3 test).

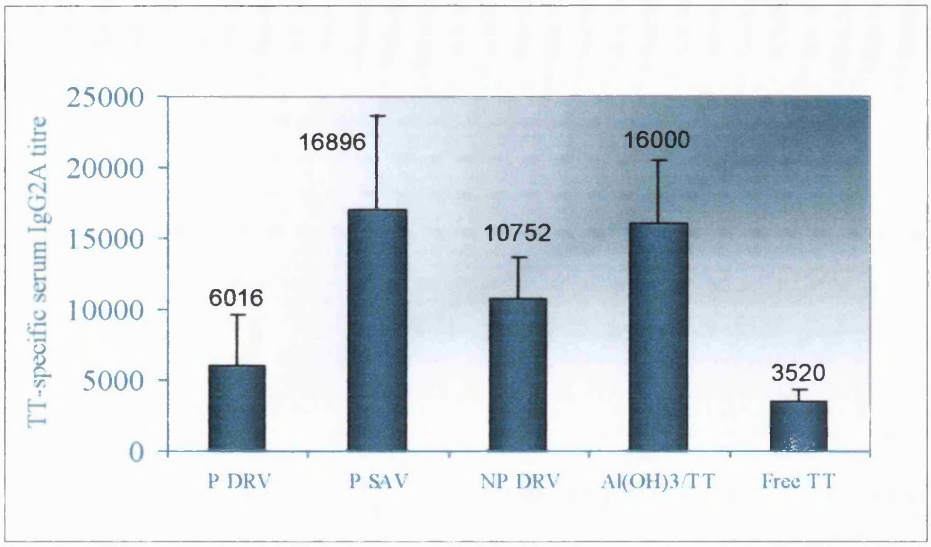

Figure 3.6: TT-specific serum IgG_{2a} titres (day 49) raised in mice in response to intramuscular administration of liposomal and non-liposomal formulations of TT (n=5). Mice were primed with 10 µg of formulated TT and boosted on day 35 with 5 µg of formulated TT. Results are mean titres (± SE)

Key (refer to table 3.1 for further information): P =polymerised, NP = non-polymerised, DRV = dehydration-rehydration vesicle, SAV = surface adsorbed vesicle

The administration of TT associated with P DRVs resulted in a higher (not significantly (p<0.05)) mean TT-specific serum IgG_{2a} antibody titre (on day 49) than when free TT was given (6,016 ± 3,618 and 3,520 ± 784 respectively) even though this was not the case for total TT-specific serum IgG and TT-specific serum IgG_1 antibody titres. The mean TT-specific serum IgG_{2a} antibody titre elicited following delivery of TT with $Al(OH)_3$/TT was also similar to the mean TT-specific serum $IgG2_a$ antibody titre for P SAVs (16,000 ± 4,480 and 16,896 ± 6,656 respectively) but higher, though not significantly (p<0.05) than the mean TT-specific serum $IgG2_a$ antibody titre for NP DRVs (10, 752 ± 2,851). Significance between groups (for mean TT-specific serum IgG_{2a} antibody titres) was analysed using a one-way ANOVA.

The overall antigen-specific serum antibody response to all formulations was a higher TT-specific serum IgG_1 antibody titres in each case (day 49) than TT-specific serum IgG_{2a} antibody titres (i.e. an indication of the dominance of a T_h2 response). The

antigen-specific serum antibody immune response elicited by $Al(OH)_3$/TT is high across all antibodies (IgG, IgG_1 and IgG_{2a}) investigated when compared to the antibody responses elicited either by liposomal formulations or free TT.

In considering the magnitude of the T_h1 response following administration of the formulations (as indicated indirectly by IgG_{2a} antibody responses), it can be seen that the liposomal formulations result in a T_h1 response lower than or equal to that elicited by $Al(OH)_3$/TT. Since it is known that aluminium salts activate the cell-mediated arm of the immune response (T_h1 response) to a low extent (Gupta and Siber, 1995; Kenney and Edelman, 2003), it can be concluded that the delivery of TT in liposomal formulations (polymerised and non-polymerised) seems to be no more effective in activating a cell-mediated response than the administration of TT with $Al(OH)_3$.

The polymerisation of DRVs (from NP DRVs) resulted in a reduction of both antigen-specific IgG_1 and IgG_{2a} antibody responses. The immune response to P DRVs (more 'solid' membrane than non-polymerised liposomes) was in agreement with the studies of Davis and Gregoriadis (1987) who administered DSPC (T_c of 54 °C) and equimolar cholesterol liposomes containing TT intramuscularly to male BALB/c mice and observed a lower TT-specific serum IgG_1 and IgG_{2b} than when free TT or phospholipids with a lower T_c with equimolar cholesterol were given.

Though significant differences ($p<0.05$) were not found between many of the liposomal formulations on analysis of antigen-specific serum IgG subclasses, the analysis of trends by comparing IgG_1 and IgG_{2a} antibody titres elicited can indicate the possibility of T_h1 and T_h2 shifts. The administration of surface adsorbed liposomal TT (P SAVs) as opposed to delivery of both liposomally adsorbed and possibly encapsulated TT (P DRVs) resulted in a further T_h2 shift (as determined by analysing the ratios of antigen-specific serum IgG_1 and IgG_{2a}). This is in agreement to the results obtained by Thérien and co-workers (Thérien *et al.*, 1991) who administered DMPC/DPPE/cholesterol liposomes with either surface-linked (chemically) or encapsulated BSA intraperitoneally to BALB/c mice. The authors reported that surface-linked BSA was more effective at evoking BSA-specific antibodies than liposome encapsulated BSA. They also detected a difference in the production of BSA-specific serum IgG subclass antibodies with surface-linked BSA leading to a higher IgG_{2a} antibody response than when BSA was encapsulated (in agreement to our results) which was observed to be dependent on the

theoretical loading of the antigen. Although the surface adsorption of TT to liposomes (P SAVs) resulted in a higher (not significantly ($p<0.05$) mean TT-specific serum IgG antibody response than when TT was associated to liposomes using the DRV method (P DRVs), which is in agreement to the results of Thérien and co-workers (Thérien *et al.*, 1991), on analysis of antigen-specific serum IgG_1 antibody levels it seems that delivery of TT using P SAVs did not show a T_h1 shift in the type of immune response elicited when compared to administration of TT using P DRVs. This agrees with the studies conducted by Gregoriadis and co-workers (Gregoriadis *et al.*, 1987) described above using PC/cholesterol or DSPC liposomes with either entrapped or surface-linked TT who also showed that animals given liposomally surface-linked TT did not result in any differences in TT-specific serum IgG_1 and IgG_{2b} antibody titres and the authors concluded that the humoral response to surface-linked antigen was similar to the humoral response to encapsulated antigen. However, the studies conducted both by Thérien and co-workers and Gregoriadis and co-workers (cited above), compared the adjuvanticity of surface-linked antigen to encapsulated antigen whereas in the course of the studies reported in this thesis using P SAVs, antigen was surface adsorbed only (not surface-linked) onto liposomes and so the presentation of the antigen to immunocompetent cells may be different.

3.4.4 Systemic (humoral and cellular) immune responses to liposomal and non-liposomal TT formulations following intranasal administration

Serum IgM production is an early indication of the activation of an immune response after the administration of an immunogenic agent (Playfair and Chain, 2001) (see section 1.1.2). Figure 3.7 shows the TT-specific serum IgM titres (determined using an ELISA) following the intranasal administration of 10 μg of TT as a priming dose (as either free in solution or in various liposomal formulations) and boosting intranasally on day 35 and 59 with 5 μg of TT in the same formulations (by diluting priming formulations with an equal volume of saline).

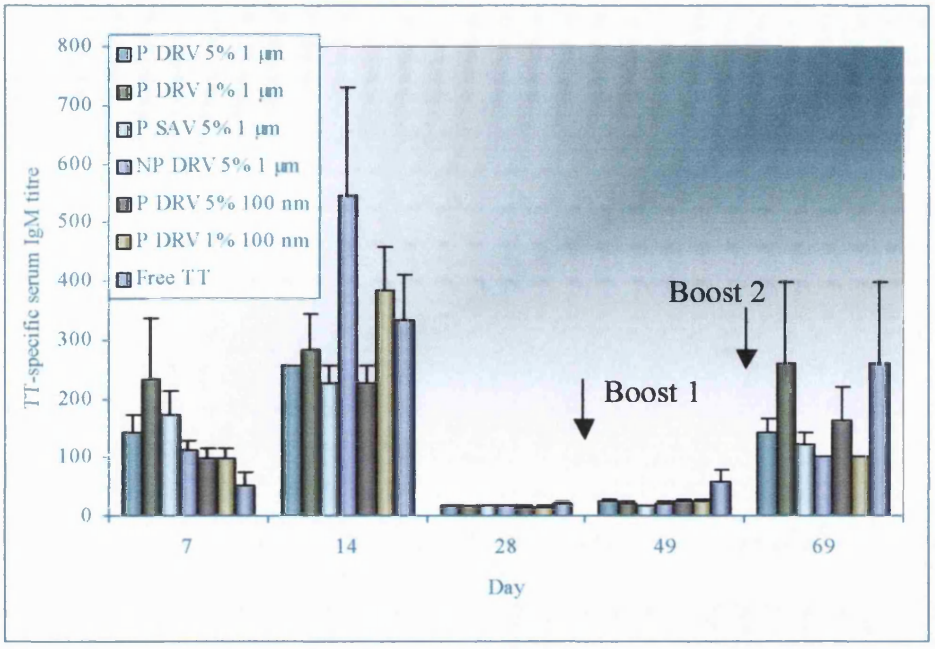

Figure 3.7: TT-specific serum IgM antibody titres raised in mice (n=5) in response to intranasal administration of TT in various liposomal formulations or free in solution. Mice were primed with 10 µg of formulated TT and boosted on days 35 and 59 with 5 µg of formulated TT. Results are mean titres (± SE)

Key (refer to table 3.2 for further information): P =polymerised, NP = non-polymerised, DRV = dehydration-rehydration vesicle, SAV = surface adsorbed vesicle, 5 $\%^{m}/_{m}$ or 1 $\%^{m}/_{m}$ = theoretical loading (mg TT/ mg phospholipid), 1 µm = liposomes with a wide particle size distribution prepared using an extruder filter with 1 µm pore diameter, 100 nm = liposomes with a narrow particle size distribution prepared using an extruder filter with 0.1 µm final pore diameter

Day 7 mean TT-specific serum IgM antibody titres were highest (though not significantly (p<0.05)) for polymerised vesicle formulations with a wide particle size distribution and the least for free TT. However, by day 14 the NP DRV formulation (wide particle size distribution) had elicited a markedly higher, though not significantly higher (p<0.05) mean TT-specific serum IgM titre (544 ± 184) than all other formulations. As expected, the TT-specific serum IgM titres then decreased to base-line levels by day 28 for all formulations. The mice were boosted on day 35 and serum collected on day 49 showed no increase in TT-specific serum IgM titres. Mice were boosted for the second time on day 59 and serum analysed on day 69 showed detectable TT-specific serum IgM antibody titres.

The TT-specific serum IgM antibody responses for P DRVs, P SAVs and NP DRVs with 5 $\%^{m}/_{m}$ theoretical loading (TL) and a wide particle size distribution were similar to those observed after intramuscular administration of the same formulations in that antibody titres decreased to base-line levels by day 28. However, unlike the intramuscularly administered formulations, there was no increase in TT-specific serum IgM titres following the boost on day 35. IgM production is usually observed following the first exposure of an antigen or if there remains no 'memory' of the previous presentation (Crowther, 1995). Thus, in the case of intranasal administration, where antigen-specific serum IgM titres increased after the 2nd boost, but not the 1st, the immunological memory elicited may be longer than that produced following intramuscular administration of the formulations given.

Serum IgG production in response to an antigenic stimulus is generally observed following the early IgM response (see section 1.1.2). Figure 3.8 represents the total TT-specific serum IgG response (determined using an ELISA) following the intranasal administration of 10 μg of TT as a priming dose (as either free in solution or in various liposomal formulations) and boosting intranasally on day 35 and 59 with 5 μg of TT in the same formulations (by diluting priming formulations with an equal volume of saline). TT-specific serum IgG levels on days 7, 14 and 28 were shown to be at base-line levels in response to the priming dose. Day 49 serum analysis (following boosting on day 35) revealed only a small increase, though not significantly higher ($p<0.05$) in TT-specific serum IgG titres for all formulations except polymerised liposomes with a narrow particle size distribution (1 $\%^{m}/_{m}$ theoretical loading) which produced an antigen-specific serum IgG antibody response (1,024 ± 0) significantly higher ($p<0.05$) than that elicited by all other formulations. After a second boost on day 59, serum samples taken on day 69 showed TT-specific serum IgG trends between formulations, though significance ($p<0.05$) was not achieved.

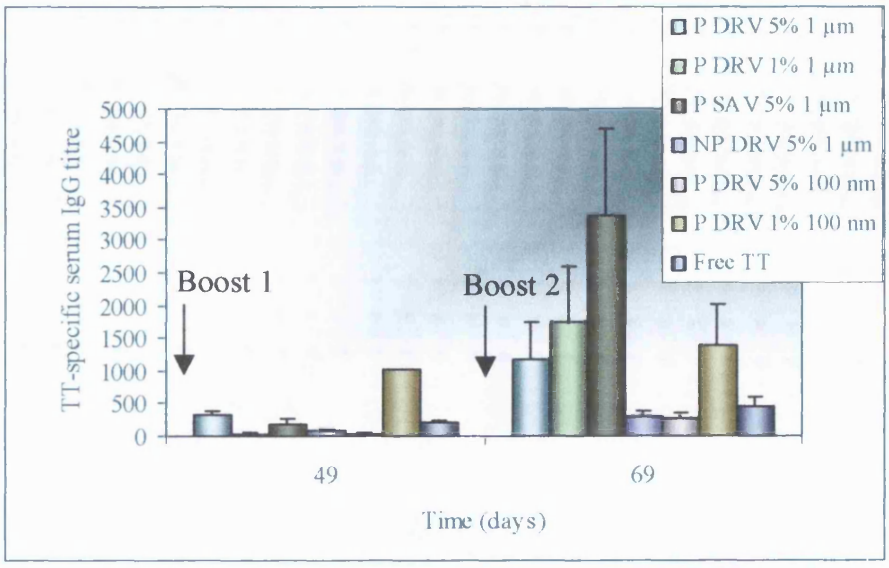

Figure 3.8: TT-specific serum IgG titres raised in mice (n=5) in response to intranasal administration of TT in various liposomal formulations or free in solution. Mice were primed with 10 µg of formulated TT and boosted on days 35 and 59 with 5 µg of formulated TT. Results are means (± SE)

Key (refer to table 3.2 for further information): P =polymerised, NP = non-polymerised, DRV = dehydration-rehydration vesicle, SAV = surface adsorbed vesicle, 5 $\%^{m}/_{m}$ and 1 $\%^{m}/_{m}$ = theoretical loading (mg TT/ mg phospholipid), 1 µm = liposomes with a wide particle size distribution prepared using an extruder filter with 1 µm pore diameter, 100 nm = liposomes with a narrow particle size distribution prepared using an extruder filter with 0.1 µm final pore diameter

Animals immunised with P SAVs produced the highest (not significantly (p<0.05)) mean TT-specific serum IgG titre (3,360 ± 1,342) compared to animals given all other formulations and was approximately 7.3 times greater than the mean titre elicited by administration of free TT (460 ± 147). Administration of P DRVs (with a wide and narrow particle size distribution) (1 $\%^{m}/_{m}$ theoretical loading) resulted in the next highest TT-specific serum IgG titres (1,750 ± 838 and 1,400 ± 622 respectively) which represents 3.8 and 3.0 times higher a response than that elicited by free TT. Immunising with the same formulations using a higher theoretical loading (P DRV 5 $\%^{m}/_{m}$ for liposomes with a wide and narrow particle size distribution) resulted in a TT-specific serum IgG titre of 1,160 ± 580 (2.5 times the free TT) for vesicles with a wide particle size distribution and no higher than free TT for liposomes with a narrow particle size distribution (275 ± 75). The antigen-specific serum IgG antibody titre elicited by

non-polymerised vesicles was also not higher than that elicited by free TT (300 ± 100). Significance between groups (for mean TT-specific serum IgG antibody titres) was analysed using a one-way ANOVA and Tukey's HSD test.

The increase in TT-specific serum IgG titres observed in response to the administration of liposomal formulations with a lower protein theoretical loading is in agreement with the results obtained by Thérien and co-workers (Thérien *et al.*, 1991). The authors reported that after intraperitoneal injection of dimyristoylphosphatidylcholine liposomes encapsulating BSA to BALB/c mice, that formulations with a lower theoretical loading produced higher BSA-specific serum IgG titres than formulations with higher theoretical loadings. Davis and Gregoriadis (1987) also showed using TT encapsulated in either PC/cholesterol or DSPC/cholesterol liposomes that both TT-specific serum IgG_1 and IgG_{2b} antibody levels increased after intramuscular administration to male BALB/c mice when lower protein:phospholipid theoretical loadings were used. The authors also reported a decrease in adjuvanticity when much lower theoretical loadings were used and suggested that this may have been due to the requirement of an optimum/minimum quantity of protein being delivered (in liposomal formulation) to immunocompetent cells. Investigation into the effect of protein:particulate theoretical loading after intranasal delivery was conducted by Somavarapu and co-workers (Somavarapu *et al.*, 1998). The administration of 0.9 $\%^m/_m$ and 5.0 $\%^m/_m$ (mg protein/ mg polymer) ovalbumin loaded polylactide nanoparticles containing Pluronic® F-68 with a mean hydrodynamic diameter of 0.32 μm to female BALB/c mice resulted in higher ovalbumin-specific serum IgG antibody levels for the lower loaded (0.9 $\%^m/_m$) formulation.

