Optimisation and mechanistic assessment of an oral influenza vaccine

By

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Declaration of the author's rights

I, Ewan Murdoch Bennett, hereby certify that this thesis has been composed by me. It is a record of my own work and has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

Signed…… Date……..

I was admitted in the faculty of Engineering, Department of Bioengineering, University of Strathclyde for an Eng.D course in the 2006-2007 session.

Signed…… Date……..

Publications attained during my Eng.D

1. Bennett, E., A.B. Mullen, and V.A. Ferro, (2009) Translational modifications to improve vaccine efficacy in an oral influenza vaccine. *Methods*. 49(*4*): p. 322-327.

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Abstract

The aim of this thesis was to improve the formulation processes of an existing oral vaccine delivery system, the bilosome, and to investigate its mechanism of action. There were three main areas of research; (1) refinement and adaptation of the existing homogenisation melt method, (2) development of a new formulation process, and (3) investigation of the mechanism of action. The results from (1) showed that lyophilisation has no detrimental effect on the bilosome, allowing improved storage characteristics; this was proven in a 9-month study which showed that the immunogenicity of the lyophilised formulation was retained after this time. With a view to developing a system which could be more easily mass-produced, a new formulation process using a microwave reactor was developed in (2). This gave bilosomes with equal immunogenicity to those in (1), in fewer steps and 1/5th of the time; these also allowed incorporation of inexpensive surfactants, which was not possible with the original method. As the formulation process had been successfully streamlined, the mechanism of action was examined in (3). It was thought that further understanding of this could provide information which would allow enhancement of the bilosomes immunogenicity. Results showed that no enhancement of immunogenicity was possible using bilosomes incorporating squalene, or with suppression of gastric acid pre-administration. Investigation of uptake in the intestine showed uptake in both the villi and the Peyer's patches of the small intestine, which may prove useful in the development of future vaccine delivery systems. The study in the lungs was less successful, and a number of issues meant that no significant conclusions could be made; however, the groundwork has been laid for future work in this area.

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List of abbreviations

Ab	Antibodies
AFC	Antibody-forming cells
AG	Antigen
AMPB	2-4′-amino-3′-methylphenyl benzothiazole
APC	Antigen presenting cells
BALB	Bagg Albino
BALT	Bronchus-associated lymphoid tissue
BCG	Bacillus Calmette Guerin
BP	British Pharmacopeia
B2M	Beta-2-microglobulin
CAIV	Cold adapted influenza vaccine
CDC	Centre for Disease Control and Prevention
cDNA	complementary Deoxyribonucleic acid
CHO	Cholesterol
CMC	Critical micelle concentration
CMIS	Common mucosal immune system
CO_2	Carbon dioxide
CPP	Critical packing parameter
CRBC	Chicken red blood cell ghosts
CTL	Cytotoxic T lymphocytes
DCP	Dicetyl phosphate
DLS	Dynamic light scattering
DMSO	Dimethylsulphoxide
DOD	Dodecylgluconamide
DRV	Dehydration-rehydration vesicle
DSC	Differential Scanning Calorimetry

DNA	Deoxyribonucleic acid
DTH	Delayed-type hypersensitivity
E.coli HLT	*Eschericia coli* heat-labile toxin
EDTA	Ethylenediaminetetraacetic acid
EE	Entrapment efficiency
ELISA	Enzyme linked immunosorbant assay
FAE	Follicle-associated epithelium
FD	Freeze dried (lyophilised)
FFEM	Freeze fracture electron microscopy
GALT	Gut-associated lymphoid tissue
GAVI	Global Alliance for Vaccines and Immunization
GI	Gastrointestinal
GSK	Glaxo SmithKline
HA	Hemagglutinin
HBV	Hepatitis B virus
HIV	Human immunodeficiency virus
HM	Homogenisation melt
HSV-1	Herpes simplex virus-1
HPV	Human papillomavirus
HX	Hexylgluconamide
HXD	Hexadecylgluconamide
ID	Intradermally
IEL	Intraepithelial lymphocytes
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IM	Intramuscular

IR	Infra-Red
ISCOM	Immune stimulatory complex
LAIV	Live attenuated Influenza vaccine
LUV	Large unilamellar vesicle
MALT	Mucosal-associated lymphoid tissue
MLN	Mesenteric lymph node
MHC	Major histocompatibility complex
MMR	Measles, mumps, rubella vaccine
MPG	1-Monopalmitoyl glycerol
MW	Microwave method
NA	Neuraminidase
NALT	Nasopharynx-associated lymphoid tissue
NISV	Non-ionic surfactant vesicles
NK	Natural killer
NMR	Nuclear Magnetic Resonance
NT	No treatment
OCT	Octadecylgluconamide
OVI	Organic volatile impurity
PBS	Phosphate buffered saline
PLGA	Poly(lactic-co-glycolic acid)
PP	Peyer's patches
RNA	Ribonucleic acid
RQI	RNA quality indicator
RT-PCR	Real-Time Polymerase Chain Reaction
SC	Subcutaneous
S.D.	Standard deviation from the mean
SDS-PAGE	Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis

SED	Subepithelial dome
sIgA	Secretory IgA
TGF	Transforming growth factor
T_H	T helper
TMB	Tetramethylbenzidine
TNF-α	Tumour necrosis factor alpha
UK	United Kingdom
USA	United States of America
VLPs	Virus-like particles
WH	Water/Homogenisation method
WHO	World Health Organisation
w/v	Weight / Volume