Studies on the effect of hydrophobicity on adjuvanticity of various orally administered microspheres were conducted on mixed sex BALB/c mice by Eldridge and colleagues (Eldridge *et al.*, 1990). Microspheres composed of relatively more hydrophobic polymers (such as polystyrene) were found to be absorbed by the Peyer's patches to a greater extent then less hydrophobic polymers such as poly(DL-lactide-*co*-glycolide). Thus, hydrophobic particles may possibly be taken up more by Peyer's patches in comparison to less hydrophobic particles and thus lead to a higher systemic and mucosal response (see section 1.4.6.7.2). Though, the studies by Eldridge and co-workers (Eldridge *et al.*, 1990) were conducted on the Peyer's patches of the gastrointestinal tract, the results may be applicable to the M cells and lymphoid tissues

of the nose (NALT). Alpar and Almeida (1994) administered TT-adsorbed latex particles and TT-adsorbed poly(lactide) microspheres (both approximately 8 μm in diameter) intranasally (in a volume of 0.1 mL) to Dunkin Hartley guinea pigs. Animals were primed with 60 μg of TT in formulation and boosted on day 35. TT-specific serum IgG antibody levels were shown to be higher for animals given TT-adsorbed latex microspheres than animals immunised with TT-adsorbed poly(lactide) microspheres on day 50. The humoral immune response for poly(lactide) microspheres had decreased to lower levels at week 10 whereas antibody titres remained high for latex microspheres even at week 15. The findings of this intranasal study are in agreement with the results of Eldridge and colleagues (1990) in that more hydrophobic particles (latex particles) induced a higher humoral immune response than the administration of a less hydrophobic particulate formulation (poly(lactide) microspheres). In section 2.4.9 (table 2.10) it was shown that polymerised liposomes were more hydrophobic than non-polymerised liposomes. The levels of TT-specific serum IgG antibody titres in response to the nasal administration of these formulations (figure 3.8) revealed that polymerised (more hydrophobic) liposomes elicited a higher humoral antibody response than non-polymerised (less hydrophobic) liposomes which may possibly be due to a higher uptake of these particles by M cells in the NALT and/or attraction of APCs, due to their preference for hydrophobic surfaces (reviewed by Raghuvanshi *et al.*, 2002).

The effect on particle size uptake by M cells of the NALT and GALT was discussed in sections 1.4.6.3 and 1.4.6.4 respectively. Tomizawa and colleagues (Tomizawa *et al.*, 1993) indicated that 2 h following intraluminal administration to male Wistar rats, anionic liposomes composed of PC, PS and cholesterol with mean diameters of 374 nm and 855 nm demonstrated a higher uptake into the Peyer's patches than smaller liposomes with a mean diameter of 162 nm. Howard and co-workers (Howard *et al.*, 1994) administered fluorescent latex microparticles of two sizes (0.11 μm and 0.94 μm) intraduodenally to adult male white and Netherland Dwarf rabbits and observed that the larger particles (0.94 μm) remained mainly on the surface of domes (above the lymphoid follicles) whereas the smaller particles (0.11 μm) were found mainly at the serosal surface. The larger particles were detected to a higher extent in the mesenteric lymph than the smaller particles. The authors also reviewed that particles between 0.5-1 μm displayed a greater absorption than particle below or above this size. Studies by Ghirardelli and colleagues (Ghirardelli *et al.*, 1999) on the sub-epithelial tissue of the

upper turbinate and the central septum of male New Zealand rabbits showed that after application of insulin coated fluorescent polystyrene nanoparticles (0.5 μm), nanoparticles were largely found bound to non-ciliated microvillar cells overlying lymphoid aggregates whereas none were found bound to ciliated cells. The TT-specific serum IgG antibody levels detected after the administration of P DRVs with a wide and narrow particle size distribution (both 1 $\%^{m}/_{m}$ and 5 $\%^{m}/_{m}$ theoretical loadings) did not result in a clear pattern in the immune response. DRVs with a wide particle size distribution resulted in raised TT-specific serum IgG for both formulations with 5 $\%^{m}/_{m}$ and 1 $\%^{m}/_{m}$ theoretical loadings whereas DRVs with a narrow particle size distribution resulted in raised antibody levels for liposomes with 1 $\%^{m}/_{m}$ theoretical loading only (liposomes with 5 $\%^{m}/_{m}$ theoretical loading resulted in a TT-specific serum IgG response no higher than when free TT was administered). Thus, both sizes of formulations prepared (under 1 μm) resulted in the formation of TT-specific serum IgG antibodies after intranasal administration. This is also in agreement with the work of Jung and colleagues (Jung *et al.*, 2001) who delivered TT-absorbed sulfobutylated poly(vinyl alcohol)-graft-poly(lactide-*co*-glycolide nanoparticles with mean hydrodynamic diameters of approximately 100 nm, 500 nm and 1500 nm intranasally to female BALB/c mice and found that nanoparticles of smallest (100 nm) and medium (500 nm) size elicited high and similar TT-specific serum IgG responses. Particles with a mean hydrodynamic of approximately 1500 nm elicited low antigen-specific serum IgG antibody responses.

Antibodies of the IgG isotype can be further divided into subclasses that possess differing biological activities. The occurrence of antibodies of the IgG_1 subclass are a good indicator of the activity of a T_h2 response whereas the presence of antibodies of the IgG_{2a} subclass are an indicator of the activity of a T_h1 response (cell-mediated immunity) (section 1.1.2). Figures 3.9 and 3.10 show the mean TT-specific serum IgG_1 and IgG_{2a} antibody titres respectively, on day 69 (determined using an ELISA) following the intranasal administration of 10 μg of TT as a priming dose (as either free in solution or in various liposomal formulations) and then boosted intranasally on day 35 and 59 with 5 μg of TT in the same formulations (by diluting priming formulations with an equal volume of saline).

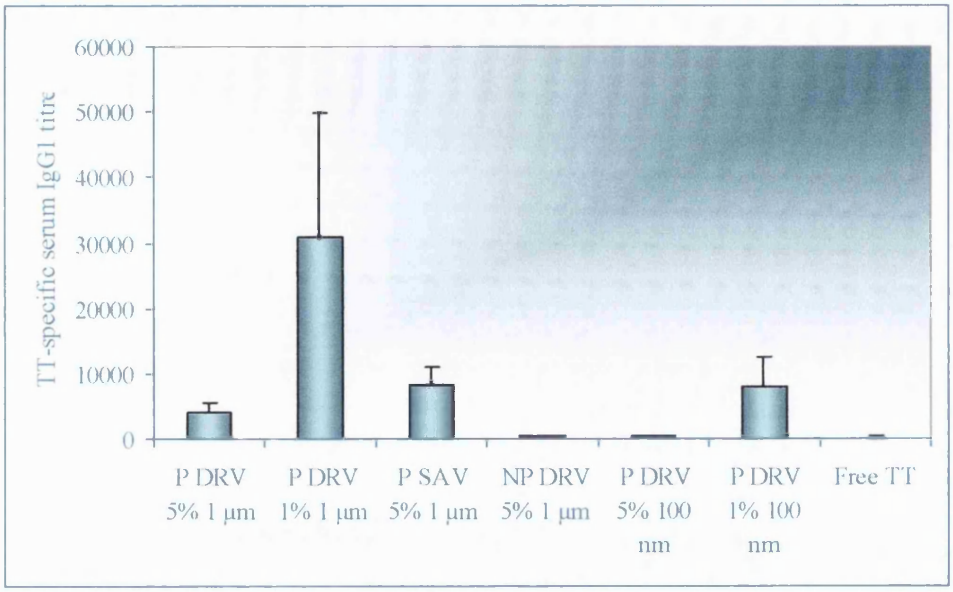

Figure 3.9: TT-specific serum IgG_1 titres (day 69) raised in mice (n=5) in response to intranasal administration of TT in various liposomal formulations or free in solution. Mice were primed with 10 µg of formulated TT and boosted on days 35 and 59 with 5 µg of formulated TT. Results are mean titres (± SE)

Key (refer to table 3.2 for further information): P =polymerised, NP = non-polymerised, DRV = dehydration-rehydration vesicle, SAV = surface adsorbed vesicle, 5 $\%^m/_m$ and 1 $\%^m/_m$ = theoretical loading (mg TT/ mg phospholipid), 1 µm = liposomes with a wide particle size distribution prepared using an extruder filter with 1 µm pore diameter, 100 nm = liposomes with a narrow particle size distribution prepared using an extruder filter with 0.1 µm final pore diameter

Animals immunised with P DRVs (1 $\%^m/_m$ theoretical loading, wide particle size distribution) produced the highest (though not significantly higher (p<0.05)) mean TT-specific serum IgG_1 response (30,900 ± 18,739) compared to responses to all other formulations, and decreased when the protein theoretical loading was increased to 5 $\%^m/_m$ (3,800 ± 1,510). This was also mirrored for animals immunised with P DRVs with a narrow particle size distribution when protein theoretical loading was increased from 1 $\%^m/_m$ to 5 $\%^m/_m$ (7,760 ± 4,560 and 250 ± 87 respectively). Thus, the administration of an increased quantity of polymerised phospholipid (in formulations with a lower protein theoretical loading) resulted in a higher TT-specific serum IgG_1 response. Surface adsorption of TT (P SAVs) as opposed to protein association through the DRV method also resulted in a higher TT-specific serum IgG_1 response, though this was not significant (p<0.05) (8,160 ± 2,844 and 3,800 ± 1,510 respectively). NP DRVs

produced a TT-specific serum IgG_1 titre similar to that elicited following the administration of free TT (233 ± 88 and 150 ± 58 respectively). Significance between groups (for mean TT-specific serum IgG_{2a} antibody titres) was determined using a one-way ANOVA and Dunnett's T3 test.

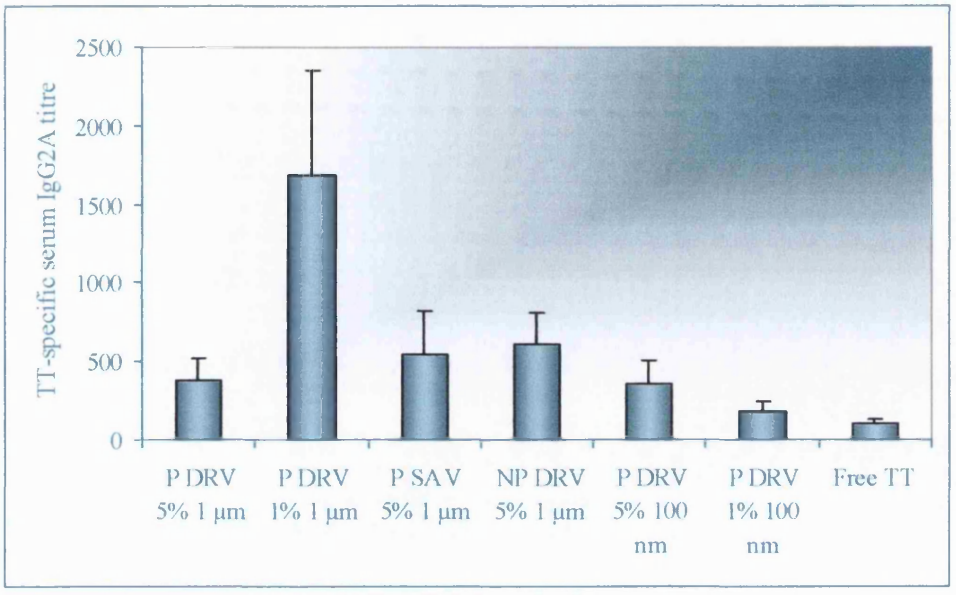

Figure 3.10: TT-specific serum IgG_{2a} antibody titres (day 69) raised in mice (n=5) in response to intranasal administration of TT in various liposomal formulations or free in solution. Mice were primed with 10 µg of formulated TT and boosted on days and 35 and 59 with 5 µg of formulated TT. Results are mean titres (± SE)

Key (refer to table 3.2 for further information): P =polymerised, NP = non-polymerised, DRV = dehydration-rehydration vesicle, SAV = surface adsorbed vesicle, 5 $\%^m/_m$ and 1 $\%^m/_m$ = theoretical loading (mg TT/ mg phospholipid), 1 µm = liposomes with a wide particle size distribution prepared using an extruder filter with 1 µm pore diameter, 100 nm = liposomes with a narrow particle size distribution prepared using an extruder filter with 0.1 µm final pore diameter

Animals immunised with P DRVs (1 $\%^m/_m$ theoretical loading) with a wide particle size distribution produced the highest (though not significantly higher (p<0.05)) TT-specific serum IgG_{2a} antibody response (1,680 ± 674). Any trends between the theoretical loading of P DRVs to antigen-specific serum IgG_{2a} antibody levels produced were not clear as results were contradictory between the responses to liposomes with a narrow and wide particle size distribution. Administration of TT using P SAVs and NP DRVs (5 $\%^m/_m$ theoretical loading) with a wide particle size distribution evoked similar mean TT-specific serum IgG_{2a} antibody responses (540 ± 271 and 600 ± 200 respectively) whereas the mean response for P DRVs (5 $\%^m/_m$ theoretical loading) with a wide

particle size distribution was even lower (375 ± 138), though not significantly lower (p<0.05). Significance was analysed using a one-way ANOVA and Dunnett's T3 test.

The overall response to all formulations (on day 69) was a higher antigen-specific serum IgG_1 antibody titres in each case than antigen-specific serum IgG_{2a} antibody titres (i.e. T_h2 response was indicated to be dominant over T_h1 response) except for non-polymerised vesicles where the opposite was true (though overall immune response was not high). This was also mirrored at the earlier time point (day 49 bleed) though the overall responses in terms of antibody titres were lower for each formulation (data not shown). The comparison of titres within antibody classes can help to indicate T_h1 or T_h2 shifts amongst formulations.

On day 69, animals given P DRV (1 $\%^m/_m$ theoretical loading) with a wide particle size distribution elicited the highest antigen-specific serum IgG_1 and IgG_{2a} antibody levels (indicative of high T_h2 and T_h1 responses respectively) in comparison to all other formulations. The theoretical loading decrease from 5 $\%^m/_m$ to 1 $\%^m/_m$ for P DRVs with a wide particle size distribution resulted in an increase in the TT-specific serum IgG_1 and TT-specific serum IgG_{2a} antibody levels. On analysis of subclass responses, in the case of the P DRVs with a wide particle size distribution, a lower theoretical loading (and hence increased amount of polymerised phospholipid) increased the magnitude of both T_h1 and T_h2 responses and resulted in further enhancement of the T_h2 skew.

Administration of NP DRVs resulted in a noticeably different response when compared to P DRVs with the same particle size distribution (wide) and theoretical loading (5 $\%^m/_m$) in that there was a greater TT-specific serum IgG_{2a} response (i.e. indication of T_h1 activity) than IgG_1 response. Animals immunised with P SAVs produced both a higher TT-specific serum IgG_1 and IgG_{2a} antibody response when compared to P DRVs and NP DRVs with the same particle size distribution (wide) and theoretical loading (5 $\%^m/_m$) indicating a higher overall humoral antibody response when animals received P SAVs as opposed to P DRVs.

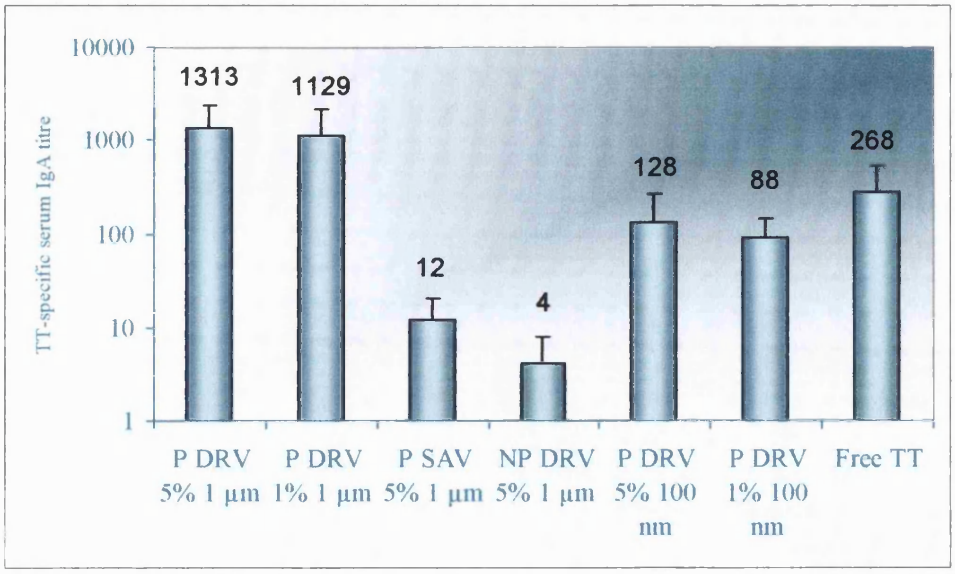

Figure 3.11: TT-specific serum IgA titres (day 69) raised in mice (n=5) in response to intranasal administration of TT in various liposomal formulations or free in solution. Mice were primed with 10 µg of formulated TT and boosted on days 35 and 59 with 5 µg of formulated TT. Results are mean titres (± SE)

Key (refer to table 3.2 for further information): P =polymerised, NP = non-polymerised, DRV = dehydration-rehydration vesicle, SAV = surface adsorbed vesicle, 5 $\%^{m}/_{m}$ and 1 $\%^{m}/_{m}$ = theoretical loading (mg TT/ mg phospholipid), 1 µm = liposomes with a wide particle size distribution prepared using an extruder filter with 1 µm pore diameter, 100 nm = liposomes with a narrow particle size distribution prepared using an extruder filter with 0.1 µm final pore diameter

Figure 3.11 shows the TT-specific serum IgA response on the day 69. The mean TT-specific serum IgA antibody titre was highest for polymerised DRVs with a wide particle size distribution. Both polymerised DRVs with wide and narrow particle size distribution with a higher theoretical loading (i.e. less phospholipid per same amount of antigen) resulted in an increased antigen-specific serum IgA antibody response. P DRVs with a narrow particle size distribution produced TT-specific serum IgA antibody levels lower than that attained by the administration of free TT. Animals given P SAVs and NP DRVs produced only base-line TT-specific serum IgA antibody responses.

Interferon gamma production by mixed spleen cells of immunised and non-immunised mice in response to antigen stimulation (see section 3.3.7 and section 3.3.8) is shown in figure 3.12. IFN-γ production is indicative of a T_h1 response (effects include decreasing IgG_1 levels and increasing IgG_{2a} levels) and hence the possible activation of a cell-mediated immune response. Mean IFN-γ levels were higher, though not significantly higher ($p<0.05$) (one-way ANOVA) for spleen cells derived from mice immunised with

NP DRVs when compared to the response elicited by naïve spleen cells. For all other formulations including free TT, the IFN-γ production levels following stimulation of spleen cells were similar to that of spleen cells from non-immunised (naïve) mice.

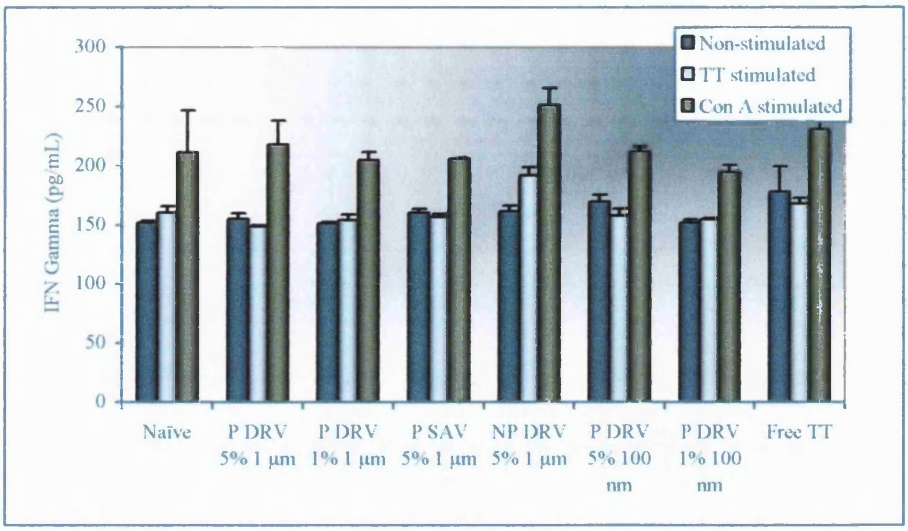

Figure 3.12: IFN-γ production by mixed spleen cells stimulated with free TT or with PBS (pH 7.4). Results are means of pooled spleens cultured in triplicate (± SE)

Key (refer to table 3.2 for further information): P =polymerised, NP = non-polymerised, DRV = dehydration-rehydration vesicle, SAV = surface adsorbed vesicle, 5 $\%^{m}/_{m}$ and 1 $\%^{m}/_{m}$ = theoretical loading (mg TT/ mg phospholipid), 1 μm = liposomes with a wide particle size distribution prepared using an extruder filter with 1 μm pore diameter, 100 nm = liposomes with a narrow particle size distribution prepared using an extruder filter with 0.1 μm final pore diameter

The higher mean IFN-γ production by non-polymerised vesicles is in agreement to the lower TT-specific serum IgG_1 levels (figure 3.9) and higher TT-specific serum IgG_{2a} levels (figure 3.10) detected when compared to the response by polymerised vesicles with the same protein theoretical loading and, size and size distribution. There were no identifiable trends between polymerised and non-polymerised liposomal formulations when production of IL-2, IL-4, IL-6, IL-12 and TNF-α were analysed and compared against the production of cytokines by spleen cells from non-immunised (naïve) mice (data not shown).

3.5 Conclusions

The adjuvant effect of aluminium salts following intramuscular administration has been attributed to the creation of a depot effect by retarding the release of antigen from the vaccine formulation. Inflammation in the area may lead to an increased infiltration of APCs and there is the possibility of the antigen-adjuvant complex migrating to a regional lymph node for presentation to T cells. The adjuvant action of liposomes when administered parenterally has also been thought to be due to a depot effect. However, unlike aluminium salts, it has been reported that liposomal formulations possess the ability to induce CMI (reviewed by Gregoriadis, 1990). Studies by de Haan and colleagues (de Haan *et al.*, 1995a) reported that liposomal adjuvanticity was not just due to the 'carrier' effect but also due to another unspecified mechanism.

To date, numerous articles have been published regarding the synthesis of polymerisable phospholipids for the preparation of liposomes (O'Brien *et al.*, 1985; Hayward *et al.*, 1985; Regen, 1987 and Freeman and Chapman, 1988) and the more robust nature of polymerised liposomes in comparison to conventional non-polymerised liposomes (see table 1.5). However, the application of polymerised liposomes for vaccine delivery has been limited to only a few published articles. Chen and Langer (1998) reported that the oral administration of polymerised DODPC liposomes encapsulating diphtheria toxoid resulted in higher antigen-specific serum IgG antibody levels than when polymerised liposomes were mixed with the antigen only. Further details of these experiments were obtained from the primary authors doctoral thesis (Chen, 1997). Polymerised DODPC liposomes were prepared containing UEA I (an M-cell specific lectin) and were either encapsulated with or were mixed with 30 μg of diphtheria toxoid. After oral administration to mice (in a volume of 200 μL) as a priming dose, mice were boosted four weeks later using the same formulations and same dose. Mean antigen-specific serum IgG antibody levels at 2 weeks, 4 weeks and 8 weeks were higher for lectin modified polymerised liposomes encapsulating diphtheria toxoid than for lectin modified polymerised liposomes simply mixed with diphtheria toxoid (which elicited an antibody response no higher than that elicited in response to administration of free diphtheria toxoid in PBS).

Jeong and colleagues (Jeong *et al.*, 2002) prepared liposomes using the polymerisable lipid 1,2-bis[12-(lipoyloxy)dodecanoyl]-*sn*-glycero-3-phosphorylcholine (DLL) and

reported that the encapsulation of lysozyme resulted in higher antigen-specific serum IgG antibodies (after intraperitoneal injection) 10 weeks post-dosing than when lysozyme was encapsulated and administered in conventional DSPC liposomes or as free lysozyme in solution. The nature of the immune response may not solely be due to the use of polymerised liposomes as opposed to non-polymerised liposomes, as the diameter of DLL liposomes was 240 nm and the diameter of DSPC liposomes was 530 nm. Thus, the difference in liposomal diameters between the formulations may have affected immune responses in terms of antigen presentation to APCs.

Studies conducted in chapter 2 (figure 2.12) and in this chapter (figure 3.1) investigating the initial BSA and TT association to polymerised and non-polymerised liposomes prepared using the DRV or SAV method showed that there were marked differences between the two proteins investigated in their initial association to and subsequent release from liposomal formulations. The initial association of BSA was higher to polymerised liposomes (P DRVs and P SAVs) and lower for NP DRVs whereas in the case of TT, liposomal association was highest for formulations prepared using the DRV method irrespective of whether the bi-layers were polymerised or not. The difference in the initial associations and subsequent release of proteins was most probably due to factors such as variation between proteins in the degree of van der Waals interactions, hydrophobic interactions and hydrogen bond formation between the protein and the carrier surface and differences in protein molecular weights. Mean BSA association to all liposomal formulations was found to be higher than mean TT-association to liposomal formulations which was in agreement to the results of Alpar and Almeida (1994) who investigated the association of BSA and TT to poly(lactide) microspheres.

Even though intramuscular administration of $Al(OH)_3$/TT elicited the best overall TT-specific humoral response, both P SAVs and NP DRVs also elicited a considerable response when compared to that produced by delivery of free TT. The effect of intranasal administration clearly was to reduce overall IgG titres for all formulations. The lower overall immune response (and hence the need for additional boosting in the intranasal study) made direct analyses between routes difficult. However, it is seen that P SAVs elicited the highest mean antigen-specific serum IgG response amongst liposomal formulations irrespective of the route of delivery (i.e. intramuscular or intranasal). In contrast, animals given NP DRVs elicited a high mean TT-specific serum IgG antibody titre after intramuscular administration but amongst the lowest

antigen-specific serum IgG antibody titres after intranasal administration. Tetanus toxoid delivered intranasally with NP DRVs however, elicited the greatest T_h1 skew (compared to free TT) amongst all formulations but the overall humoral immune response was very low.

This study stresses the differing modes of liposomal adjuvanticity depending on route of administration. P SAVs seem to be the most promising formulation from those investigated for intranasal administration of TT, as both the humoral immune response and the ease of formulation are favourable aspects. Formulations containing liposomes with a wide particle size distribution and TT theoretical loadings of 1 $\%^m/_m$ are seen to give highest mean TT-specific serum IgG antibody responses and so further investigations delivering TT intranasally using P SAVs with a wide particle size distribution and with a protein theoretical loading of 1 $\%^m/_m$ should be conducted.

4.0 Poloxamer- and chitosan-coated polymerised liposomes for the intranasal delivery of vaccine antigens: effect on immune response

4.1 Introduction

The adjuvant properties of chitosan (see section 1.3.19) are thought to be due to a number of mechanisms including mucoadhesion (on application to mucosal surfaces), the transient opening of tight junctions (Illum *et al.*, 2001 and van der Lubben *et al.*, 2001) and also due to the immunostimulatory properties of the molecule. Seferian and Martinez (2001) briefly reviewed this immunostimulatory property of chitosan following intramuscular delivery, including the stimulation of APCs such as macrophages and polymorphonuclear cells, augmenting delayed type hypersensitivity and CTL responses and inducing cytokine release. High molecular weight hydrophobic poloxamers have also been shown to exhibit adjuvant properties which has been suggested to be linked to their surfactant properties (see section 1.3.18). Poloxamers are thought to act as an intermediary in the adsorption/binding of the antigen to the carrier (such as polymerised liposomes) and do so in a way that maintains more of the antigen's natural protein configuration (and hence possibly maintaining immunogenicity) than if it were to adsorb to the carrier without the poloxamer or to other competing hydrophobic surfaces such as the walls of containers (Hunter *et al.*, (1994).

The studies conducted in this chapter aim to increase the adjuvanticity of P SAVs (polymerise surface-adsorbed vesicles) when administered intranasally (as concluded in section 3.4) by potentiating the formulation through co-delivery with other known adjuvants such as chitosan chloride, chitosan glutamate, poloxamer 331 (L101) or poloxamer 401 (L121).

4.2 Materials

4.2.1 Animals used for intranasal immunisation studies

Female BALB/c mice (6-8 weeks old) (n = 5) were purchased from Harlan Olac (Blackthorn, U.K.) and were acclimatised for 1 week prior to use. The animals were allowed food and water *ad libitum* during the course of the experiments.

4.2.2 Chemicals and reagents

Ninhydrin reagent was purchased from Sigma-Aldrich Company Ltd. (Poole, UK). Synperonic™ PE/L101 (poloxamer 331) and Synperonic™ PE/L121 (poloxamer 401) were a gift from Ellis & Everard (Bradford, U.K.) and Protasan™ UP CL 213 and Protasan™ UP G 213 were purchased from FMC BioPolymer (Drammen, Norway). Table 4.1 lists the physical properties of the chitosan salts used in this study.

Table 4.1: Physical properties of chitosan salts used in experimental studies. The degree of deacetylation indicates the positive charge density of the polymer.

Characteristics	Protasan™ UP CL 213	Protasan™ UP G 213
Salt	Chitosan chloride	Chitosan glutamate
Deacetylation (%)	84	86
Molecular mass (g/mol)	272 000	460 000
Viscosity (mPas)	133	108

4.3 Methods

4.3.1 Procedure for determining the mass of chitosan adsorbed to liposomal surfaces

The degree of chitosan association to vesicles was determined using a method as used by Prochazkova and co-workers (Prochazkova *et al.*, 1999) using Ninhydrin reagent. Ninhydrin reagent contains 20 g/L ninhydrin and 3 g/L hydrindantin dissolved in 75 $\%^{v}/_{v}$ dimethylsulfoxide in 1 M lithium acetate buffer (pH 5.2). The procedure used was as follows:

1/ Quantification of chitosan association to vesicles was measured indirectly by determining concentration of non-associated chitosan remaining in the supernatant when vesicles (with associated chitosan) were pelleted after centrifuging (150 000 g, 40 min, 4 °C). Ninhydrin reagent (1 mL) was mixed with 1 mL of chitosan standard or sample (supernatant) in glass tubes with screw caps and mixed by vortexing for 1 min.

2/ The tubes were then heated for 30 min in a boiling water bath before being immediately cooled to below 30 °C in a water bath at room temperature.

3/ To each tube, 5 mL of 50 $\%^{v}/_{v}$ ethanol (in water) was added and the mixture thoroughly vortexed for 15 seconds. This step ensured oxidation of excess hydrindantin.

4/ The absorbance of the samples against a Ninhydrin reagent-P SAV blank was measured spectrophotometrically at 560 nm and chitosan concentration determined by calculation against a calibration curve (figure 4.1).

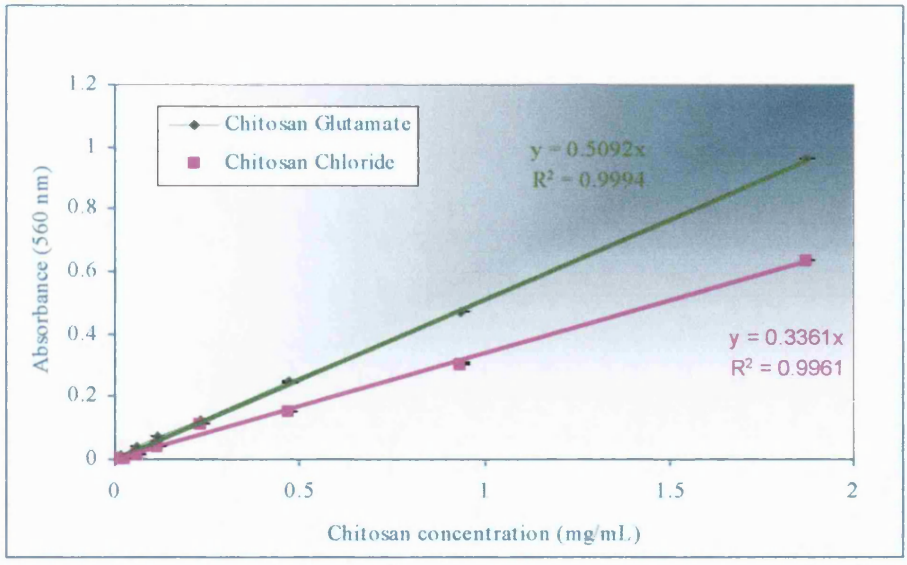

Figure 4.1: Calibration curve (n=3) for the determination of chitosan chloride and chitosan glutamate (± SD)

Nb: error bars present though range too small to be seen on graph

4.3.2 Procedure for determining the amount of poloxamer adsorbed to liposomal surfaces

The extent of poloxamer association to vesicles was determined using a method reported by Baleux and co-workers (Baleux *et al.*, 1972). Quantification was measured indirectly by calculating the concentration of non-associated poloxamer remaining in the supernatant when the liposomes (with associated poloxamer) were pelleted after centrifugation (150 000 g, 40 min, 4 °C). The following method was used to determine the mass of poloxamer in the supernatants:

1/ An aqueous solution of potassium iodide and iodine (2 mg KI and 1 mg I_2 (s) diluted to 100 mL using water) was prepared and stirred overnight and protected from light. Both L101 and L121 were measured by weight for all experimental protocols, as accuracy of measures was not possible using pipettes due to viscous nature of concentrate. The working concentrations were made up in water and stirred overnight at 2-8 °C.

2/ 100 μL of supernatants or standards were diluted to 2 mL in water (diluted 20 times) and 0.05 mL of KI_3 (potassium iodide and iodine) solution was added to each sample and incubated at room temperature for 10 min.

3/ The absorbance of the samples against a KI_3-P SAV blank was measured spectrophotometrically at 500 nm and poloxamer concentration determined by calculation against a calibration curve (figure 4.2).

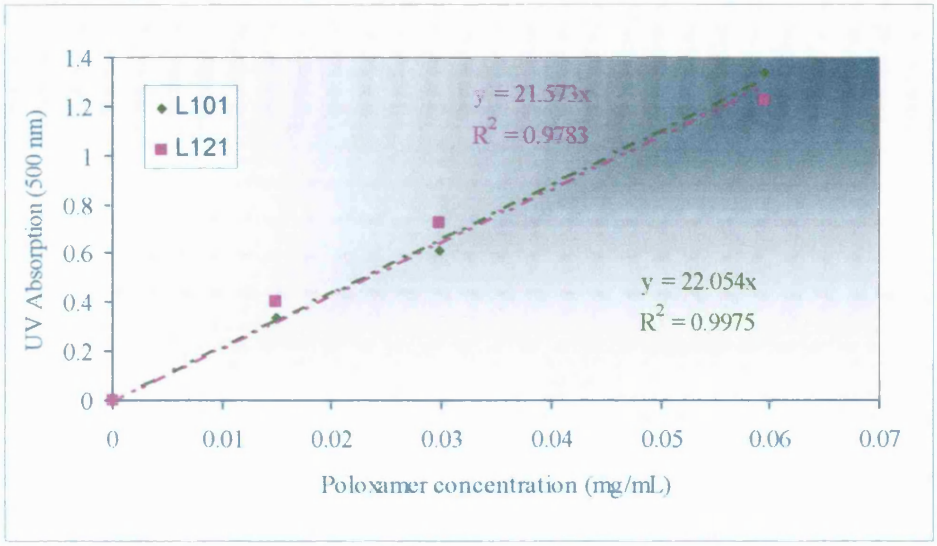

Figure 4.2: Calibration curve (n=3) for the determination of L101 and L121 concentration (± SD)

Nb: error bars present though range too small to be seen on graph

4.3.3 Poloxamer- or chitosan-coated and non-coated polymerised liposomal TT formulations for intranasal delivery

Poloxamer- or chitosan-coated polymerised DODPC liposomes were formulated to contain 10 µg of TT in total (liposome-associated and free) as a primer by incubating the antigen with the chitosan-/poloxamer-coated P SAV. Non-coated polymerised DODPC liposomes (P SAVs) were prepared as described in detail in section 2.3.1. In brief, 600 mg DODPC was added to 150 mL pre-warmed (50 °C) *t*-butanol (4 mg phospholipid/mL) and stirred until dissolved. Aliquots of 5 mL (containing 20 mg of DODPC) were added to 20 mL clear freeze-drying vials and sealed using 20 mm freeze-drying stoppers and frozen in liquid nitrogen (<195.79 °C, 1 min). The stoppers were then removed and the vials covered with Parafilm® M film, perforated with 12 needle width holes and lyophilised for 48 h using the Edwards Micro-Modulyo under vacuum drawn by an Edwards E2M5 high vacuum pump. The resulting phospholipid 'cake' was then hydrated with water to 10 mg/mL and stirred at 360 rpm for 2 h at room temperature using a magnetic stirrer plate and bead. The mean particle size, size distribution and structure of the liposome suspension thus formed were modified (section 2.3.2) using freeze-thawing and extrusion. Liposomes were extruded 10 times through filter membranes with pore diameters of 1 µm and were then freeze-thawed 10

times before repeating the extrusion cycles to yield uni-/oligo-lamellar DODPC liposomes. Liposomes were then polymerised by placing vesicle suspensions into quartz vessels before subjecting to UV light (254 nm) using the Rayonet photochemical mini-reactor (RMR-600) for 3 h 15 min (section 2.3.4 and 2.4.6).

The vesicles were then lyophilised (using 125 mM trehalose as a cryoprotectant) by freezing in liquid nitrogen (<-195.79 °C, 1 min) and attaching to the manifold of an Edwards Micro-Modulyo drier, under vacuum drawn by an Edwards E2M5 high vacuum pump for 48 h. When required, the liposomes were redispersed by rehydration with saline at room temperature and the resultant suspension rocked at 20 rpm for 1 h to allow complete liposome formation. In the case of non-coated P SAVs, TT was added to the liposomal suspensions at this stage and allowed to adsorb for 1 h at room temperature with a protein to phospholipid theoretical loading of 1 $\%^{m}/_{m}$ (mg protein to mg phospholipid). In the case of polymer-coated liposomes, chitosan or poloxamer was added to P SAVs before addition of TT to the formulation. Theoretical loading of 1 $\%^{m}/_{m}$ were investigated as studies conducted in section 3.4.4 showed that intranasal administration of liposomal formulations containing TT with a theoretical loading of 1 $\%^{m}/_{m}$ (mg protein to mg phospholipid) induced higher (though not significantly higher ($p<0.05$)) mean antigen-specific serum IgG antibody responses than when theoretical loading of 5 $\%^{m}/_{m}$ were delivered. Polymer coating of polymerised liposomes with chitosan or poloxamer was achieved using methods as detailed in section 4.4.1 and 4.4.3 respectively.