Chapter 1 – Introduction

1.1 Immunology in its infancy

The origins of vaccination can be traced back to the beginning of the second millennium, where there is some evidence in Chinese literature that variola scabs were being administered nasally to protect against smallpox, a practice known as variolation [1-3]. This practice continued to spread through Asia, into the Middle East and Africa, and eventually into Turkey, where it came to the attention of the English aristocrat Lady Mary Montague in 1721, who had been severely scarred after contracting smallpox at an early age [3-5]. During her time in Istanbul, where her husband was a British ambassador, she observed that ladies of the harem did not have any smallpox scars. It was explained that they were protected from the disease by a process in which scratches or punctures were made on the arm, and material from pustules from a person with a mild case of smallpox applied topically [4, 5]. This led to her ordering the embassy doctor to variolate her own two children, and she was to become a strong proponent of the practice, bringing it back to Britain [4, 5]. The process became accepted, with one of the earliest medical statistical examinations showing a reduction in fatalities in the UK due to the disease from 1 in 14 to 1 in 91 [4]. The practice spread quickly throughout Europe, due mainly to an organised medical profession, as well as a few high profile deaths caused by smallpox. Many of the Royal families of the time inoculated their children, and soldiers in the British army were variolated during the American Revolution [2-4]. However, due to the fact that a live, unattenuated form of the virus was used, variolation could cause severe reactions, sometimes causing fatalities, thus preventing the practice from becoming universal.

Edward Jenner, a surgeon from the Royal Society, was one of those variolated as a child, after which he suffered post-inoculation fever. His hypothesis was that the beneficial aspects of variolation could be retained, whilst minimising the side-effects he had suffered, and reducing the risk of death. This formed the basis of his work on smallpox in the last decade of the 18th century [2, 4].

During his time as an apprentice surgeon, Jenner heard a milkmaid remark that "I shall never have smallpox, for I have had cowpox. I shall never have an ugly pockmarked face," a common belief at the time, though without any scientific backing. This, in addition to anecdotal evidence he may have received of inoculations performed by Benjamin Jesty and Peter Plett, led Jenner to develop his hypothesis that inoculation with the cowpox virus provided immunity against the smallpox virus [3, 4]. The opportunity came in 1796, when a local milkmaid, Sarah Nelmes, developed cowpox, causing pustules on her hand from which Jenner was able to extract fluid. He used this to inoculate his gardener's eight year old son, James Phipps, via two half-inch incisions on his arm [3-5]. After inoculation the boy developed a mild fever and some discomfort in the armpit, both of which lasted only a few days. After two months had passed, Jenner again inoculated Phipps, this time with fluid from a smallpox pustule, which had no effect, leading Jenner to conclude that the boy was protected from the disease. This procedure was named vaccination, from the Latin *vacca*, meaning cow [3, 4]. At the end of the year Jenner submitted his account of the Phipps experiment, along with accounts of persons who had previously had cowpox in whom variolation induced no reaction, for publication in Philosophical Transactions of the Royal Society, but was rejected. After substantial revisions he published the

manuscript himself under the title "*An Inquiry into the Causes and Effects of the Variolae Vaccinae, a disease discovered in some of the western counties of England, particularly Gloucestershire and Known by the Name of Cow Pox"* [6]. The reaction of the scientific community was mixed, however, several other physicians took up the cause, including Henry Cline, George Pearson and Henry Woodville. Jenner himself conducted a nationwide survey into immunity against smallpox of those previously infected with cowpox, the results of which confirmed his theory. This lead to widespread vaccination in London, and Woodville immunised upwards of 600 people in the first half of 1799 [3, 4]. Vaccination disseminated quickly across Europe and the New World as its efficacy was proven, and many nations were to pass laws making vaccination a requirement in the next fifty years, though these were not always widely enforced [5]. Though Jenner did not himself discover vaccination, his work was the first to put it in a scientific context, which culminated in the 1966 World Health Organisation (WHO) global programme to eradicate smallpox. The result of this was that over the next 15 years smallpox outbreaks were drastically reduced as a result of mass vaccination, with the last reported case in Somalia in 1977, leading to a statement from WHO in 1980 that smallpox had been wiped from the face of the earth [4, 5, 7]. Today the virus survives only in laboratories, and vaccinations for smallpox are no longer given. Smallpox remains the only human infectious disease to have been eradicated by human intervention.

After Jenner's use of live, unattenuated virus to combat smallpox, the next major advance in Immunology came in the 1880s through Louis Pasteur, a French chemist considered one of the forefathers of Microbiology, who himself coined the term vaccination [8]. Whilst working on fowl cholera in 1879, a bacterial respiratory disease affecting the poultry industry, Pasteur

had developed a method for culturing the bacterium which gave a product that proved lethal in all inoculated animals [8]. When Pasteur and his assistant left for vacation one of the cultures was left on the laboratory bench exposed to the air, causing it to become acidic; on his return this culture was inoculated into chickens, but did not prove fatal [8, 9]. The animals were then inoculated with a fresh culture, shown to be fatal in naïve chickens, and were resistant to this challenge as well, leading to the conclusion that the aged strain had conferred immunity. From this, Pasteur developed the theory that pathogen virulence could be attenuated by environmental factors such as chemicals, oxygen exposure and high temperature, which lead to veterinary vaccines against both fowl cholera and anthrax (in sheep), and eventually a human vaccine against rabies [2, 8, 9].

Since the times of Jenner and Pasteur, vaccination has eradicated smallpox, reduced the incidence of polio by 99% worldwide, and grown into an industry worth around €11.5 billion in 2008, with the average child in the UK receiving 10 vaccines, against diseases such as measles, rubella and mumps, by the age of 15 [10, 11]. It is now considered imperative to not only develop new vaccines, but to also improve the way in which they are delivered, using routes other than the traditional intramuscular injection.