The TT-specific systemic and mucosal immune response to intranasal administration of chitosan (chloride or glutamate) and poloxamer (L101 or L121) adsorbed polymerised liposomes were compared to non-coated polymerised liposomes. A summary of formulations administered intranasally to each group of animals is given in table 4.2. All formulations were administered in saline in a volume of 20 μL.

Table 4.2: Poloxamer- or chitosan-coated and non-coated polymerised liposomal formulations used for the intranasal administration of tetanus toxoid

Abbreviation	Formulation
P SAV-ChCl	100 μL (added in 10 μL portions) 0.01 $\%^{m}/_{v}$ chitosan chloride:300 μL P SAVs (20 mg)
P SAV-ChGl	100 μL (added in 10 μL portions) 0.01 $\%^{m}/_{v}$ chitosan glutamate:300 μL P SAVs (20 mg)
P SAV-L101	400 μL 1 $\%^{m}/_{v}$ poloxamer L101: P SAVs (20 mg)
P SAV-L121	400 μL 1 $\%^{m}/_{v}$ poloxamer L121: P SAVs (20 mg)
P SAV	Not coated with polymer (400 μL P SAVs (20 mg))
Free TT	TT in saline solution

Key: P SAV = polymerised surface adsorbed vesicle, ChCl = chitosan chloride, ChGl = chitosan glutamate, L101 = poloxamer 331, L121 = poloxamer 401

4.3.4 Immunisation protocol for the delivery of poloxamer- and chitosan-coated and non-coated liposomal TT formulations by the intranasal route

Groups of five mice were placed into an inhalation anaesthesia chamber and anaesthetised using isoflurane (a volatile liquid anaesthetic) at a rate of 2-3 L *per* minute using oxygen as a carrier gas (1.5 L *per* minute). The mice were anaesthetised to prevent the gag reflex on intranasal application of formulations. TT (either as free in saline or liposomally formulated) was administered in a 20 μL volume by drop-wise application to alternate nostrils using a 20 μL pipette. The mice were primed with 10 μg of free or formulated TT in saline (as detailed in table 4.2) and boosted on day 35 and 59 with 5 μg of TT in the same formulation (by diluting priming formulations with an equal volume of saline solution). Tail bleeds were taken on days 14, 28, 49 and 69 and sera prepared for analysis of anti-TT antibodies as described in section 3.3.5. Faecal pellets were collected on day 69 and processed as detailed in section 3.3.6. At the end of the study, spleens of immunised and control mice were removed and processed as detailed in section 3.3.7 for the analysis of cytokine production.

4.4 Results and discussion

4.4.1 Optimisation of chitosan adsorption onto P SAVs without changing size distribution of liposomes

The adsorption of chitosan chloride and chitosan glutamate to P SAVs was investigated. The aim of the study was to produce a formulation of chitosan-coated P SAVs without affecting the mean liposomal size (due to aggregation/flocculation). Non-coated P SAVs were prepared using the procedure as outlined in section 4.3.3, mean vesicle size and size distribution was measured using PCS (section 2.3.6.1) and zeta potential determined using laser Doppler velocimetry (section 2.3.8). The zeta potential of non-coated polymerised DODPC liposomes (31.6 ± 0.6 mV) (mean ± SD) and also of non-polymerised liposomes following rehydration of lyophilised formulation was lower than that observed in section 2.4.9 (table 2.10) (-22.0 ± 0.7 mV) which was thought to be due to a change in water supply (for purposes of zeta potential determination) and also due to the addition of trehalose (as a cryoprotectant) in these formulations.

The studies on the optimisation of chitosan addition to polymerised liposomes are summarised in table 4.3, table 4.4 and table 4.5 and were based on previous studies by Henriksen and colleagues (Henriksen *et al.*, 1994 and 1997). The authors used chitosan chloride (mwt = 220,000; percentage deacetylation = 91.6 %) and investigated the interaction of this polymer with EPC/PG anionic liposomes. It was demonstrated that to avoid aggregation of chitosan-liposome vesicles that an excess of chitosan was desirable and that it was necessary to add the liposome suspension to the chitosan solution (and not the other way around), which also meant that chitosan, remained in excess throughout the procedure. The authors used various concentrations of chitosan and showed that the addition of 0.5 mL of liposomes (5.5 mg phospholipid/mL aqueous solution) to 2 mL of a 2000 parts *per* million (0.2 $\%^{m}/_{v}$) chitosan solution, resulted in a good candidate formulation. This is equal to a chitosan to phospholipid ratio of 4 mg chitosan:2.75 mg phospholipid in 2.5 mL total volume. However, for intranasal administration, the total dose volume must not exceed 10-20 μL (section 1.4.6.5) and so a more concentrated formulation is required to maintain a high mass of phospholipid *per* dose. However, the quantity of chitosan that can be added to liposomes to form an end volume of 10-20 μL is limited by the solubility of chitosan in the aqueous solution used and so, though the concentration of phospholipid can be increased, the

concentration of chitosan cannot, resulting in lower chitosan:phospholipid ratios. On the basis of the above obstacles it was not possible to use an excess of chitosan (in relation to phospholipid).

Henriksen and co-workers (Henriksen *et al.*, 1994) also showed that the interaction of 2 mL of a lower concentration (less than 10 parts *per* million (0.001 $\%^{m}/_{v}$)) of chitosan chloride (percentage deacetylation = 91.6 %; mwt = 220,000) to 0.5 mL PC/PG liposomes (5.5 mg phospholipid/ mL aqueous solution) did not result in size increase for liposomes. Therefore, neither the addition of a low concentrations of chitosan or an excess of chitosan resulted in liposomal aggregation/flocculation. The absence of aggregation when using high concentrations of chitosan may possibly be due to a charge reversal on the surface of the anionic liposomes resulting in no further negative 'sites' available for chitosan adsorption, leading to chitosan chains being extended from liposomal surfaces into the suspension, resulting possibly in an electrostatic repulsion between liposomes. This was not the case for low molecular weight chitosans where aggregation was observed when high concentrations of chitosan were added. Aggregation/flocculation occurred irrespective of the order of mixing (addition of chitosan to liposome suspension or *vice-versa*) when using either low or high concentrations of chitosan (when adding 2 mL of 0.001 $\%^{m}/_{v}$ (or lower) or 0.05 $\%^{m}/_{v}$ (or above) of chitosan solution to 0.5 mL phospholipid solution (5.5 mg phospholipid/mL)). This formed the basis of the data for the studies shown in table 4.3, table 4.4 and table 4.5. Chitosan in solution was added to liposomal formulations (rather than the other way around) to minimise loss of phospholipid in glassware and pipettes and hence maintaining constant phospholipid mass amongst the formulations that were to be dosed to animals at a later stage.

As chitosan glutamate and chloride have a poor solubility in water (at high pH), a higher volume of the dilute chitosan solution needs to be added to the liposomes in order for a medium to high chitosan:phospholipid ratio. This however, results in the final chitosan-liposome formulation being too dilute and thus would require being concentrated to achieve sufficient delivery of liposomes and chitosan in a 20 μL volume (intranasal dosing volume). A solution to this problem is to add the chitosan to P SAVs pre-lyophilisation so that the chitosan-P SAVs can be rehydrated to required volume post-lyophilisation. This would also be an appropriate manufacturing approach, as in the interests of scaling-up, the vaccine would be fully formulated before being dispatched

from the factory and would require only rehydration at the bedside prior to its use. Table 4.3 shows the results derived from the addition of either 4 mg chitosan to 20 mg phospholipid or 0.8 mg chitosan to 20 mg phospholipid. Both visual inspection and PCS measurement of the samples immediately following chitosan addition revealed that aggregation had occurred which was not reversed following stirring (using a magnetic bead and magnetic stirrer plate) or vortexing. The samples were lyophilised and rehydrated and as was expected, the vesicle remained aggregated. The presence of visible aggregation meant that particle size exceeded 1 μm and that in this case, PCS was not appropriate for accurately assessing the vesicle mean size and size distribution. Probe sonication (Sanyo MSE Soniprep 150 MSE) (using 30 s sonication cycles on ice with a titanium probe followed by 30 s rest) was used as a tool for separating aggregates and PCS analysis revealed that mean hydrodynamic diameter of sonicated liposomes (241.8 ± 8.1 nm) was similar to the mean hydrodynamic diameter of liposomes before the addition of chitosan (222.6 ± 2.5 nm). However, the polydispersity of sonicated liposomes was much lower (0.272) than that of the liposomes before chitosan addition (0.685), which indicated that the size distributions of the formulations were markedly different. On viewing liposomal formulations using TEM., it could be seen that the process of sonication had damaged the integrity of the liposomes (pictures not shown). Reformation of liposomes post-sonication may not have occurred due to bi-layer formation possibly being more 'difficult' with polymerised membrane fragments. Decreasing the chitosan:phospholipid ratio from 4:20 to 0.8:20 lowered the zeta potential of the vesicles from $^{+}$45.8 mV to $^{+}$13.3 mV for chitosan chloride and $^{+}$42.4 mV to $^{+}$8.5 mV for chitosan glutamate liposomes.

The addition of chitosan to liposomes post-lyophilisation was also investigated (table 4.4) as an alternative to pre-lyophilisation addition. The quantity of chitosan added to liposomes was decreased due to the limitation on the maximum volume that could be used for rehydration of lyophilised liposomes due to the volume for intranasal administration being restricted to a maximum of 20 μL. The upper limits of chitosan solubility in deionised water (pH 5.5) restricted the amount of chitosan that could be added to the rehydration volume. The addition of 0.01 mg chitosan in solution to 20 mg phospholipid resulted in a degree of reversible aggregation (after vortexing) but still, this state of 'non-aggregation' was short-lived. The use of 0.005 mg chitosan in solution to 20 mg phospholipid created a formulation where aggregation was minimal, however there was no marked change in vesicle zeta potential.

In order to increase the amount of chitosan that could be added to liposomes for the preparation of a formulation with a positive zeta potential without the occurrence of aggregation, the effect of chitosan solution addition in small portions to liposomes was investigated as an alternative to a 'one-step' addition as previously employed (table 4.5). Chitosan was introduced in 10 μL portions to the vesicles allowing for vortexing of samples in between each addition. This procedure allowed for the previously unstable preparation (0.01 mg chitosan: 20 mg phospholipid) to remain non-aggregated. However, still no change in measured zeta potential was observed. This quantity of chitosan input (both for chloride and glutamate) into the formulation (0.01 mg:20 mg phospholipid) was the highest possible, without major changes in size (370.9 ± 36.2 nm and 370.4 ± 21.8 nm respectively) or size distribution as indicated by polydispersity index (0.490 and 0.461 respectively) compared to liposomal size and size distribution before chitosan addition (220.5 ± 4.6 nm and 0.399 respectively) yet was far less than that reported by Henriksen (1994) and hence a liposomal zeta potential change at such low levels of chitosan was not detected.

The polymerised DODPC liposomes prepared had a mean zeta potential of -31 mV and were similar to the anionic PC/PG liposomes prepared by Henriksen (1994), which possessed a zeta potential of approximately –25 mV (adjusted using hydrochloric acid to pH 5 ± 0.1, 30 mM sodium chloride). The authors showed that PC/PG liposomes were more susceptible to aggregation on interaction with chitosan (as measured by PCS) than neutral liposomes composed of PC (zeta potential approximately –0.8 to -3.4 mV measured in the same medium). As polymerised DODPC liposomes (in the medium used) possessed a more negative zeta potential than the PC/PG liposomal system, the effect of chitosan addition in terms of aggregation may be more pronounced which would explain the increased occurrence of aggregation.

Table 4.3: The effect of pre-lyophilisation chitosan addition to P SAVs on mean hydrodynamic diameter and zeta potential

Chitosan:lipid mass ratio (mg:mg)	Formulation	Type of chitosan	Observations	Observation stage	Mean hydrodynamic diameter (nm) (± SD) (n = 3)	Polydispersity index (± SD)	Zeta potential (mV) (± SD) (n = 5)
4:20	4 mL 0.15 %($^{m}/_{v}$) chitosan solution added to 3 ml lipid (10 mg/mL) (0.086 % $^{m}/_{v}$ final chitosan concentration)			Pre-addition	222.6 (2.5)	0.685 (0.008)	-25.86 (0.24)
		ChCl	Aggregation	Post-addition	549.2 (21.7)	0.463 (0.052)	+ 45.8 (1.3)
				Post-10 min sonication	241.8 (8.1)	0.272 (0.048)	-
		ChGl	Aggregation	Post-addition	508.5 (9.2)	0.072 (0.073)	+ 42.4 (0.7)
				Post-5 min sonication	291.1 (4.8)	0.274 (0.020)	-
0.8:20	8 mL 0.01 %($^{m}/_{v}$) chitosan solution added to 4 mL lipid (5 mg/mL)(0.007% $^{m}/_{v}$ final chitosan concentration)			Pre-addition	211.9 (2.4)	0.604 (0.01)	-25.86 (0.24)
		ChCl	Aggregation	Post-addition	493.2 (14.4)	0.042 (0.051)	+ 13.3 (0.7)
		ChGl	Aggregation	Post-addition	594.1 (41.8)	0.357 (0.278)	+ 8.5 (1.7)

Key: P SAV = polymerised surface adsorbed vesicles, ChCl = chitosan chloride ChGl = chitosan glutamate

Table 4.4: The effect of post-lyophilisation chitosan addition to P SAVs on mean hydrodynamic diameter and zeta potential

Chitosan:lipid mass ratio (mg:mg)	Formulation	Type of chitosan	Observations	Observation stage	Mean hydrodynamic diameter (nm) (± SD) (n = 3)	Polydispersity index (± SD)	Zeta potential (mV) (± SD) n = 5)
0.02:20	200 μL Ch 0.01 $\%^{m}/_{v}$ to 200 μL (100mg/mL lipid)	ChCl	Aggregates	-	-	-	-
		ChGl	Aggregates				
0.01:20	100 μL Ch 0.01 $\%^{m}/_{v}$ to 300 μL (66.7 mg/mL lipid)	ChCl	Aggregates but decreases during vortexing but unstable and reaggregates	-	-	-	-
		ChGl	Aggregates but decreases during vortexing but unstable and reaggregates				
0.005:20	100 μL Ch 0.005 $\%^{m}/_{v}$ to 300 μL (66.7 mg/mL lipid)			Pre-addition	220.5 (4.6)	0.399 (0.004)	-31.0 (0.6)
		ChCl	No Aggregation but no zeta change	Post-addition	294.0 (4.1)	0.444 (0.038)	-30.9 (0.2)
		ChGl	No Aggregation but no zeta change	Post-addition	273.6 (11.2)	0.469 (0.026)	-27.9 (0.2)

Key: P SAV = polymerised surface adsorbed vesicles Ch = chitosan, ChCl = chitosan chloride ChGl = chitosan glutamate

Table 4.5: The effect of pre-lyophilisation chitosan addition (in portions) to P SAVs on mean hydrodynamic diameter and zeta potential

Chitosan:lipid mass ratio (mg:mg)	Formulation	Type of chitosan	Observations	Observation stage	Mean hydrodynamic diameter (± SD) (n = 3)	Polydispersity index (± SD)	Zeta potential (mV) (± SD) (n = 5)
0.2:20	200 μL(in 10 μL portions) Ch 0.1 % to 200 μL (100 mg/mL lipid)	ChGl	Aggregation				
0.1:20	100 μL(in 10μL portions) Ch 0.1 % to 300 μL (66.7mg/mL lipid)	ChGl	Aggregation				
0.05:20	50 μL (in 10 μL portions) Ch 0.1 % to 350 μL (57.14 mg/mL lipid)	ChGl	Aggregation				
0.025:20	50 μL (in 10 μL portions) Ch 0.05 % to 350 μL (57.14 mg/mL lipid)	ChGl	Some aggregation	Pre-addition	220.5 (4.6)	0.399 (0.004)	-31.0 (0.6)
				Post addition	403.2 (49.7)	0.445 (0.046)	-30.9 (0.2)
0.01:20	100 μL (in 10μL portions) Ch 0.01 % to 300 μL (66.7mg/mL lipid)	ChCl	Little aggregation	Pre-addition	220.5 (4.6)	0.399 (0.004)	-31.0 (0.6)
				Post addition	370.9 (36.2)	0.490 (0.062)	-33.5 (0.5)
		ChGl	Little aggregation	Pre-addition	220.5 (4.6)	0.399 (0.004)	-31.0 (0.6)
				Post addition	370.4 (21.8)	0.461 (0.042)	-32.9 (0.2)

Key: P SAV = polymerised surface adsorbed vesicles Ch = chitosan, ChCl = chitosan chloride ChGl = chitosan glutamate

4.4.2 Quantification of the amount of chitosan adsorbed to P SAVs

Chitosan chloride- and chitosan glutamate-coated polymerised liposomes (0.01 mg chitosan: 20 mg phospholipid) were constructed by addition of 100 μL of 0.01 $\%^{m}/_{v}$ chitosan solution in a step-wise manner (in 10 μL portions) to 300 μL polymerised liposome suspension (66.7 mg/mL phospholipid) (prepared as detailed in section 4.4.1). The chitosan was allowed to adsorb to the vesicles for 1 h before the samples were assayed for quantification of chitosan as described in section 4.3.1.

It was found that at such a low concentration of chitosan (initial concentration $0.01\%^{m}/_{v}$) the level was below the assay's sensitivity for detecting the molecule. However, as aggregation was seen when the same quantity of chitosan (100 μL of 0.01 $\%^{m}/_{v}$ chitosan) was added (for both salts) in only one step, there still remains sufficient chitosan within the preparation to render the formulation with different characteristics than if no chitosan were present. Also, as the surface of the polymerised vesicles are negatively charged it is expected that possibly a large portion of the chitosan added to the liposomes would be associated/adsorbed to vesicle surfaces.

4.4.3 Optimisation of poloxamer adsorption onto P SAVs without changing size distribution of liposomes

The aim of this study was to produce a formulation of poloxamer-coated P SAVs and to optimise such coating without altering vesicle size or size distribution (due to surfactant nature of poloxamers interfering with bi-layer integrity). Non-coated P SAVs were prepared using the procedure as outlined in section 4.3.3, mean vesicle size and size distribution was monitored using PCS (section 2.3.6.1) and surface charge estimated through zeta potential determination using laser Doppler velocimetry (section 2.3.8) (table 4.6 and 4.7).