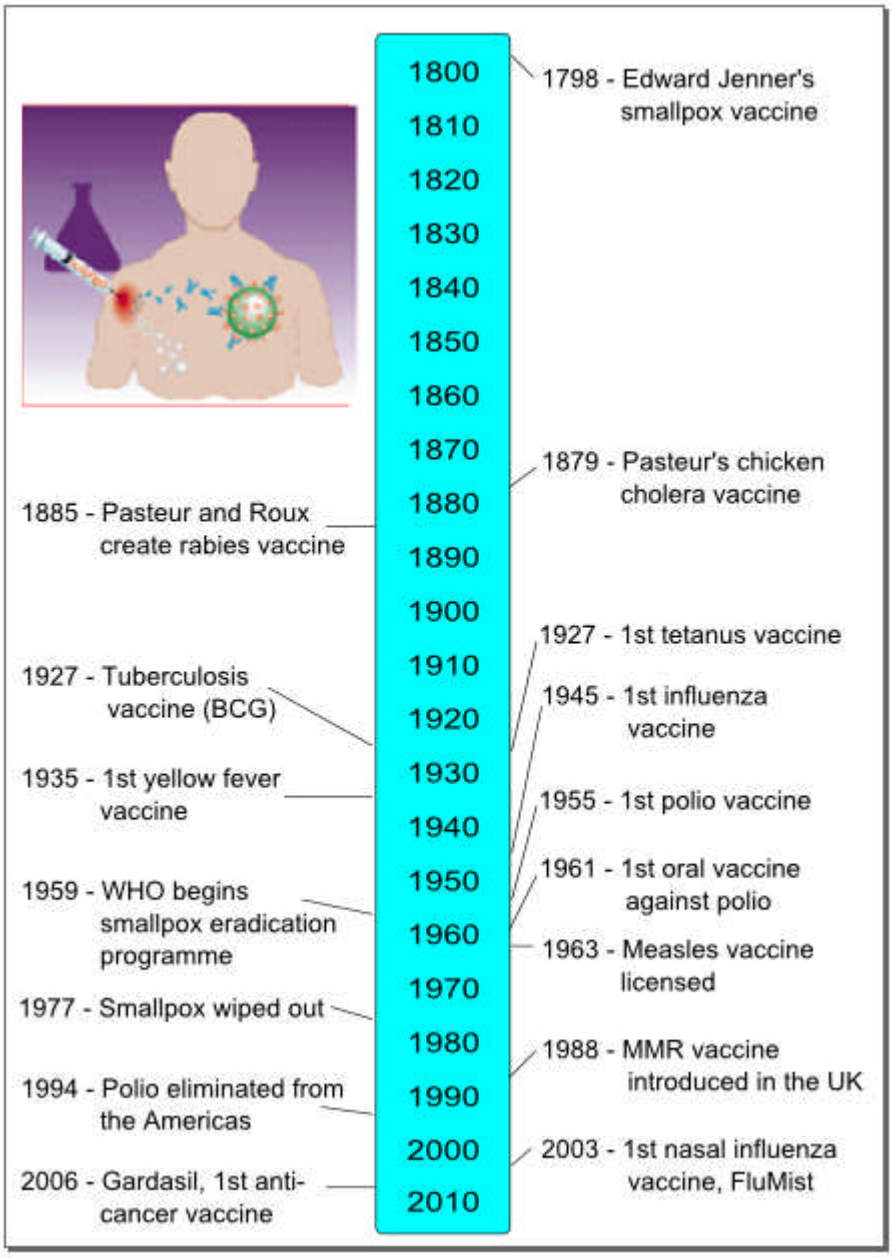


Figure 1 – A timeline of some of the significant events in vaccination history. Constructed from [1-5, 9]

1.2 Vaccination today

Since the 1950s, when cell culture was introduced for virus production, the number of vaccines has increased significantly, and vaccines are now available for a range of diseases such as rubella, influenza, and rotavirus [2, 12]. There are currently two major types of vaccine [live (attenuated) or killed (inactivated) whole organisms], although other types exist, such as subunit recombinant vaccines and DNA vaccines [13].

1.2.1 Live attenuated vaccines

Edward Jenner's smallpox vaccine started the concept of using a live organism, in this case cowpox, to create an immune response in a patient without causing the disease. This method removes the virulence of the pathogen, whilst retaining its ability to stimulate a protective response, and is effective as the product retains its natural invasiveness, antigens and adjuvant effect, and the immune system effectively treats it in the same way as the infectious pathogen [13]. Pathogenic attenuation is often achieved by cultivating the virus through a number of cell lines, which can create mutations rendering the virus less virulent, used for the measles and polio vaccines [14, 15], or create a strain which is adapted to growth at lower temperatures [16]. Attenuation of bacterial pathogens is also possible, and in 1921 Calmette and Guerin developed one of the most well known vaccines, the Bacillus Calmette Guerin, or BCG, by attenuating *Mycobacterium bovis* through 231 subcultures over 13 years to create a vaccine against tuberculosis, an infection of the pulmonary system [16, 17]. Whilst live vaccines are useful due to their ability to mimic natural disease progression, the factors that allow this also enable reversion to virulence. This is the case with the poliomyelitis vaccine, where vaccine associated paralysis is found in

around 1 in 2 million vaccinees [13, 18], and there is also a possibility of contamination with endogenous retroviruses [19].