Table 4.6 depicts the effect on mean hydrodynamic diameter and zeta potential on addition of poloxamer L101 or L121 to liposomes in a mass ratio of 0.02 mg poloxamer to 20 mg phospholipid (a similar weight ratio of poloxamer to phospholipid as that of chitosan to phospholipid (section 4.4.1)). Mean vesicle hydrodynamic diameter (initially 222.7 ± 4.5 nm) (mean ± SD) was shown not to increase significantly ($p<0.05$) (paired Student's t-test) after the addition of either poloxamer L101 or L121 in solution

(224.4 ± 5.7 nm and 216.0 ± 4.1 nm respectively). The addition of a larger mass of poloxamer (L101 or L121) to the same mass of phospholipid (table 4.7) resulted in a significant increase ($p<0.05$) (paired Student's t-test) in the mean liposomal hydrodynamic diameter (initially 222.7 ± 4.5 nm) though the mean hydrodynamic diameter (257.5 ± 4.3 nm and 324.6 ± 8.4 nm respectively) remained acceptable for the means of the study. The increase in liposome size is, as expected and is thought to be due to the insertion of the hydrophobic tails of the poloxamer (PPO chains) into liposomal bi-layers (increasing bi-layer volume) and surface exposure of the hydrophilic portion (PEO chains) creating the appearance of a larger sphere on particle size analysis. The greater size increase seen after poloxamer coating with L121 compared to L101 may be explained by the presence of the longer hydrophobic tails present on L121 (Alexandridis and Hatton, 1995) which would insert into the bi-layer and hence increase bi-layer volume and the longer hydrophilic PEO chain which due to greater surface exposure than L101 would create the appearance of a larger particle. The zeta potential of the vesicles remained approximately the same after addition of the poloxamer and so could not be used as an indication for the extent of poloxamer adsorption.

Table 4.6: The effect on the mean hydrodynamic diameter, polydispersity and zeta potential of P SAVs on addition of poloxamer solution to mass ratio 0.02 mg poloxamer: 20 mg phospholipid

Abbreviation for formulations	Description of formulation (20 μL=1 mg phospholipid, 0.001 mg poloxamer)	Mean hydrodynamic diameter (nm) (± SD) (n = 3)	Poly-dispersity index (± SD)	Zeta potential (mV) (± SD) (n = 5)
P SAV		222.7 (4.5)	0.397 (0.023)	-33.2 (0.1)
P SAV-L 101	200μL 0.01 $\%^{m}/_{v}$ poloxamer aqueous to 20 mg phospholipid	224.4 (5.7)	0.426 (0.024)	-34.2 (0.3)
P SAV-L 121	200μL 0.01% $^{m}/_{v}$ poloxamer aqueous to 20 mg phospholipid	216.0 (4.1)	0.412 (0.022)	-32.8 (0.5)

Key: P SAV = polymerised surface adsorbed vesicle, L101 = poloxamer 331, L121 = poloxamer 401

Table 4.7: The effect on the mean hydrodynamic diameter, polydispersity and zeta potential of P SAVs on addition of poloxamer solution to mass ratio 4 mg poloxamer: 20 mg phospholipid

Abbreviation for formulations	Description of formulation (20 μL=1 mg phospholipid, 0.2 mg poloxamer)	Mean hydrodynamic diameter (nm) (± SD) (n = 3)	Polydispersity index (± SD)	Zeta Potential (mV)(± SD) (n = 5)
P SAV		222.7 (4.5)	0.397 (0.023)	-33.2 (0.1)
P SAV-L 101	400 μL 1 $\%^{m}/_{v}$ poloxamer solution to 20 mg phospholipid	257.5 (4.3)	0.502 (0.028)	-35.2 (0.5)
P SAV-L 121	400 μL 1 $\%^{m}/_{v}$ poloxamer solution to 20 mg phospholipid	324.6 (8.4)	0.476 (0.051)	-35.1 (0.4)

Key: P SAV = polymerised surface adsorbed vesicle, L101 = poloxamer 331, L121 = poloxamer 401

The nature of poloxamer association to liposomes was investigated by Jamshaid and co-workers (Jamshaid *et al.*, 1988). The authors noted that on addition of Synperonic® F127 to polystyrene latex microspheres, the mean hydrodynamic diameter as calculated using PCS increased, indicating the thickness of the adsorbed poloxamer layer. At higher poloxamer concentrations, a second peak at approximately 20 nm appeared which may be due to the formation of poloxamer micelles. At the poloxamer concentrations used in our studies, such a peak was not present, indicating that poloxamer micelles were not forming to a high extent. Jamshaid and colleagues also reported that the coating of F127 onto egg PC liposomal formulations resulted in a smaller adsorbed layer of poloxamer as measured using PCS than when latex particles were used. This may either have been due to a lower mass of poloxamer adsorbed onto liposomes compared to latex particles or due to penetration of portions of the poloxamer chains into liposomal bi-layers. The interpretation of data from the measurement of the thickness of adsorbed polymer layers onto a particulate surface using PCS must be undertaken with care as changes in sample polydispersity due to the formation of aggregates or micelles will change the values of the mean hydrodynamic diameters determined (Min *et al.*, 2002). Jamshaid and co-workers (Jamshaid *et al.*, 1988) also reported that the loss of encapsulant (6-carboxyfluorescein) from SUVs was higher than MLVs when liposomes were incubated in F127 and lowest when liposomes were incubated in PBS only. These results indicate that on addition of poloxamers to

liposomes, regions of the membrane are weakened, possibly by creation of pores by hydrophobic poloxamer chains penetrating the bi-layer.

Studies by the same group (Khattab *et al.*, 1995) showed that liposomes composed of DPPC/cholesterol released only small quantities of desferrioxamine when incubated with poloxamer 338 (F108) when compared to non-coated liposomes and was thought to be due to the more 'solid' nature of the DPPC/cholesterol bi-layer due to a higher transition temperature. Castile and co-workers (Castile *et al.*, 1999) showed using high sensitivity differential scanning calorimetry that when DPPC and DMPC liposomes were incubated with poloxamer 338 and poloxamer 407 that the poloxamers were incorporated into the bi-layer to some extent. In further studies, the authors showed that liposomes were more susceptible to the effects of poloxamer incubation at temperatures near to liposomal transition temperature where the bi-layers were more 'fluid' (Castile *et al.*, 2001). In our studies using DODPC liposomes, the effect of polymerisation of the phospholipid membranes on the transition temperature was not evaluated (non-polymerised DODPC liposomes have a transition temperature of 18.7 ± 2.8 °C (Koynova and Caffrey, 1998)). Polymerised DODPC liposomes may have a higher transition temperature (> 18.7 °C) than non-polymerised DODPC liposomes or may not possess one at all (Gaub *et al.*, 1984). So, though it would be expected that poloxamer adsorption onto non-polymerised DODPC liposomes would result in bi-layer penetration by the hydrophobic chains of the poloxamer, this situation may not be mirrored for polymerised liposomes and the majority of poloxamer may be rendered adsorbed to the surface.

Zigterman and co-workers (Zigterman *et al.*, 1987) showed that carboxyfluorescein incorporated liposomes (phosphatidylethanolamine/DPPC/SA/cholesterol) released their load to higher extents on addition of poloxamers with lower HLB values such as L101 and L121 than on addition of poloxamers with lower molecular weights and higher HLB values. The extent of release also increased when the liposomes were incubated with higher concentrations of poloxamers. These results should not have an impact on the release of TT from the poloxamer-coated liposomes in our studies as the protein is adsorbed to the surface of poloxamer-coated liposomes and so neither L101 nor L121 would increase it's release from an internal liposomal compartment.

4.4.4 Quantification of the amount of poloxamer adsorbed to P SAVs

Poloxamer-coated P SAVs containing 0.2 mg poloxamer: 1 mg phospholipid were chosen as candidate formulations for intranasal delivery (prepared as detailed in section 4.4.3). Table 4.8 shows the mean percent of poloxamer adsorbed to the polymerised vesicles when 4 mg of poloxamer was added to 20 mg of DODPC liposomes and allowed to react for 24 h at 37 °C (100 rpm) for 24 h using the Sanyo Gallenkamp IOX400.XX2.C incubator (Loughborough, U.K.). Non-associated poloxamer was separated from liposome-associated poloxamer by centrifugation (150,000g, 40 min, 4 °C) and percentage association determined indirectly by analysis of supernatants for non-associated poloxamer as detailed in section 4.3.2. Though both L101 and L121 adsorbed to P SAVs to a high percentage (90.5 ± 0.019 $\%^{m}/_{m}$ and 96.2 ± 0.003$\%^{m}/_{m}$ respectively) (mean (mg poloxamer associated/ mg total poloxamer in formulation) ± SD), it was calculated that L121 adsorbed significantly higher ($p<0.05$) (Student's t-test: two sampled assuming equal variances) to P SAVs than L101. On imaging both L101- and L121-coated liposomes using TEM, it could be seen that vesicles remained intact on the addition of poloxamers to P SAVs (0.2 mg poloxamer: 1 mg phospholipid).

Table 4.8 Quantification of L101 and L121 adsorption to polymerised vesicles (400 μL 1 $\%^{m}/_{v}$ poloxamer solution to 20 mg lyophilised polymerised vesicles) (n = 3)

Formulation	Mean association ($\%^{m}/_{m}$) (± SD)
P SAV-L101	90.5 (0.019)
P SAV-L121	96.2 (0.003)

Key: P SAV = polymerised surface adsorbed vesicle, L101 = poloxamer 331, L121 = poloxamer 401

4.4.5 *In vitro* release of surface-adsorbed TT from chitosan and poloxamer adsorbed polymerised vesicles

The retention of TT with non-coated and chitosan- and poloxamer-coated polymerised liposomal formulations (which contains both liposomally associated and free TT as well as liposomally associated and free chitosan or poloxamer depending on the formulation) was investigated following incubation (37 °C, 100 rpm) in PBS (pH 7.4) (see section 2.3.10) (figure 4.3). Non-coated P SAVs were prepared using the procedure as outlined in section 4.3.3 and coated with ChCl or ChGl (section 4.4.1 and section 4.4.2) and

L101 and L121 (section 4.4.3 and section 4.4.4). TT was mixed with radio-labelled TT as a tracer for the determination of the quantity of protein retained over various time-points.

Initial (0 h) mean percentage of TT associated with polymerised SAVs coated with either ChCl or ChGl was shown to be 29.4 ± 4.6 $\%^{m}/_{m}$ and 30.7 ± 4.4 $\%^{m}/_{m}$ respectively (mean (mg protein associated to liposome/mg total protein in formulation) ± SD) which was not significantly different ($p<0.05$) than the mean percentage of TT associated to non-coated P SAVs (25.9 ± 5.1 $\%^{m}/_{m}$). This may be due to the very small quantity of chitosan (chloride and glutamate) added to the P SAV formulations not altering the surface characteristics of polymerised liposomes in a manner that would affect TT adsorption to and subsequent release from it. However, mean initial percentage of TT associated to P SAVs coated with either L101 or L121 (9.3 ± 1.6 $\%^{m}/_{m}$ and 11.0 ± 3.2 $\%^{m}/_{m}$ respectively) were shown to be significantly lower ($p<0.05$) than TT associated to either the chitosan-coated P SAVs or non-coated P SAVs. There were no significant differences ($p<0.05$) in percentage of initial TT associated to liposomal formulations between either the two chitosan preparations or between the two poloxamer preparations. At all other time points (2 h and 24 h) there were no significant difference between formulations in respect of percentage remaining TT associated to liposomal formulations.

The analysis of the release data indicated a rapid initial desorption/release of associated TT from non-coated P SAVs and ChCl- and ChGl-coated P SAVs within 2 h (11.1 ± 7.8 $\%^{m}/_{m}$, 8.8 ± 4.0 $\%^{m}/_{m}$ and 16.3 ± 3.0 $\%^{m}/_{m}$ respectively) and a higher mean percentage of remaining TT associated to these vesicles at 24 h (10.6 ± 4.7 $\%^{m}/_{m}$, 13.6 ± 1.9 $\%^{m}/_{m}$ and 11.7 ± 6.4 $\%^{m}/_{m}$ respectively) compared to L101- and L121-coated P SAVs (6.5 ± 5.7 $\%^{m}/_{m}$ and 4.5 ± 1.6 $\%^{m}/_{m}$ respectively) though this was not significantly different ($p<0.05$). Significance between groups was analysed using a one-way ANOVA and Tukey's HSD test.

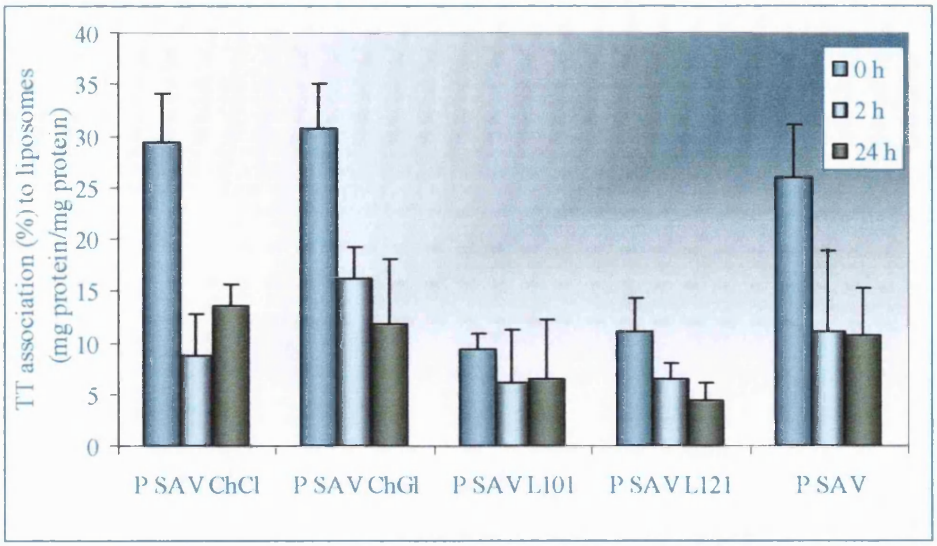

Figure 4.3 The TT retention profile (expressed as the percentage of TT associated to liposomes as opposed to free in solution) for various liposomal formulations in PBS pH 7.4, 37 °C, 100 rpm (n = 3). Results are means (± SD)

Key: P SAV = polymerised surface adsorbed vesicle, ChCl = chitosan chloride, ChGl = chitosan glutamate, L101 = poloxamer 331, L121 = poloxamer 401

The *in vitro* TT retention profiles by liposomal formulations may not be mirrored in the *in vivo* situation, as demonstrated by Hunter and Bennett (1984). The authors showed that L101 and L121 oil-in-water emulsions containing BSA retained more (both 98 %) protein when injected into the hind footpad of ICR mice after 4 days than when stability *in vitro* was determined after 4 days (22 % and 31 % protein retention respectively). L101 and L121 formulations were subsequently shown to evoke the highest BSA-specific serum IgG antibody levels (6 weeks post-priming) in comparison to formulations containing L81, L92 or L122. Thus, in the case of poloxamer surfactants, the *in vitro* protein retention and release profile may not give a good indication for the *in vivo* release and immune response characteristics.

4.4.6 Systemic (humoral and cellular) and mucosal immune response to poloxamer- and chitosan-coated and non-coated liposomal TT formulations following intranasal administration

Figure 4.4 shows the TT-specific serum IgG antibody titres following the intranasal administration of 10 µg of TT as a priming dose (as either free in solution or in various liposomal formulations). Mice were boosted intranasally on day 35 and 59 with 5 µg of

TT in the same formulations (by diluting priming formulations with an equal volume of saline).

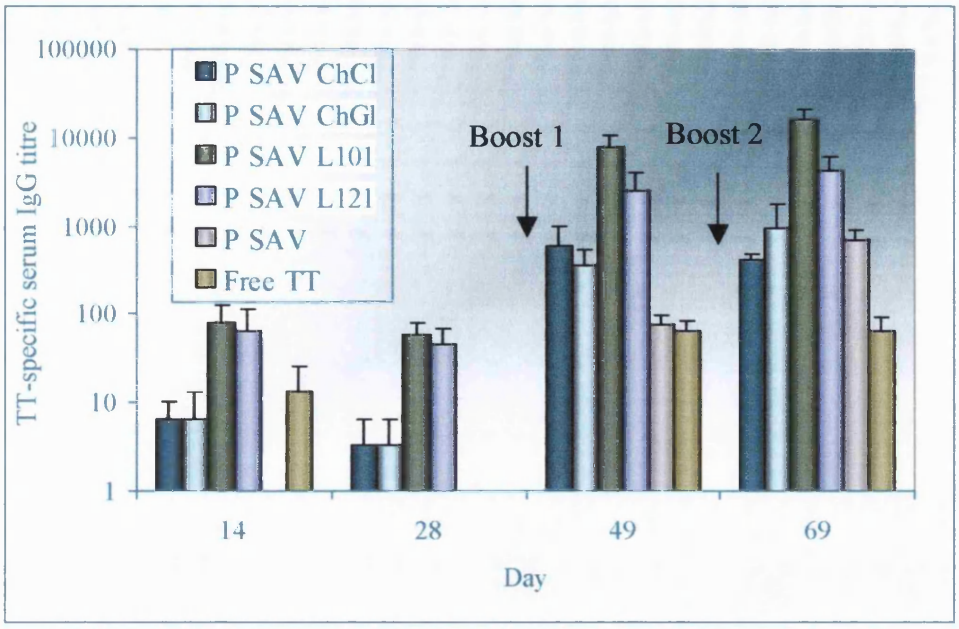

Figure 4.4 TT-specific serum IgG titres raised in mice (n=5) in response to intranasal administration of TT in various liposomal formulations or free in solution. Mice were primed with 10 μg of formulated TT and boosted on days 35 and 59 with 5 μg of formulated TT. Results are means (± SE)

Key (refer to table 4.2 for further information): P SAV = polymerised surface adsorbed vesicle, ChCl = chitosan chloride, ChGl = chitosan glutamate, L101 = poloxamer 331, L121 = poloxamer 401

The primary response to administration of TT (either free in solution or in various liposomal formulations) observed on days 14 and 28 showed only base-line levels of TT-specific serum IgG antibody levels (no significant differences $p<0.05$). Mean TT-specific serum IgG titres on day 14 elicited in response to the administration of both L101 and L121 containing formulations (80 and 64 respectively) were higher than that evoked in response to all other formulations. This was also mirrored on day 28 where, although the mean titres for all formulations had decreased, L101 and L121 maintained the highest mean TT-specific serum IgG antibody levels (58 and 45 respectively).

Day 49 serum analysis (following boosting on day 35) showed that all animals produced an increase in the mean TT-specific serum IgG titres in response to the administration of all formulations (though significant differences were not achieved ($p<0.05$)). Animals immunised with L101-coated liposomes achieved the highest mean TT-specific serum IgG titre (7,782 ± 2,458) followed by L121 coated liposomes (2,490 ± 1,492).

Administration of ChCl- and ChGl-coated liposomes evoked mean TT-specific serum IgG responses of only 588 ± 372 and 346 ± 191only, yet were still higher than when P SAVs were administered alone (77 ± 22) which evoked an antibody response similar to free TT (64 ± 18).

After a second boost on day 59, tail bleeds taken on day 69 showed that the trends seen on day 49 were further accentuated (though significant differences were still not achieved ($p<0.05$)). Animals given L101-coated liposomes produced a mean TT-specific serum IgG antibody titre that was 243.2 times higher (15,565 ± 4,915) than that elicited by the administration of free TT, followed by the response to L121-coated liposomes (4,147 ± 1,803) which was 64.8 times the antigen-specific serum IgG titre produced by free TT. ChCl-, ChGl- and non-coated P SAVs evoked lower responses (410 ± 62.7, 934 ± 791 and 691.2 ± 204.8 respectively) (6.4, 14.6 and 10.8 times the mean TT-specific serum IgG antibody response to free TT respectively).

The mean TT-specific serum IgG titre for P SAVs containing L101 on day 49 (7,782 ± 2,458) can be compared to TT-specific serum IgG titres evoked when free TT was administered intramuscularly using the same dosing regime (13,926 ± 5,116). When the intranasal group administered P SAVs containing L101 were boosted for the second time on day 59, bleeds on day 69 revealed that the mean TT-specific serum IgG titre was 15,565 ± 4,915 which was higher (though not significantly higher) ($p<0.05$) than for the intramuscular group with one boost. The length of elevation of the titres remains to be extensively investigated as it can be seen that where TT-specific serum IgG titres at earlier time points (day 14 = 83 and day 28 = 284) were still rising for free TT when administered intramuscularly pre-boosting that the intranasal response for all liposomal formulations were in decline. The mean TT-specific serum IgG antibody titre for non-coated P SAVs (1 $\%^{m}/_{m}$ theoretical loading (mg protein/mg phospholipid)) on day 69 was 10.8 times higher than the antibody titre elicited by intranasal administration of free TT only. Studies in section 3.4.4 showed that animals immunised intranasally with P SAVs (5 $\%^{m}/_{m}$ theoretical loading (mg protein/mg phospholipid)) possessed TT-specific serum IgG antibody levels 7.3 times higher than free TT. Thus, though the results were not directly analysed for levels of significance, the decrease in theoretical loading from 5 $\%^{m}/_{m}$ to 1 $\%^{m}/_{m}$ resulted in a small mean increase in TT-specific serum IgG antibody levels when compared to the antibody response to free TT administration alone.

The adjuvanticity of L101 and L121 was previously demonstrated by Snippe *et al.* (1981) who primed female BALB/c mice with 30μg dinitrophenyl-BSA mixed with various poloxamers (100 μg *per* dose) intraperitoneally and boosted animals intravenously on day 40 with 30 μg of dinitrophenyl-BSA free in solution. The authors reported that animals immunised with poloxamers with a low HLB (L101 and L121 formulated dinitrophenyl-BSA) produced a higher secondary antibody response than those animals immunised with higher HLB values such as F68 (HLB=29) and L31 (HLB=3.5).

Studies by the same group (Hunter *et al.*, 1981) showed that after rear footpad immunisation of ICR white mice with 25 μg of BSA in various poloxamer (2.5 mg poloxamer *per* dose) formulations in an oil-in-water emulsion containing Drakeol 6VR or eicosane as a mineral oil, a higher antibody immune response was achieved by formulations containing L121 and L101 than those formulations containing poloxamers with higher HLB values such as L31, L33 and P38.

In both studies (Snippe *et al.*, 1981 and Hunter *et al.*, 1981), L121 was a better adjuvant for antibody formation than L101 which is in contrast to the antigen-specific serum IgG antibody responses observed in our studies, where L101 formulations produced higher antigen-specific serum IgG antibody levels than L121. The stronger adjuvant action of L101 in comparison to L121 was in agreement with further studies reported by Hunter and Bennett (1984) and Hunter and co-workers (Hunter *et al.*, 1991). In the first study the authors injected 25 μg of BSA in an oil-in-water emulsion containing 2.5 mg Drakeol 6VR mineral oil, 0.2 % Tween® 80 and 1.25 mg of poloxamer in a 50 μL volume to the hind footpad of ICR mice and showed that formulating BSA with L101 evoked a higher mean BSA-specific serum IgG antibody titre than formulating BSA with L121. In the second study (Hunter *et al.*, 1991) female ICR mice were given a subcutaneous injection to the hind footpad of 50 μg of trinitrophenyl conjugated hen egg albumin in an oil-in-water emulsion containing 2 % squalane and 1 mg of poloxamer and boosted on day 90. Animals receiving albumin in L101 formulation produced higher primary and secondary antigen-specific serum IgG antibody response than albumin in L121 formulations.

The studies conducted using L101- and L121-coated liposomes can be compared to the investigations of Zigterman and co-workers (Zigterman *et al.*, 1987) who replaced the

poloxamer-$^{O}/_{W}$ emulsion described above with haptenated (*N*-(2,4-dinitrophenyl-)-*β*-alanylglycylglycine)-liposomes composed of haptenated PE and DPPC. The authors mixed the liposomal formulations with 0.2 nmol of various poloxamers immediately before intraperitoneal administration to 10 week old female BALB/c mice and showed that all non-ionic block co-polymer formulations resulted in a higher humoral response than animals who received haptenated liposomes alone. However, contrary to their earlier findings (Snippe *et al.*, 1981), higher molecular weight hydrophobic poloxamers (such as L101 and L121) displayed weaker adjuvant properties than lower molecular weight hydrophobic poloxamers such as L81. In the same way, L122 (high molecular weight) showed weaker adjuvant properties than L72 and L92 (lower molecular weights) even though the percentage of hydrophilic polymer (PEO) remained the same.

These opposing results on the role of a low HLB and high molecular weight on adjuvanticity may be due to a number of factors. Zigterman and colleagues (Zigterman *et al.*, 1987) used liposomally formulated membrane bound antigens whereas the studies by Snippe and colleagues (Snippe *et al.*, 1981); Hunter and colleagues (Hunter *et al.*, 1981); Hunter and Bennett (1984) and Hunter and colleagues (Hunter *et al.*, 1991) used $^{o}/_{w}$ emulsions containing BSA in the oil phase. Another factor may be the difference in the route of administration of the formulations. Although Snippe and colleagues (Snippe *et al.*, 1981) primed using the intraperitoneal route, the boosting dose was given intravenously whereas mice in the studies by Zigterman and colleagues (Zigterman *et al.*, 1987) received immunisations intraperitoneally only and formulations were administered subcutaneously and by injection to the rear footpad in the studies published by Hunter *et al.* (1981); Hunter and Bennett (1984) and Hunter and co-workers (Hunter *et al.*, 1991). Therefore, the differences in opinion about the role of HLB and high molecular weight poloxamers in adjuvanticity may be dependent on the route of administration as well as variations in antigen physicochemical properties.

Previous studies conducted by our own group (Eyles *et al.*, 1999) showed that the use of L121 as a stabiliser in the preparation of co-encapsulated F1 and V antigen poly DL-lactide (mwt 124 kDa) nanoparticles resulted in higher V-specific serum IgG antibody levels after intranasal administration to mice than when polyvinyl acetate stabilised poly L lactide (mwt 100 kDa) nanoparticles with co-encapsulated F1 and V antigen were given. The authors also reported that the L101 formulated nanoparticles evoked a V-specific sIgA response in lung washings, which was comparable to that when

'conventional' nanoparticles were administered. However, the volume mean diameter of L101 formulated nanoparticles was much lower (150 nm) than that of the conventionally prepared nanoparticles (500 nm). This particle size difference may have had an impact on particle uptake from the nasal tract (see section 1.4.6.3, section 1.4.6.4 and section 1.4.6.5) and so the differences in immune responses seen between the formulations may not only be directly due to L101.

The high TT-specific serum IgG antibody levels produced after administration of L101- and L121-coated polymerised liposomes was as expected though the low humoral antibody response observed in animals given TT with ChCl- and ChGl-coated polymerised liposomes was surprising. Administration of the poloxamer-coated P SAVs resulted in higher antigen-specific serum IgG antibody levels compared to chitosan-coated liposomes though the amount of initial TT association (mg protein detected/ mg initial protein added) to coated P SAVs was higher for non-coated P SAVs and chitosan-coated P SAVs (approximately 30 $\%^{m}/_{m}$) and lower for poloxamer-coated P SAVs (approximately 10 $\%^{m}/_{m}$) (section 4.4.5). The mechanism of poloxamer adjuvanticity is thought to be due to the adsorption of the protein to the poloxamer attachment of the poloxamer to a hydrophobic surface such as a liposomes (Hunter, 2002). Such interactions are less denaturing to the protein than when proteins directly interact with hydrophobic surfaces such as plastic (e.g. containers and syringes) and so the antigenicity of proteins is maintained. This may possibly be one reason as to why a low TT association to poloxamer-coated liposomes resulted in a strong immune response. Newman and colleagues (Newman *et al.*, 1998a and Newman *et al.* 1998b) review other mechanisms by which certain poloxamers exert immuno-modulatory effects.

The adjuvanticity of chitosan preparations, especially after nasal delivery has been reviewed by van der Lubben and colleagues (van der Lubben *et al.*, 2001) and Illum and colleagues (Illum *et al.*, 2001). Mice given chitosan glutamate-coated liposomes produced a higher mean TT-specific serum IgG titre than when non-coated liposomes were administered (primary response and response after boosting) which is in agreement with studies by Bramwell and co-workers (Bramwell *et al.*, 1999). The authors showed that chitosan-coated DPPC/DCP liposomes (anionic) containing TT evoked a significantly higher ($p< 0.05$) primary TT-specific serum IgG antibody response than non-coated liposomes and free antigen after intranasal administration.

After boosting, the chitosan-coated liposomes maintained mean TT-specific serum IgG antibody levels higher (though no longer significantly higher (p<0.05)) than all other formulations investigated.

Antibodies of the IgG isotype can be further divided into subclasses, which possess differing biological activities. The occurrence of antibodies of the IgG_1 subclass are a good indicator of the activity of a T_h2 response whereas the presence of antibodies of the IgG_{2a} subclass are an indicator of the activity of a T_h1 response (cell-mediated immunity) (section 1.1.2). Figure 4.5 and figure 4.6 show the mean TT-specific serum IgG_1 and IgG_{2a} antibody titres respectively on day 69, following the intranasal administration of 10 μg of TT as a priming dose (as either free in solution or in various liposomal formulations). Mice were boosted intranasally on day 35 and 59 with 5 μg of TT in the same formulations (by diluting priming formulations with an equal volume of saline).

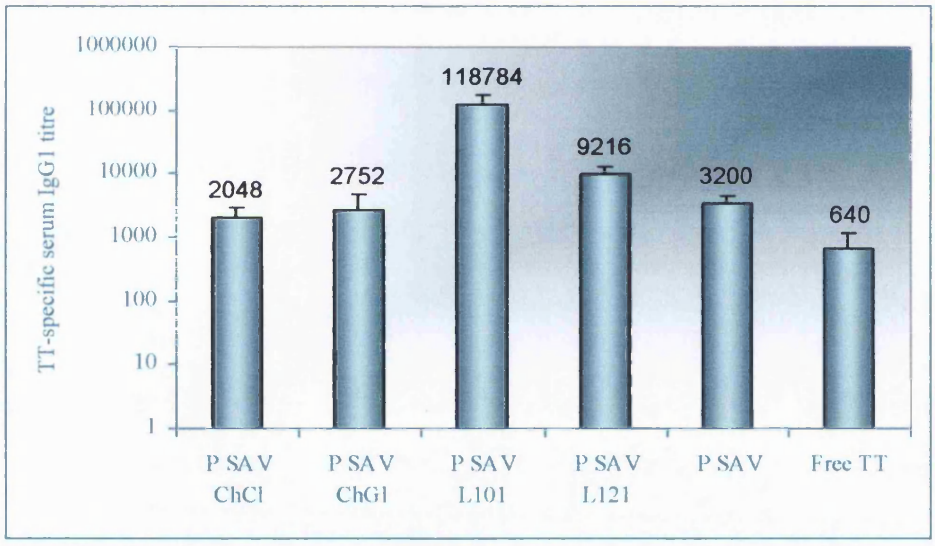

Figure 4.5 TT-specific serum IgG_1 titres (day 69) raised in mice (n=5) in response to intranasal administration of TT in various liposomal formulations or free in solution. Mice were primed with 10 μg of formulated TT and boosted on days 35 and 59 with 5 μg of formulated TT. Results are means (± SE)

Key (refer to table 4.2 for further information): P SAV = polymerised surface adsorbed vesicle, ChCl = chitosan chloride, ChGl = chitosan glutamate, L101 = poloxamer 331, L121 = poloxamer 401

Animals immunised with TT delivered with L101-coated P SAVs demonstrated the highest TT-specific serum IgG_1 response (mean titre ± SE) (118,784 ± 53,562) followed by L121-coated P SAVs (9,216 ± 3,396). Animals given TT in non-coated P SAVs achieved a higher mean TT-specific serum IgG_1 titres (3,200 ± 1,180) (even though in

similar orders of magnitude) than P SAVs coated with ChGl (2,752 ± 1,922) and ChCl (2,048 ± 868). The TT-specific serum IgG_1 response to free TT was the lowest. Significant differences ($p<0.05$) between groups could not be found on determination using a one-way ANOVA.

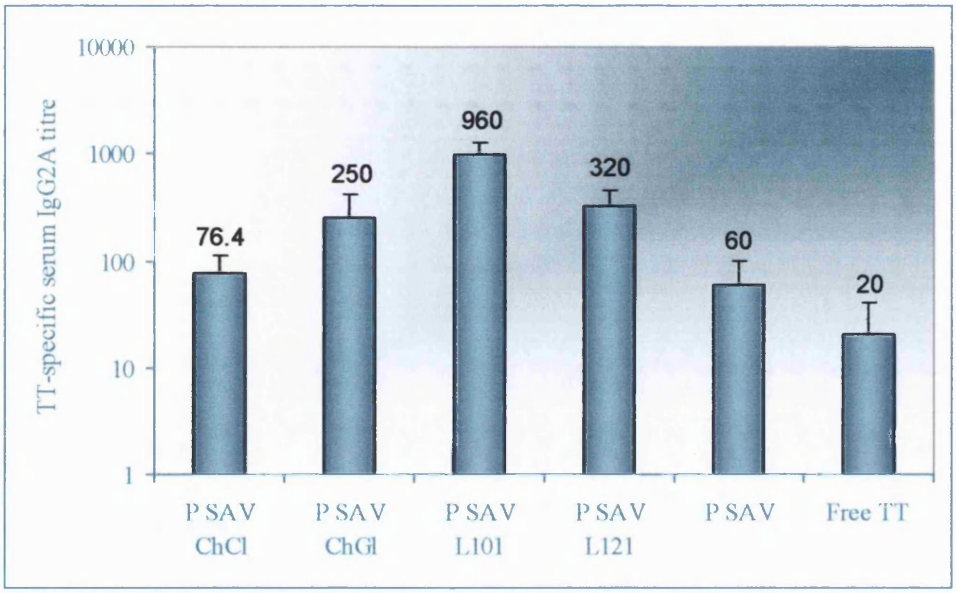

Figure 4.6 TT-specific serum IgG_{2a} titres (day 69) raised in mice (n=5) in response to intranasal administration of TT in various liposomal formulations or free in solution. Mice were primed with 10 µg of formulated TT and boosted on days 35 and 59 with 5 µg of formulated TT. Results are means (± SE)

Key (refer to table 4.2 for further information): P SAV = polymerised surface adsorbed vesicle, ChCl = chitosan chloride, ChGl = chitosan glutamate, L101 = poloxamer 331, L121 = poloxamer 401

Animals receiving L101-coated P SAVs produced the highest TT-specific serum IgG_{2a} antibody levels (960 ± 299) (mean titre ± SE) followed by L121-coated P SAVs (320 ± 135.6) and ChGl-coated P SAVs (250 ± 156.5). When P SAVs were coated with ChCl, the TT-specific serum IgG_{2a} antibody levels elicited following intranasal administration were similar to the response seen when non-modified P SAVs alone were given (76.4 ± 34.9 and 89.4 ± 40 respectively), though remained higher than that evoked by free TT alone (44.7 ± 20). Significant differences ($p<0.05$) between groups could not be found on determination using a one-way ANOVA.

The overall antibody response to all formulations was a larger antigen-specific serum IgG_1 antibody titre in each case than antigen-specific serum IgG_{2a} titre (i.e. an indication of the dominance of a T_h2 response). Though significant differences ($p<0.05$) did not

exist between groups on analysis of antigen-specific serum IgG_1 and IgG_{2a}, the analysis of trends using mean titres can elucidate important information regarding T_h1 and T_h2 shifts. On day 69, animals given TT delivered with L101-coated P SAVs showed the highest T_h1 and T_h2 response in comparison to all other formulations.

The results reported in figure 4.5 and figure 4.6 in respect of the poloxamer containing formulations are in agreement with the reports of Hunter and co-workers (Hunter *et al.*, 1991) who administered 50 μg of trinitrophenyl conjugated hen egg albumin in an oil-in-water emulsion containing 2 % squalane and 1 mg of poloxamer to the hind footpad of ICR mice *via* subcutaneous injection. Animals receiving albumin in L101 formulation produced a predominant albumin-specific IgG_1 response, which is in agreement to our results with the administration of the L101 formulation with TT. The authors also showed that the administration of L121 in a vaccine formulation resulted in a lower antigen-specific serum IgG_1 response than when L101 formulations were given which also concurs with our results. As the albumin-specific serum IgG_{2a} antibody levels were approximately the same when L101 and L121 formulations were given, there was an enhanced T_h2 shift in the response when L101 was administered in comparison to when L101 was given which mirrors the trend observed in our results.

These findings are in agreement to the work of Newman and colleagues (Newman *et al.*, 1998a; Newman *et al.*, 1998b) who concluded from their studies using ovalbumin and various poloxamers, that after subcutaneous administration in female C57BL/6 mice, formulations containing poloxamers with 5 % PEO (more hydrophobic) induced a mixed T_h1/T_h2 response whereas formulations containing poloxamers with a PEO content of 10 % (less hydrophobic) resulted in a predominantly T_h2 response (as determined after antigen stimulated *in vitro* cytokine production by splenocytes of immunised mice). Both L101 and L121 contain PEO blocks that are approximately 10 % of the total poloxamer molecular weight though L121 has a lower HLB than L101 (0.5 and 1 respectively). Thus, as can be deduced from figure 4.5 and figure 4.6, animals immunised with L121-coated polymerised liposomes produced a more mixed T_h1/T_h2 response (though still T_h2 dominant as can be seen from taking ratios between IgG_1 and IgG_{2a} antibody titres) than animals given L101-coated polymerised liposomes which showed an even stronger T_h2 response.