1.2.2 Inactivated vaccines

While Jenner's smallpox vaccine marked the starting point for all future vaccines, Pasteur's cholera vaccine marks the inception of inactivated vaccines. These vaccines retain the pathogenic antigens, but cannot reproduce within the body, and are usually produced in a similar manner to attenuated, live vaccines, then inactivated by chemical means such as treatment with formaldehyde or phenol [13, 20]. Vaccines produced by this method are generally poorly immunogenic compared to live, attenuated vaccines as they lack the ability to replicate *in vivo*, and are often administered with adjuvants such as alum to increase potency [13]. Killed pathogens are also unable to elicit a significant cell-mediated response, but they do allow effective presentation of surface antibody epitopes, creating an antibody-mediated response [13]. Whilst the use of this method is problematic for bacterial vaccines, due to short-lived immunity and possible contamination with toxic bacterial products, when a virus requires an antibody-mediated response it is highly effective, and is used for diseases such as influenza, hepatitis A and rabies [13, 20].

1.2.3 Subunit and recombinant vaccines

Attenuated vaccines are effective, but administration of the whole organism may lead to side-effects or a reduction in immune response, which could be avoided by using only immunologically significant components, the antigens. These have been used since the early 20th century, when it was recognised that bacterial toxins could be detoxified and administered,

creating an immune response which then protected against the disease; a method used in two of the most prevalent vaccines in the developing world, against diphtheria and tetanus [13]. Toxins are not the only bacterial target; polysaccharides extracted from *Neisseria meningitidis* and *Streptococcus pneumoniae* have been successfully used to protect against the diseases, although these vaccines are ineffective in children under two due to their immature immune system, where they must be used in conjunction with carrier proteins [13]. The approach for viral vaccines is rather different, relying on recombinant DNA technology to identify open reading frames in the viral DNA sequence, which can be used to produce viral proteins that are tested for immunogenicity in an animal model, allowing rapid identification of antigens [13].

1.2.4 DNA vaccines

Reducing the amount of pathogen used for immunisation clearly has advantages; using only the antigenic components reduces the risk of side effects, and the amount of material needed. This is taken to its logical conclusion with DNA vaccines, where the vaccine consists of the genes encoding the protein antigens, which are inserted into an expression vector system, usually a plasmid [21]. DNA can then be delivered by several different methods such as traditional intramuscular (IM) injection, intradermally (ID), or directly to antigen presenting cells (APC) by a gene gun [21-23]. After delivery, the plasmid uses the host cells own machinery to enter the nucleus and initiate transcription, resulting in encoded proteins being produced in the cytoplasm, which then induce both cellular and humoral immune responses. The induction of both immune responses is a significant advantage, and DNA vaccines have many others including;

minimal risk of contamination or reversion to virulence, ease of manufacture and storage, application of the system to a range of diseases, and can be used for multivalent vaccines [21-23]. Due to these, and early successful animal studies, a great deal of interest was generated in DNA vaccines, particularly for their use against cancer and human immunodeficiency virus (HIV). Results in humans have been less encouraging however, and at present they are only licensed for veterinary immunisation [21-23], though this is expected to change in the near future [24].

1.3 Needle-free vaccine delivery

For the vast majority of vaccines the only route of administration used is IM or subcutaneous (SC) injection, as this has been shown to be effective in eliciting protective immune responses. However, whilst effective, needle-based vaccination comes with a number of disadvantages; including: risk of needle-stick injuries, associated pain and therefore decreased patient compliance, necessity for delivery by medical professional and cost of paying these professionals [25, 26]. New routes and methods for vaccine delivery are consequently a major area of research, and needle-free vaccination is supported by key global players such as WHO, the Global Alliance for Vaccines and Immunization (GAVI) and the Centre for Disease Control and Prevention (CDC) [27].

While the use of needles can cause problems, the reproducible protective immune responses to antigens given in this way means transcutaneous administration is an alternative possibility. To use this route the skin must obviously be penetrated in some way; this has led to the development of jet injectors, which deliver vaccine at high pressure through a nozzle, allowing

either ID, SC or IM administration depending on the pressure used [25]. These devices have been in use since the 1950s and have proved useful in mass vaccination programmes, where they allow vaccination of up to 1000 individuals per hour, making them effective tools in any bio-terrorism or pandemic outbreaks [25]. It was found in the 1980s that a small but significant number of vaccinations resulted in contamination of the device with blood from the patient, and in one case this was linked to an outbreak of hepatitis B [25, 28]. This led to a discontinuation of their use, though with recent improved developments in single-use disposable cartridges and powder injection (eliminating the need for cold chain storage) they have again become popular, despite the high cost of the device remaining a limiting factor [25]. Another transcutaneous method does retain the use of needles, however, they are reduced in size to less than 1mm and up to several hundred are used. With microneedle arrays the theory is that penetration of the stratum corneum, the outermost layer of the skin which is 15-20μm thick, is all that is required to deliver an antigen [25, 29]. Microneedles would potentially allow self-administration, and the reduced size would make the risk of needle-stick injury negligible [25, 29]. Studies have demonstrated their effectiveness in influenza vaccination [30-32], however, the cost of fabrication is high compared with IM injection and is likely to remain a limiting factor in their use.

The other route of administration which research has focussed on has been via the mucosae, with nasal and oral administration regarded as the main sites. At present there is only one licensed mucosal influenza vaccine, FluMist ™ from Medimmune, which is delivered nasally as a spray by a pre-filled single use device [33, 34], discussed further in section 1.9.4. Oral

vaccination remains a more attractive route, as the highest degree of patient compliance and simplicity is expected here with patients having used this route for drug administration for many years. Vaccination in this way may be most attractive, but the harsh conditions of the gastrointestinal tract are such that it can be problematic, and protective carrier systems are therefore required.

1.4 Vaccine adjuvants and oral delivery systems

Vaccines have proven to be useful tools in dealing with infectious diseases, however, many new vaccines, such as subunit and DNA, are poorly immunogenic and in most cases vaccines only elicit a systemic immune response, leaving the main pathway for infection, the mucosal surfaces, unprotected [35, 36]. The efficacy of vaccines can be enhanced by the use of adjuvants, agents which can increase immune responses, some of which can be used as vaccine delivery systems to allow mucosal immunisation [37]. Derived from the Latin *adjuvare*, meaning "to help", adjuvants have been known as useful tools in vaccine delivery since 1926, when Glenny *et al.*, (1926) observed that diphtheria toxoid adsorbed to alum induced a greater immune response than diphtheria toxoid alone [38]. Since this time, aluminium based adjuvants have dominated the market, with billions of doses administered in child vaccination programmes for pertussis, diphtheria, poliomyelitis and tetanus [39], where the mechanism of action is thought to be depot formation at the site of injection [40]. However, alum adjuvants can cause severe local reactions such as erythema and contact hypersensitivity [36], are ineffective at inducing cell-based immunity with peptide antigens [37], and do not protect antigens for oral delivery; thus there is great interest in developing alternative adjuvants with alternative

mechanisms of action. There are various pathways by which an adjuvant may act, classified into four groups by Schijns [41] as a) upregulation of antigen presentation, b) increased antigen presentation and localisation to the lymph nodes, c) cellular distress and d) depot formation. Examples can be seen in Table 1 . Particulate based systems, such as liposomes, non-ionic surfactant vesicles (NISV) and bilosomes, are of great interest as they can mimic the particulate nature of viruses. This therefore allows targeting of the antigen-presenting cells of the immune system [36], and the possibility of antigen encapsulation allows transportation and protection of soluble antigens through the gastrointestinal system.

(a) Upregulation of antigen presentation	Poly(lactic-co-glycolic acid**)** (PLGA) Liposomes Non-ionic surfactant vesicles (NISV)
(b) Increased antigen presentation	Dehydration-rehydration vesicle (DRV) liposomal DNA polymer-based nanoparticles
(c) Cellular distress	Oil emulsions Surfactants Aluminium salts
(d) Depot formation	Liposomes Polymer-based nanoparticles Oil emulsions Aluminium salts Gels

Table 1 – Examples of adjuvants and their mode of action. Adapted from [41].

1.4.1 Adjuvants currently licensed for human use

Despite the potential advantages of adjuvants, very few have actually been licensed for clinical use. Many potential candidates fail in the development stage due to issues such as poor antigen stability and inconsistent formulation, with some causing systemic or local adverse events [42]. In addition, the mechanism of action of those that are licensed is still poorly understood, and further work is required to establish a basis for their interaction with the immune system and thus to facilitate further adjuvant development [43]. Licensed adjuvants include alum, the most widely used, oil in water emulsions for influenza vaccinations, and a combination adjuvant where monophosphoryl lipid A is adsorbed to alum, used in hepatitis B (HBV) and human papillomavirus (HPV) vaccines [42, 43]. A summary of licensed vaccines is given in Table 2.

Adjuvant	**Company**	**Class**	**Disease**
Alum	Various	Mineral Salts	Various
ASO3	Glaxo SmithKline (GSK)	Oil/water emulsion and tocopherol	Influenza
ASO4	GSK	monophosphoryl lipid A absorbed to alum	HBV, HPV
Liposomes	Crucell	Oil/water emulsion	Influenza, Hepatitis A
MF59	Novartis	Oil/water emulsion	Influenza

Table 2 – Vaccine adjuvants currently licensed for human use, adapted from [42].

1.4.2 Liposomes

Since the discovery by Alec Bangham in 1961 that in an aqueous system phospholipids will spontaneously form a closed bilayered system [44], liposomes have become of great interest to the pharmaceutical industry as a carrier system. They have been used for vaccine delivery in human trials with various antigens, against diseases such as malaria, hepatitis A [45] and colorectal cancer [46], although none have yet reached the market.

Liposomes can be prepared by a number of methods, producing either multilamellar or unilamellar vesicles, which range in size from 0.05-5μm, allowing encapsulation, or embedding in the membrane of the antigen in a biocompatible vehicle [44]. After administration, liposomes are quickly cleared by the reticulo-endothelial system (although residence time can be increased by surface coating), and the major antigen presenting cells are believed to be macrophages. These may later mature into dendritic cells (DC), although direct uptake by DC has been shown *in vitro* [47].

Disadvantages of the liposome include their instability in the presence of bile salts present in the gastrointestinal (GI) tract, meaning that they are ineffective in delivery of peptide antigens via an oral route due to a lack of protection [48], and a lack of inherent immunogenicity. However, incorporation of a particular low concentration of bile salt or immunopotentiator during liposomal formulation can overcome these problems, dealt with in section 1.4.4.

1.4.3 Immune stimulatory complexes (ISCOMs)

The principle of creating a lamellar vesicle to protect the antigen is again used in ISCOMs, which address the issue of poor immunogenicity of liposomes by incorporation of saponin, an immunopotentiator. ISCOMs comprise of cholesterol, phospholipid, saponin and antigen, which when formed without antigen are known as ISCOMATRIX™, a particulate adjuvant, which can then be combined with an antigen to form an ISCOMATRIX vaccine. Both are around 40nm in diameter [49, 50]. Saponins are known to have adjuvant properties, and are derived from the bark of the *Quillaja saponaria* tree, with the most commonly used being Quil A, which, although considered safe for veterinary applications, is regarded as unsuitable for use in humans due to its haemolytic properties. Despite the reduction in haemolytic activity when formulated in ISCOMs, Quil A still presents a regulatory problem for use in humans. This has been alleviated through recent further characterisation, which has identified several saponin fractions that retain their immunogenicity and ability to form ISCOMs, without haemolytic activity [49, 50]. At present ISCOMs and ISCOMATRIX™ have been used in numerous trials in both animal models and humans for diseases such as influenza [50, 51], herpes HSV-1 [50, 52], rotavirus [50, 53] and tuberculosis [50, 54]. There is also potential for their use in anti-cancer vaccination due to their ability to rapidly generate antigen-specific antibodies, T helper cells and cytotoxic T lymphocytes, with studies in humans already performed against human papilloma virus with moderate success [50, 55].