The overall T_h2 type nature of the immune response to chitosan-coated polymerised liposomes concurs with the results of Bramwell and co-workers (Bramwell *et al.*, 1999) who observed predominantly TT-specific serum IgG_1 antibodies after the intranasal administration to mice of chitosan coated DPPC/DCP liposomes encapsulating TT. These findings were also mirrored in intranasal studies in humans (McNeela *et al.*, 2004) where it was shown that the administration of a mutant of diphtheria toxin (CRM_{197}) with chitosan resulted in a predominantly T_h2 response.

Figure 4.7 shows the mean antigen-specific serum IgA antibody titres on day 69 for the formulated TT preparations following intranasal administration. The mean TT-specific serum IgA antibody titres were highest for chitosan- and L101- coated P SAVs. L121-coated P SAVs evoked mean IgA titres about half of that elicited by administration of the chitosan- and L101-coated P SAVs whilst non-coated P SAVs possessed mean antigen-specific serum IgA titres no greater than that elicited by delivery of free TT. In comparison, when free TT was administered intramuscularly there were no detectable TT-specific serum IgA antibody titres (results not shown).

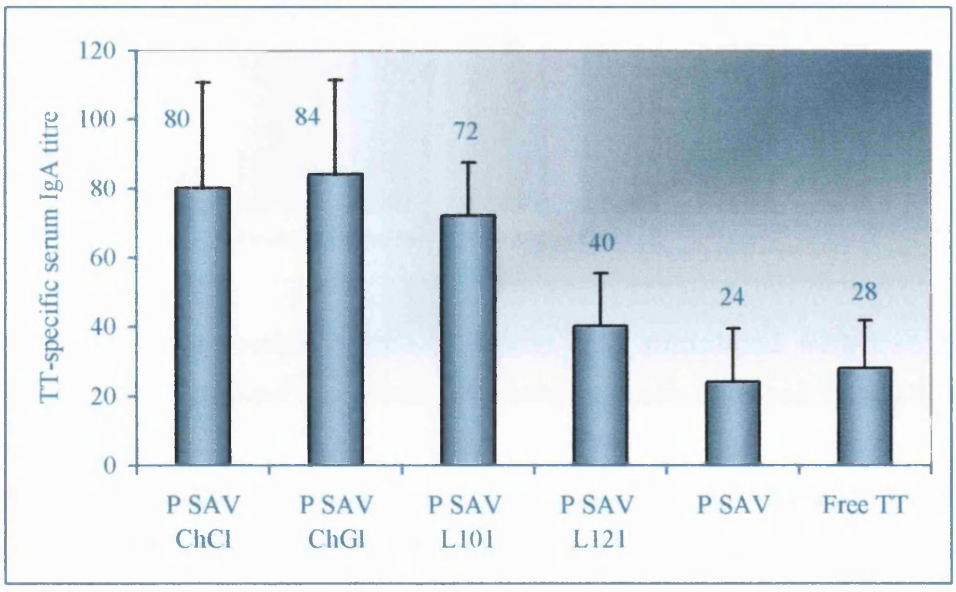

Figure 4.7 TT-specific serum IgA titres raised in mice (n=5) in response to intranasal administration of TT in various liposomal formulations or free in solution. Mice were primed with 10 μg of formulated TT and boosted on days 35 and 59 with 5 μg of formulated TT. Results are means (± SE)

Key (refer to table 4.2 for further information): P SAV = polymerised surface adsorbed vesicle, ChCl = chitosan chloride, ChGl = chitosan glutamate, L101 = poloxamer 331, L121 = poloxamer 401

Cytokine production by mixed spleen cells of immunised and non-immunised mice in response to antigen stimulation (see section 3.3.7 and section 3.3.8) are shown in figures 4.8-4.13. Spleen cells from mice given L101-coated P SAVs produced significantly higher ($p<0.05$) levels of IL-2, IL-4, IL-6 and TNF-α following *in vitro* TT stimulation when compared to all the other groups. Spleen cells from mice administered L101-coated P SAVs also produced significantly higher levels ($p<0.05$) of IFN-γ compared to that produced by chitosan-coated polymerised liposomes, free TT and naïve groups but not significantly different ($p<0.05$) to that elicited by mice given L121-coated P SAVs. There were no other significant differences amongst the groups and in the case of IL-12 production there were no significant differences ($p<0.05$) at all between groups. Significance between groups was analysed using a one-way ANOVA and Tukey's HSD test.

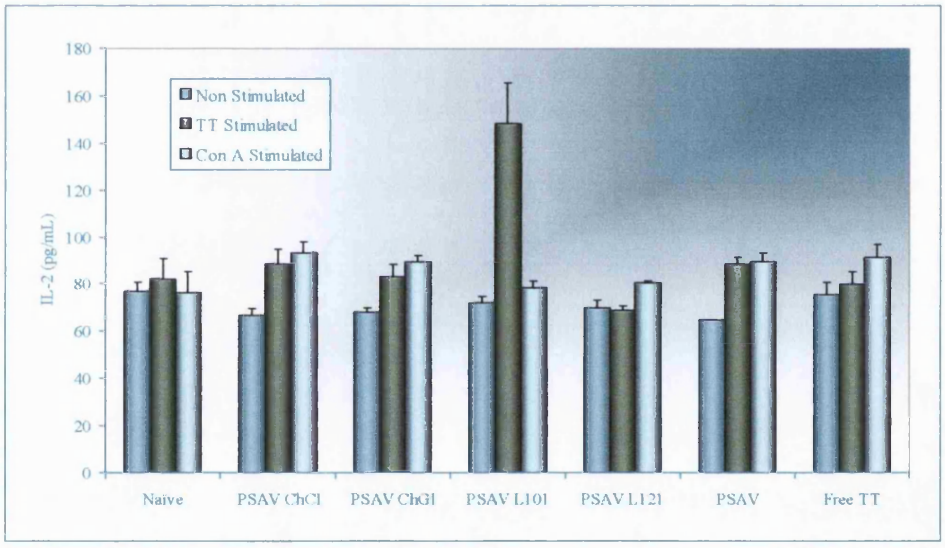

Figure 4.8: IL-2 production by mixed spleen cells stimulated with free TT or with PBS (pH 7.4). Results are means of pooled spleens cultured in triplicate (± SE)

Key (refer to table 4.2 for further information): P SAV = polymerised surface adsorbed vesicle, ChCl = chitosan chloride, ChGl = chitosan glutamate, L101 = poloxamer 331, L121 = poloxamer 401

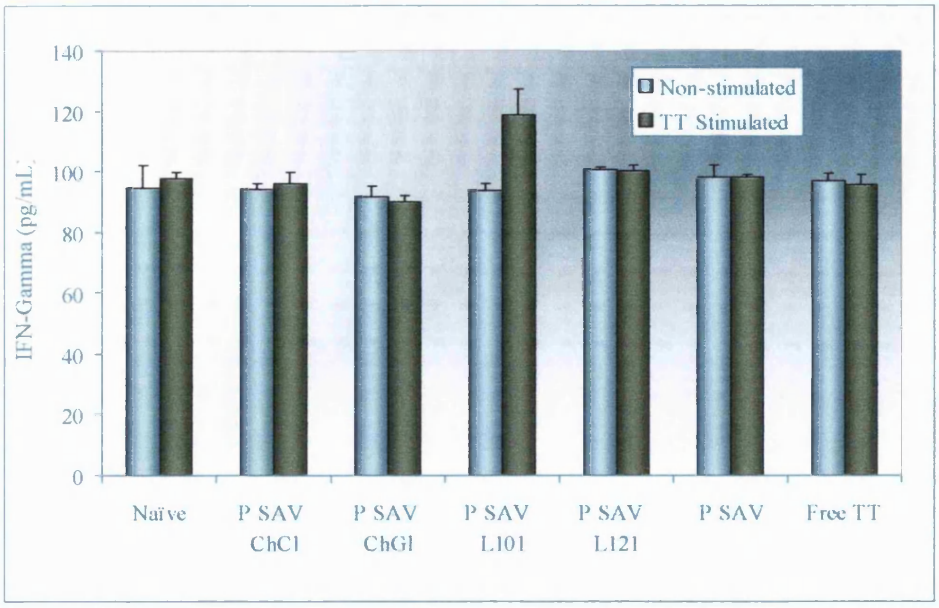

Figure 4.9: IFN-γ production by mixed spleen cells stimulated with free TT or with PBS (pH 7.4). Results are means of pooled spleens cultured in triplicate (± SE)

Key (refer to table 4.2 for further information): P SAV = polymerised surface adsorbed vesicle, ChCl = chitosan chloride, ChGl = chitosan glutamate, L101 = poloxamer 331, L121 = poloxamer 401

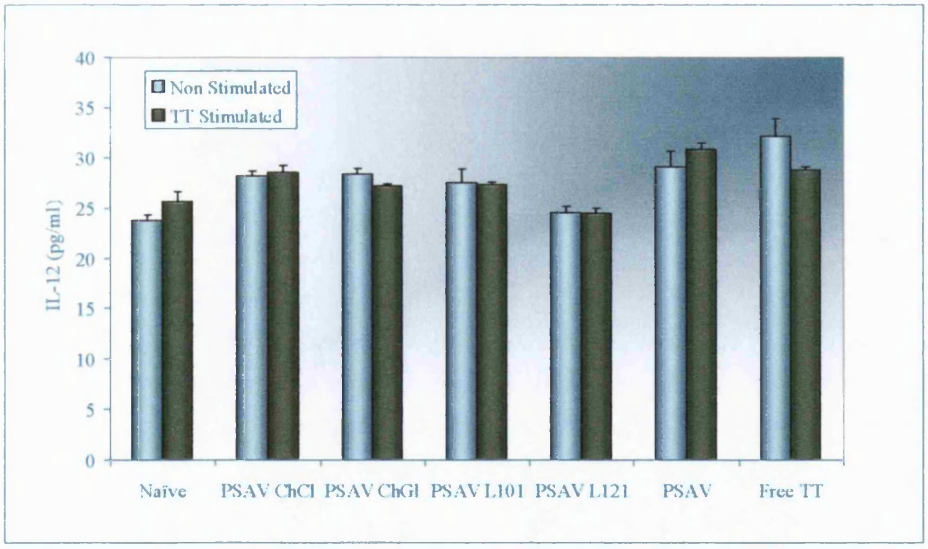

Figure 4.10: IL-12 production by mixed spleen cells stimulated with free TT or with PBS (pH 7.4). Results are means of pooled spleens cultured in triplicate (± SE)

Key (refer to table 4.2 for further information): P SAV = polymerised surface adsorbed vesicle, ChCl = chitosan chloride, ChGl = chitosan glutamate, L101 = poloxamer 331, L121 = poloxamer 401

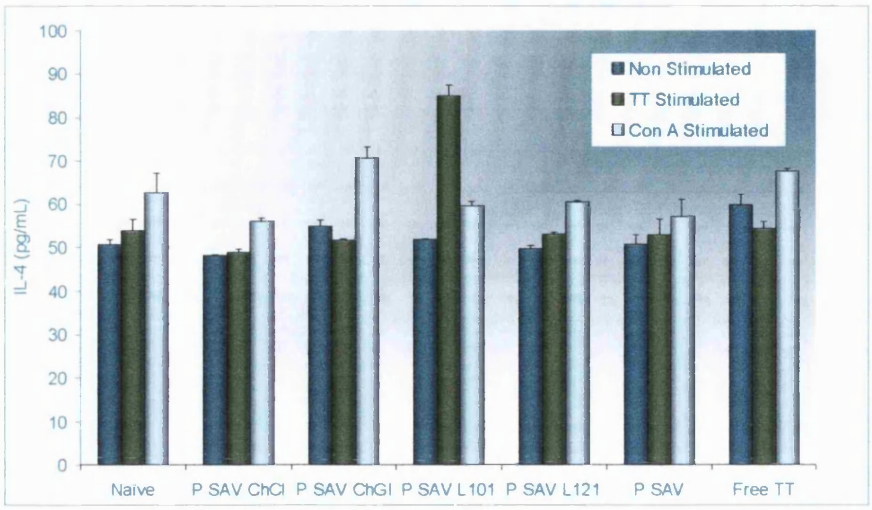

Figure 4.11: IL-4 production by mixed spleen cells stimulated with free TT or with PBS (pH 7.4). Results are means of pooled spleens cultured in triplicate (± SE)

Key (refer to table 4.2 for further information): P SAV = polymerised surface adsorbed vesicle, ChCl = chitosan chloride, ChGl = chitosan glutamate, L101 = poloxamer 331, L121 = poloxamer 401

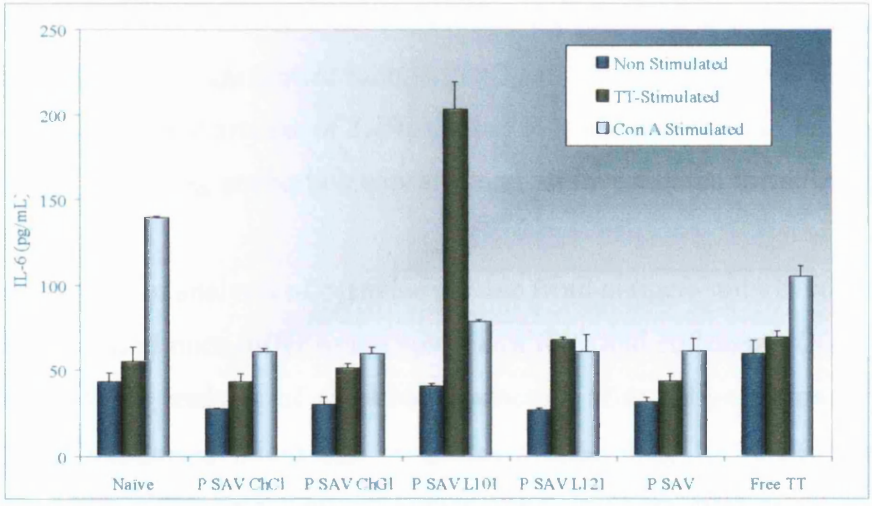

Figure 4.12: IL-6 production by mixed spleen cells stimulated with free TT or with PBS (pH 7.4). Results are means of pooled spleens cultured in triplicate (± SE)

Key (refer to table 4.2 for further information): P SAV = polymerised surface adsorbed vesicle, ChCl = chitosan chloride, ChGl = chitosan glutamate, L101 = poloxamer 331, L121 = poloxamer 401

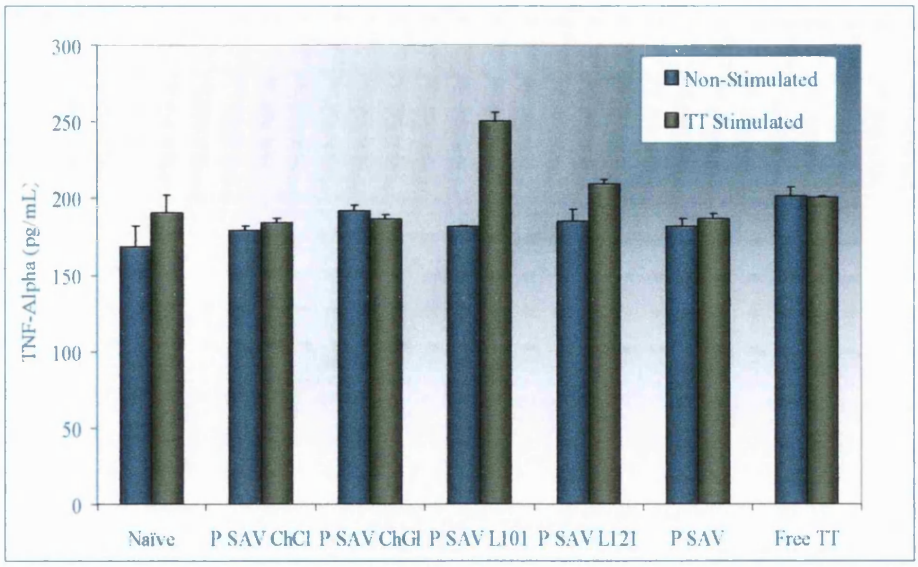

Figure 4.13: TNF-α production by mixed spleen cells stimulated with free TT or with PBS (pH 7.4). Results are means of pooled spleens cultured in triplicate (± SE)

Key (refer to table 4.2 for further information): P SAV = polymerised surface adsorbed vesicle, ChCl = chitosan chloride, ChGl = chitosan glutamate, L101 = poloxamer 331, L121 = poloxamer 401

The cytokine profile for L101-coated P SAVs revealed the presence of both a strong T_h1 and T_h2 response. This is in agreement with the IgG subclass analyses in which it was shown that intranasal administration of L101-coated P SAVs elicited the highest TT-specific serum IgG_1 and IgG_{2a} antibody levels amongst all investigated formulations.

The results seen here from analysis of cytokine release from antigen-stimulated cultured spleen cells of immunised mice differ to the opinion of Katz and colleagues (Katz *et al.*, 2000) who reported (on analysis of cytokine production after antigen stimulation of spleen cells from immunised mice) that poloxamers containing 10 % PEO tend to stimulate preferentially a T_h2 type response, which although agrees with our findings of L101-and L121-coated polymerised liposomes in terms of TT-specific serum IgG_1/IgG_{2a} analysis, differs in the results observed with regard to the types of cytokines released after antigen stimulation of cultured spleen cells of immunised mice. Mice immunised with L101-coated liposomes (L101 contains 10 % PEO) induced cytokines indicative of both a T_h1 and T_h2 response indicating a high mixed T_h1/T_h2 response. This may be due to the administration of polymerised liposomes with the poloxamers affecting the type of immune response but cannot be substantiated without conducting experiments with poloxamer administration (with TT) alone. Unlike the results seen

with L101-coated polymerised liposomes, it was surprising not to see similar cytokine release trends with L121-coated polymerised liposomes, which may be attributable to a lower overall immune response in the case of the latter.

Figure 4.14 demonstrates that low levels of faecal TT-specific secretory IgA were detected amongst all formulations. Both chitosan-coated polymerised liposomal preparations evoked the highest faecal secretory IgA response followed by poloxamer-coated polymerised liposomal formulations. Animals given non-coated P SAVs were observed to secrete the same amount of antigen-specific IgA as animals given free TT. McNeela and co-workers (McNeela *et al.*, 2001) immunised female BALB/c mice intranasally (using a 20 μL volume) with a diphtheria toxoid mutant formulated with chitosan glutamate (UPG210) and showed that chitosan formulated antigen evoked a higher antigen-specific secretory IgA response (in lung homogenate) than the administration of soluble antigen alone. This is in agreement to our studies and confirms the mucosal adjuvanticity of chitosan.