1.4.4 Non-ionic surfactant vesicles (NISV) and Bilosomes

Whilst liposomes have potential as a vaccine or drug delivery system, there exists an alternative analogous system which offers several key advantages over the liposome; the NISV. Produced in a similar manner to liposomes, but generally requiring an energy stimulus such as heating, non-ionic amphiphiles (e.g. alkyl ethers or esters [56]) form a closed bilayered system with the advantages of a liposome, but at a reduced cost, with greater stability and therefore ease of storage [57]. NISV have been shown to have adjuvant activity with several antigens, such as HSV-1 (herpes) [58] and A/Texas (influenza) [59], and modulation of the type of immune response is possible by altering the size of the vesicle, with NISV over 225nm producing a T_H1 biased response, and those under 155nm T_H2 [60]. Despite these advantages, NISV still do not allow oral administration, as they are also unstable in the presence of bile salt [61]. It has been shown that incorporation of bile salts (typically sodium deoxycholate) at a low concentration into liposomes can improve the stability of the resultant vesicles [62], and this has also been found to be the case with NISV, with the bile salt thought to provide protection against such action in the GI tract [61]; the resultant vesicle is termed a "bilosome". These have been shown to retain 85% of entrapped antigen in 20mM bile salt solution *in vitro*, whilst the corresponding NISV retained only around 40% [61]. Subsequently, bilosomes have been used successfully with antigens for tetanus [63], hepatitis B [64] and influenza [65, 66].

1.5 Formation of lipid vesicles

Interest in liposomes as vaccine or drug carriers has lead to a wide number of applications in both the pharmaceutical and cosmetic industries, and

subsequently there exist a large number of methods by which they can be produced. Many of these, however, form liposomes which give low entrapment or lack of protection of the active agent, poor stability, and have high costs involved in mass production [67]; thus to develop a commercially viable product these issues must be dealt with. Liposome formation is driven by a number of factors – electrolyte and lipid concentration, pH and temperature all play a part – but the major governing forces are hydrophobic and hydrophilic interactions at the interface between the monomer and the aqueous phase. These forces act in opposition to each other [68], and create a system where the hydrophobic nonpolar tails aggregate together to exclude water, with the hydrophilic polar heads on the outside (Table 1.1). The form that the lipids will then take is determined by two parameters, the critical micelle concentration (CMC) and the critical packing parameter (CPP), which are shown in equations 1 and 2 respectively;

$$cmc \approx \exp -[\frac{4\pi r^2 \gamma}{kT}] \quad (g/L^{-1}) \qquad \textit{Equation 1}$$

$$cpp = \frac{v}{a_0 \times I_c} \qquad \textit{Equation 2}$$

Where r = effective radius of a molecule, γ = interfacial free energy per unit area, k = Boltzmann's constant, T = temperature, v = volume of hydrocarbon chain, a_0 = head group area and I_C = maximum critical chain length [69]. The different structures which can be formed are shown in Table 3.

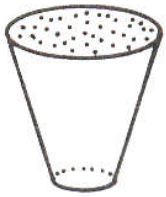
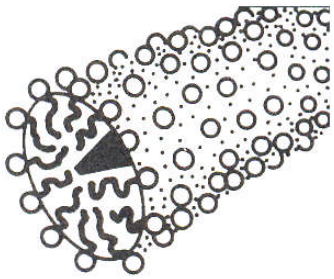
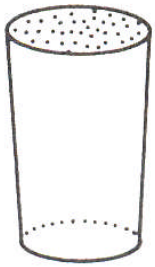
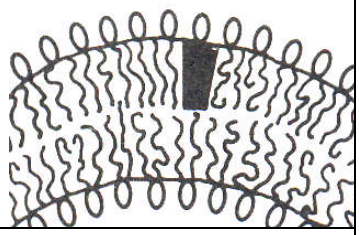
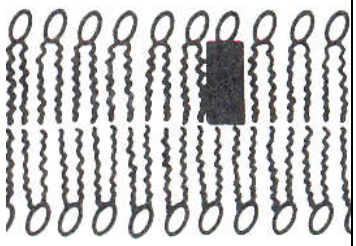
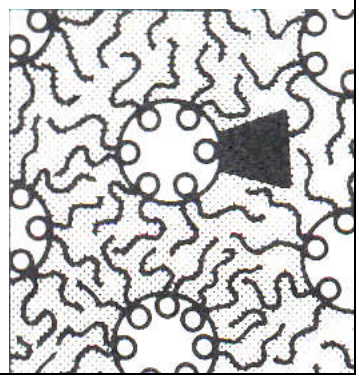
Lipid Type	CPP Value	Critical packing shape	Colloidal Structure
Single chained lipids with small head group areas, e.g. SDS in high salt, non-ionic lipids.	1/3-1/2	Truncated cone 	Cylindrical micelles 
Double chained lipids with large head group areas, fluid chains, e.g. phosphatidyl serine, sphingomyelin.	1/2 -1	Truncated cone 	Flexible bilayers, vesicles 
Double chained lipids with small head group areas, anionic lipids in high salt, e.g. phosphatidyl ethanolamine.	~1	Cylinder 	Planar bilayers 
Double chained lipids with small head group areas, non-ionic lipids, unsaturated chains, e.g. cardiolipin + Ca^{2+}, phosphatidic acid + Ca^{2+}.	>1	Inverted truncated cone 	Inverted micelles 

Table 3 - Mean packing shapes of lipids and the structures they form (adapted from [69]).

Whilst the nature of the lipids used is the major factor in the formation of a liposomal formulation, the addition of other components can also have a significant effect. Liposomes are fluid-like, with components in constant state of flux within the aggregate [69]; consequently, any entrapped agent will be subject to leakage, and the size dispersion is relatively wide around the mean. Cholesterol is known to order the liquid-crystalline phase and disorder the gel phase at concentrations above 6 mol %, and above 30 mol %. This can reduce lateral diffusion, effectively hardening the liposome, easing leakage and producing a more monodisperse formulation [70]. Other additional components can be added to enhance the system, such as charged molecules like dicetyl phosphate, which act as system stabilisers and prevent aggregation [57], or incorporation of a synthetic polyethylene-glycol derivatized phospholipid, which can increase residence time *in vivo* [71].

1.6 Preparation of vesicles

Various methods are available for the preparation of vesicular systems, including conventional techniques that have been around since the late 60s such as Bangham's original hand shaken method. More recent developments include microfluidic chambers and dense gas techniques, and each method comes with its own advantages and disadvantages

1.6.1 Hand shaken multilamellar vesicles

The earliest method of vesicle preparation, reported by Bangham *et al.* in 1965, is still one of the most widely used today, as it is relatively simple in comparison with other methods. Lipids are dissolved in an organic solvent, which is then removed under high vacuum (which can take several hours) to form a thin lipid film. This is then hydrated with a solution consisting of

aqueous media and the agent to be entrapped, with constant agitation, forming large (1-5μm) multilamellar vesicles. Whilst simple, this method suffers from poor entrapment efficiency, with only 5-10% of the hydrating solution estimated to be encapsulated, and the organic solvent residues can be hard to completely remove, leading to toxicity and regulatory problems [57, 72-74].

An adaption of this method that addresses these issues has been developed by Ran and Yalkowsky (2003), where halothane, an inhalation anaesthetic, replaces the organic solvent. Following lipid film formation and hydration, the vesicle size is reduced by sonication at 45^0C to produce small (~200nm) liposomes. This method has been successfully used with the anticancer drug 2-4'-amino-3'-methylphenyl benzothiazole with a high entrapment efficiency which remains at around 90% of the original value after storage for 3 months at 4^0C [75].

1.6.2 Injection methods

Injection methods again involve dissolution of the lipids in an organic phase, such as ethanol or ether. When using ethanol the lipid solution is injected via a fine needle into an aqueous phase containing the active agent, first achieved in 1973 by Batzri and Korn [76]. This method has the advantage of simplicity, however, not all lipids are soluble in ethanol, residual levels of which may require removal post-formation, complicating the procedure [72]. A refinement of this method replaces the ethanol with ether, a solvent immiscible in an aqueous phase, which is slowly (around 0.2ml/min) injected into a heated aqueous phase to form large unilamellar vesicles, with the ether evaporated off due to the high temperature. This solvent removal means that

higher entrapment efficiencies and more concentrated products are possible than with the ethanol injection method, though the process can be lengthy due to the slow injection rate [72]. The process for forming liposomes by ether injection has been successfully adapted for the production of ISCOMs by Pham *et al.* (2006), allowing for faster and more scalable production in comparison with currently used methods [77].

1.6.3 Emulsion method

In this method a water-in-oil emulsion is formed by introducing a small amount of aqueous phase to a bulk organic phase, in which the phospholipids are dissolved, with the resultant mixture then agitated. This drives small droplets of the aqueous phase throughout the organic phase, with a monolayer of lipid forming at the boundary. This mixture is then added to a large amount of aqueous media with further agitation, effectively creating a double emulsion of water-in-oil-in-water. Lipids again form a monolayer at the boundary, therefore creating an aqueous core surrounded by a double lipid monolayer, separated by an organic layer. At this point, unilamellar liposomes can be formed by passing a stream of nitrogen through the double emulsion, removing the organic solvent [72].

1.6.4 Reverse phase evaporation

In this method, first described by Szoka and Papahadjopoulos [78], lipids are dissolved in an organic solvent, to which a small amount of the aqueous phase is introduced. This is followed by sonication, forming inverted micelles (see Table 3) where vesicles of aqueous phase are enclosed by the lipids and surrounded by the organic phase. Rotary evaporation is then applied, forming a viscous gel, which after sufficient solvent removal,

collapses to form an aqueous suspension of heterogeneous large unilamellar vesicles. This method allows for entrapment efficiencies between 30-65%, however, the substance to be entrapped must be stable in organic solvent, meaning that it is unsuitable for certain proteins [72].

Recently, liposomes and NISV formed by reverse phase evaporation have been used in a comparative study on topical tetanus toxoid delivery. However, it was found that transfersomes, which have a highly fluid and elastic membrane, were more immunogenic [79]. Greater success has been achieved using niosomes to deliver a hepatitis B DNA vaccine topically in a mouse model, with Vyas *et al.* (2005) reporting that strong cellular and humoral immune responses were generated [80].

1.6.5 Microfluidic channel method

Developed by Jahn *et al.* (2007) [81, 82], the microfluidic channel method offers two significant advantages; size can be simply regulated, and the device can be used to prepare the formulation immediately before administration, eliminating shelf life problems. The device consists of a delta of inlet channels, approximately 100μm deep and 46-64μm wide, which intersect into one main mixing channel. The system is bonded into a silicon wafer, which allows injection of the aqueous medium into the outer channels, and injection of a lipid solution in organic solvent into the middle channel. By adjusting the flow rate, and therefore shear forces at the intersection of the channels, the size can be regulated and manipulated, allowing formation of liposomes up to 29nm in diameter. Despite the advantages of size regulation and longer shelf life, microfluidic channels are unlikely to be widely used. This is due to the high cost of channel fabrication,

representing a significant barrier to production on a commercial scale, and the need for organic solvents which must be removed post-formation [72].