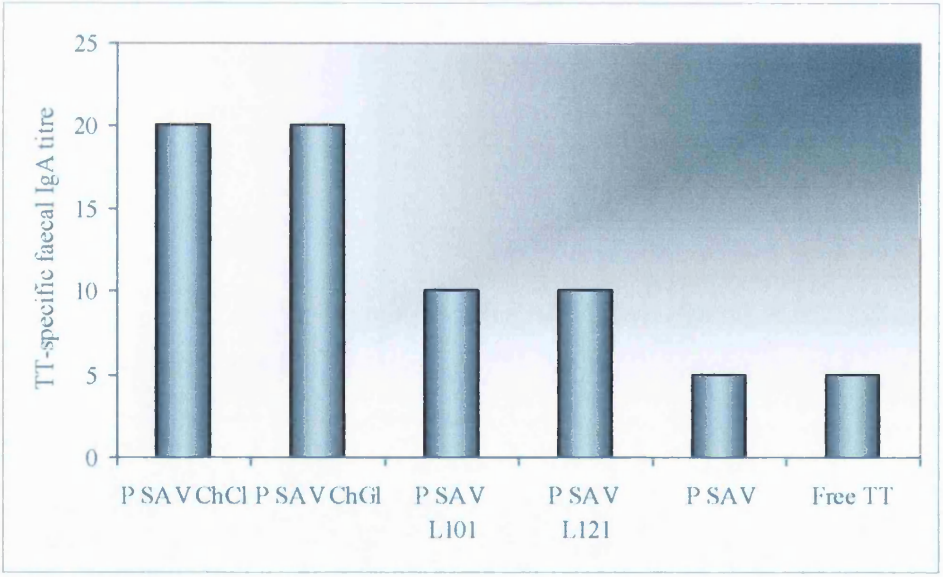

Figure 4.14 TT-specific faecal secretory IgA titres (pooled samples) raised in mice (n=5) in response to intranasal administration of TT in various liposomal formulations or free in solution. Mice were primed with 10 μg of formulated TT and boosted on days 35 and 59 with 5 μg of formulated TT. Results are means ± SE (SE = 0 for all formulations)

Key (refer to table 4.2 for further information): P SAV = polymerised surface adsorbed vesicle, ChCl = chitosan chloride, ChGl = chitosan glutamate, L101 = poloxamer 331, L121 = poloxamer 401

4.5 Conclusions

Studies conducted in chapter 3 showed that polymerised liposomes acted as a vaccine adjuvant for surface adsorbed TT after intranasal administration to female BALB/c mice (see section 3.4.4). The adjuvanticity of chitosan after intranasal administration in a liposomal formulation was demonstrated by Bramwell and co-workers (Bramwell *et al.*, 1999). The adjuvanticity of poloxamers after intranasal delivery has also been reported (Eyles *et al.* 1999). Following from this, it was deemed logical to investigate the vaccine adjuvanticity effect of chitosan or poloxamers when administered intranasally in a polymerised liposome formulation.

Section 1.4.6.3, 1.4.6.4 and 1.4.6.5 show that immune responses to the intranasal administration of a particulate formulation will vary depending on the size distribution of the formulation. Studies in section 3.4.4 investigating the immune response elicited after the administration of two liposomal formulations (one with a wide particle size distribution and one with a narrow particle size distribution) showed differences between formulations in terms of the observed immune response. With this in mind, the modification of polymerised liposomal formulations with either chitosan or poloxamers was accomplished in a manner that minimised change in particle size distribution. Poloxamers and chitosan were added after liposomal formulation in order to achieve surface adsorption only. The adsorption of TT to polymerised liposomal formulations was after chitosan and poloxamer coating in order to maintain the antigen on the surface of the particulate formulation.

The association of poloxamer 331 (L101) and poloxamer 401 (L121) to P SAVs was shown to be very high (90.5 $\%^{m}/_{v}$ and 96.2 $\%^{m}/_{v}$ respectively (mg poloxamer adsorbed to mg initial poloxamer added)), resulting in a priming dose containing 200 μg of poloxamer (with 1 mg phospholipid liposomes) being mostly adsorbed to the vesicles. In the case of chitosan formulations, a far lower quantity of chitosan was given *per* priming dose (0.5 μg chitosan to 1 mg phospholipid liposomes) as higher quantities of chitosan were found to cause liposomal aggregation (section 4.4.1). The zeta potential of both poloxamer-coated and chitosan-coated polymerised liposomes remained the same as non-coated polymerised liposomes and the particle size distributions similar (table 4.5 and 4.6). ChCl- and ChGl-coated polymerised liposomes possessed slightly higher mean hydrodynamic diameters (370.9 nm and 370.4 nm respectively) than the

mean hydrodynamic diameters of L101 and L121 coated polymerised liposomes (257.5 nm and 324.6 nm respectively) and the mean hydrodynamic diameter of non-coated polymerised liposomes (~220 nm).

The initial association of TT (mg adsorbed TT/ mg initial TT added) to ChCl- and ChGl-coated polymerised liposomes (29.4 ± 4.7 $\%^{m}/_{m}$ and 30.7 ± 4.4 $\%^{m}/_{m}$ respectively) was shown not to be significantly different ($p<0.05$) to TT association to non-coated polymerised liposomes (25.9 ± 5.1 $\%^{m}/_{m}$) which may be due to the low levels of chitosan added to the formulations (0.5 μg chitosan to 1 mg phospholipid) not being high enough to affect TT association. However, TT association (mg adsorbed TT/mg initial TT added) to L101- and L121-coated polymerised liposomes was significantly lower (9.3 ±1.6 $\%^{m}/_{m}$ and 11.0 ± 3.2 $\%^{m}/_{m}$ respectively) than TT association to both chitosan-coated and non-coated polymerised liposomes. This was probably not due to liposomal surface charge differences (as zeta potential of chitosan- and poloxamer-coated and non-coated liposomes was similar) nor due to differences in external liposomal surface areas as formulations had similar mean hydrodynamic diameters and size distributions but may be due to differences in van der Waals interactions and hydrogen bonding between the antigen and poloxamer or chitosan.

Following intranasal administration to mice, the mean TT-specific serum IgG antibody titre on day 14, 28, 49 and 69 were shown to be highest for L101- and L121-coated polymerised liposomes. On day 49, animals receiving ChCl- and ChGl-coated polymerised liposomes displayed a higher mean TT-specific serum IgG antibody response than those animals given non-coated liposomes. However, on day 69 the mean TT-specific serum IgG response was similar for animals given either chitosan-coated or non-coated liposomes. The low immune response to chitosan-coated formulations after intranasal administration is in contrast to that reported by other authors (Bramwell *et al.*, 1999; van der Lubben *et al.*, 2001 and Illum *et al.*, 2001) but may be due to the low levels of chitosan in the formulations not being enough to exert an immunostimulatory effect or the absence of a positive liposomal zeta potential decreasing the mucoadhesive action of chitosan on the nasal mucosa. The immune response to L101- and L121-coated liposomal formulations was higher in agreement to studies by Snippe *et al.*, 1981; Hunter *et al.*, 1981; Hunter *et al.*, 1991 and Hunter *et al.*, 1994.

The mechanism of the increased adjuvanticity of poloxamer-coated liposomes over non-coated liposomes (even though initial TT association to the former was lower) may be due to the poloxamer binding the protein to liposomes in a way that maintained more of the natural configuration of the protein than when higher amounts of protein were bound to liposomes directly (without poloxamers) as reported previously by Hunter (2002). It is not possible to conclude if the adjuvanticity of poloxamers was due to a co-adjuvant effect or in a synergistic way with polymerised liposomes as the administration of L101 and L121 only with TT and L101/L121 coated non-polymerised liposomes was not investigated. Analysis of TT-specific serum IgG subclass (IgG_1 and IgG_{2a}) antibody titres showed that all formulations resulted in higher levels of IgG_1 antibody levels (indicative of a dominant T_h2 response) as shown in figure 4.5 and figure 4.6.

The high immune response elicited by L101-coated polymerised liposomes was also confirmed on analysis of cytokine production by mixed spleen cells of immunised mice. The cultured spleen cells of mice given L101-coated polymerised liposomes secreted higher levels of IL-2, IFN-γ, IL-4, IL-6 and TNF-α than mice given L121-coated, chitosan-coated or non-coated polymerised liposomes.

Contrary to the high systemic response seen with poloxamer-coated liposomes, the production of sIgA in the gastro-intestinal tract (as determined in faecal stools) was lower than of that elicited when chitosan preparations were given. Intramuscular administration of free TT produced no detectable sIgA in faecal stools whereas the intranasal administration of free TT produced a low response. Intranasal administration of non-coated polymerised liposomes did not evoke a higher TT-specific sIgA than when free TT was administered alone. Mucosal secretion of IgA has been shown to be important in mediating protection against certain infections which gain entry to the host *via* mucosal surfaces (see section 1.4.6.7.2). Determination of the presence of IgG antibodies in mucosal secretions was not conducted but has also been shown (section 1.4.6.7.2) to be important in providing protection.

In conclusion, following nasal delivery, poloxamer-coated polymerised liposomes were shown to increase the immune response to TT in comparison to the administration of TT with non-coated polymerised liposomes. On the other hand, polymerised liposomes coated with chitosan did not enhance the systemic immune response to TT any more than TT delivered with non-coated polymerised liposomes but showed promising

preliminary indications for the activation of a mucosal response. The intranasal administration of TT with L101-P SAVs (primer plus two boosts) was shown to produce a mean TT-specific serum IgG antibody response higher than that produced by animals immunised intramuscularly with free TT (primer plus one boost) and thus, offers a promising alternative to the use of injections for vaccination.

5.0 General conclusions and future work

Though vaccines in current use make a significantly valuable medical and economical contribution, the current position of disease control is far from manageable. In 1998, 13.3 million deaths (25 % of all noted deaths) in the world were caused by infectious diseases (excluding deaths attributable to infection but classified under cancer, respiratory or intestinal disease). Mortality due to infectious diseases came second only to cardiovascular-linked deaths (31 %) (Dittmann, 2001). Incidence of diphtheria, malaria and tuberculosis is increasing and some relatively new diseases such as acquired immune deficiency syndrome (AIDS) are yet not only without any licensed vaccine but spreading at a rate of 16,000 new cases of HIV-1 *per* day (Goldsby *et al.*, 2000).

The benefits of vaccination without injections using alternative technology and routes are highly recognisable. The use of subunit or synthetic peptide vaccines (section 1.2.2.3) provides benefits in terms of safety over live attenuated or killed inactivated vaccines, though a major disadvantage in many cases is that they can be poorly immunogenic. The formulation of such subunit or synthetic peptides into effective delivery/targeting vehicles and/or co-administration of immunomodulatory agents will almost certainly be required to increase the immune response to the vaccine.

Particulate delivery systems such as liposomes enhance immune responses to antigen delivery and thus are recognised adjuvants (reviewed by Gregoriadis, 1990). Polymerised liposomes have been projected as more robust carriers for proteins when compared to conventional liposomes (Chen *et al.*, 1998; Okada and Langer, 1995) and have been proposed for vaccine delivery. The aim of this thesis was to establish whether polymerised liposomes possessed adjuvant activity when administered by the nasal route and whether incorporating poloxamers or chitosan into the formulation could further enhance this adjuvanticity.

After reviewing published literature it was concluded that an optimal immune response following the nasal administration of a particulate vaccine (with respect to a high systemic and mucosal immune response) would probably be achieved by administering the protein in a liposomal formulation with a particle size distribution between 0.3-1 μm in diameter. Such particle sizes could be prepared through using a freeze-thaw and

extrusion procedure in which the upper size limit could be controlled using an extrusion filter membrane with pore diameters of an appropriate size.

Entrapment of proteins within polymerised liposomes is conventionally achieved before the monomeric phospholipids of the bi-layer are polymerised (either by free radical initiation or by UV light). However, this exposes the encapsulated protein to possibly denaturing effects, which may lead to decreased immunogenicity of important epitopes. As a measure to avoid this concern, the post-polymerisation encapsulation of proteins into polymerised liposomes using a modified DRV method (Gregoriadis *et al.*, 1999) using cryoprotection was investigated. A disadvantage of the DRV method is that on rehydration of the lyophilised product the size and polydispersity of the vesicles is completely altered. Cryoprotection during lyophilisation was shown to minimise change in the particle size distribution of liposomes yet still allow protein association to polymerised liposomes. The loading of polymerised liposomes using the DRV method has not previously been reported. It would be of value to visualise the depth of protein entrapment (e.g. using a fluorescently labelled protein) when associating proteins to polymerised and non-polymerised liposomes using the DRV method with cryoprotection and to compare this to when proteins are surface adsorbed only. In order to determine the quantity of protein captured internally the removal of externally associated protein could also be achieved using proteases. Such studies would help to confirm and possibly explain the differences seen (*in vitro* and *in vivo*) in protein association to polymerised liposomes when the DRV method is used with cryoprotection.

To further understand some of the results observed when preparing polymerised liposomes it is important to determine whether the transition temperature for DODPC liposomes is altered after polymerisation of the bi-layers. This may be achieved using high sensitivity differential scanning calorimetry and would aid in developing processes where the extent of bi-layer fluidity is important such as the temperature of the rehydration medium (for rehydration of lyophilised liposomes in the DRV method) or the adsorption of poloxamers onto liposomes. Gregoriadis and colleagues (Davis and Gregoriadis, 1987; Gregoriadis *et al.*, 1988; Gregoriadis, 1990 and Gregoriadis, 1994) observed that administration of liposomes with higher transition temperatures resulted in reduced humoral immune responses to soluble antigens like TT. If the transition temperature for DODPC increases after polymerisation then bi-layers would be more

'rigid' at body temperature. This would be a possible reason why the intramuscular administration of TT with P DRVs elicited a lower TT-specific serum IgG antibody level than the administration of free TT as the 'solid' phospholipids may have hindered the presentation and processing of soluble antigen by APCs (Gregoriadis, 1990). Conversely, the administration of a more membrane soluble antigen using P DRVs should lead to a strong immune response and so further investigations using a model membrane soluble antigen are warranted.

Studies in chapter 3 (section 3.4.3) showed that though animals administered $Al(OH)_3$/TT produced the highest overall TT-specific immune response after intramuscular injection, both P SAVs and NP DRVs elicited a considerable immune response in comparison to free TT. The intramuscular administration of P DRVs however resulted in a lower immune response than when free TT alone was given.

In comparison to intramuscular administration of formulations, intranasal delivery resulted in a reduction of TT-specific serum IgG titres for all formulations investigated. However, consistent with intramuscular administration studies, animals immunised with P SAVs elicited the highest mean TT-specific serum IgG response amongst all liposomal formulations (though not significantly higher). NP DRVs evoked low TT-specific serum IgG titres after intranasal administration, which is in contrast to the results seen after intramuscular delivery. These results emphasise that there will be a large variance in the immune response elicited when vaccines are delivered by alternative routes, possibly due to differences in antigen uptake and subsequent processing (e.g. presentation of antigen to APCs).

To further enhance the immune responses seen after the intranasal administration of polymerised liposomes, the formulations were modified by surface adsorption of either chitosan or poloxamer. This was achieved in a manner that minimised change in particle size distribution as it had been previously concluded that particles size was an important factor that could influence the immune responses elicited. TT was adsorbed to polymerised liposomal formulations after chitosan and poloxamer coating in order to maintain the antigen on the surface of the particulate formulation (as with P SAVs, which were shown in chapter 3 to elicit the highest immune response amongst particulate formulations). L101- and L121-coated polymerised liposomes were shown to increase the immune response in comparison to non-coated polymerised liposomes alone following intranasal administration. Surprisingly, chitosan-coated polymerised

liposomes did not enhance the systemic immune response in comparison to non-coated polymerised liposomes but showed promising preliminary indications for the activation of a mucosal response. One reason for this finding may be that the mass of chitosan in the formulation was too low and so only a minimal immunostimulatory effect was initiated or, as the surface of coated polymerised liposomes were not cationic (following chitosan adsorption) that the mucoadhesive effect on the nasal mucosa was lacking. Hence, it is worthwhile repeating the experiments conducted using a higher quantity of chitosan or using a delivery medium with a lower pH (so that the administered liposomes have a more positive zeta potential).

It is not possible to conclude if the adjuvanticity of poloxamers and chitosans was due to a co-adjuvant effect or in a synergistic way with polymerised liposomes as the administration of L101, L121, ChCl or ChGl only with TT and L101-/L121-/ChCl- or ChGl-coated non-polymerised liposomes remains to be investigated. Analysis of TT-specific serum IgG subclass (IgG_1 and IgG_{2a}) antibody responses showed that all formulations resulted in higher IgG_1 antibody titres (indicative of a dominant T_h2 response) as shown in figure 4.5 and figure 4.6. Intranasal delivery of TT with L101-coated P SAVs (primer plus two boosts) elicited a higher humoral antibody response in comparison to animals given free TT intramuscularly (primer plus one boost) and so possibly offers an injection-free, yet effective method for vaccination.

Work by Katz and colleagues (Katz *et al.*, 2000) showed that CRL_{1005} a very high molecular weight poloxamer (12 kDa PPO core with 5 % PEO) acted as an adjuvant for the delivery of influenza A (H3N2) virus after subcutaneous administration to female BALB/c mice. The authors also showed that the response to the poloxamer based vaccine induced T_h1 indicative cytokines on stimulation of cultured spleen cells in comparison to the T_h2 type response seen with the alum based control vaccine. The use of such high molecular weight poloxamers with a very low PEO composition with polymerised liposomal systems may maintain, if not augment the high immune response seen with the use of poloxamer coated liposomal systems in this thesis and also activate a mixed T_h1/T_h2 response which may be necessary for protection against intracellular pathogens.

Mucosal secretion of IgA has been shown to be important in mediating protection against certain infectious organisms that gain entry to the host *via* mucosal surfaces (see

section 1.4.6.7.2). The determination of the secretion of sIgA at other mucosal sites such as the mucosal lining of the lung and genital tract would further substantiate the observations elucidated from determination of secretions from the gastrointestinal tract. Determination of the presence of IgG antibodies in mucosal secretions was not conducted but has also been shown (section 1.4.6.7.2) to be important in providing protection against infection.

In general, further studies need to be conducted in a second lower order animal species such as guinea pigs or in alternative mouse models to confirm the results reported here and to increase group sizes so as to decrease statistical measures of variability such as standard errors and confidence intervals. It would be of interest to observe the length of elevation of the immune response after immunisation using the various formulations investigated in order to elucidate if high antigen-specific antibody levels were sustained. Conducting challenge studies in a suitable model using the most promising formulations would provide a useful addition to the data presented in this thesis.

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