1.6.6 Organic solvents

Most of the lipid vesicle formulation methods discussed so far have one major issue in terms of clinical use; the necessity for organic solvents such as chloroform, ether or methanol to dissolve the lipids. In addition to potentially affecting the entrapped agent, it is also difficult to fully remove residual solvent from the final product. These organic volatile impurities (OVIs) can be hazardous to both the patient, causing toxicity at two levels, the molecular and the phase level, and the environment [67, 83]. OVIs within the aqueous phase are the cause of molecular toxicity, and cause a host of problems such as expansion and changes in permeability of the liposomal membrane, as well as protein denaturation and enzyme inhibition. Phase toxicity has been shown to directly correlate with the ability of the solvent to disrupt the membrane proteins of erthythrocytes [84]. This can cause toxicity by extracting cellular components or nutrients, limiting access to these nutrients by attracting red blood cells to the solvent interface, and forming emulsions and coatings around cells. As much residual solvent as possible must therefore be removed from any formulation being used in a clinical setting, using methods including vacuum removal, gel filtration and dialysis, each of which can add a significant amount of time and expense to the formulation process [67]. For these reasons it is therefore desirable to exclude organic solvents from the production of these lipid vesicular systems, and a discussion of some methods for achieving this follows.

1.6.7 Dense gas techniques

In order to eliminate the hazards of OVIs whilst retaining the basic formulation principles for organic solvent based vesicles, techniques have been developed which replace them with dense gases. These substances have solvent properties akin to liquids, with the mass transport properties of gases. A dense gas is a substance, which is formed around the critical point where the liquid, gas and supercritical fluid regions meet when the appropriate temperature and pressure conditions are met (Figure 2), with carbon dioxide (CO_2) the most widely used. CO_2 can be brought to the critical point with a relatively low energy input, requiring conditions of 31.1°C and 73.8 bar, enabling it to be used with temperature sensitive materials, and is also simple to handle, inexpensive and non-flammable [72]. The first such methods were described in 1994 by Castor and Chu [85], who developed two methods using dense gases. The first is by injection, where a mixture of dense gas, lipid, and organic co-solvent are injected into an aqueous solution, and the second is a decompression technique, where all of these components are decompressed through a nozzle, with the rate of depressurisation influencing the vesicle size. Since then, other dense gas techniques, including the supercritical reverse phase evaporation method from Otake *et al.* (2001) [86], and the supercritical fluid liposome method from Frederiksen *et al.* (1994) [87], have been developed. However, the processes themselves are complex, and therefore costly [72].

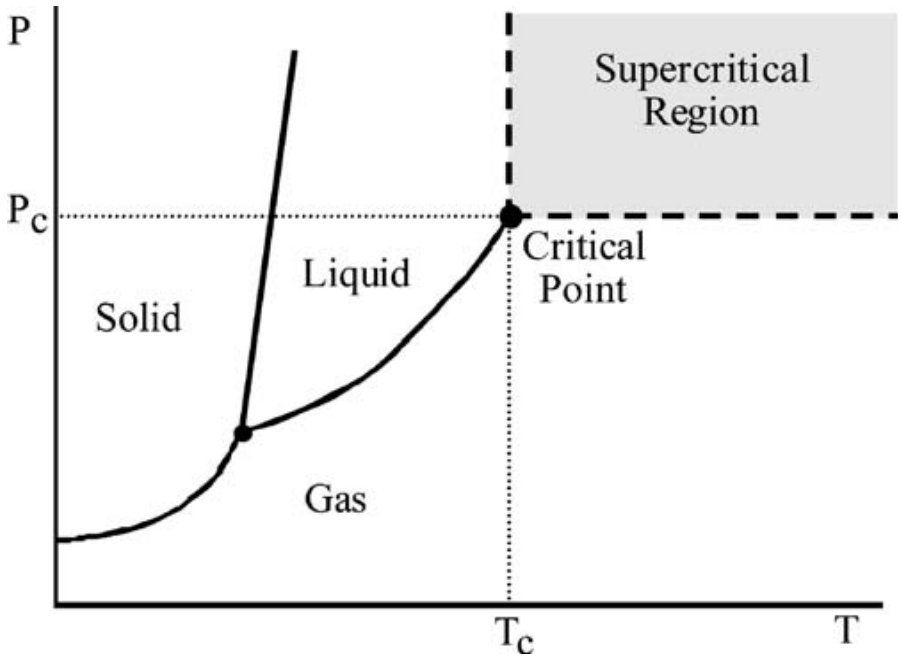


Figure 2 – Pressure – temperature diagram of a pure component where P_c is the critical pressure and T_c is the critical temperature. [62].

1.6.8 Heating methods

These methods require no organic solvents, one example of which is the technique developed by Mozafari (2005), involving hydration of the phospholipids in aqueous solution with 3% glycerol, followed by heating to 60°C or 120°C [67]. Formation of bilosomes also uses the heating principle, with lipids melted at 120°C, followed by hydration with aqueous solution, homogenisation and incubation at 30°C for approximately 3h. Aqueous solution containing the active agent is then added and the system homogenised again, forming small unilamellar vesicles [66]. This formulation method has been successfully used in a murine model for oral vaccine delivery against both influenza (A/Panama, N/Cal [65, 66]) and tetanus toxoid [63], and the work to further refine the method forms the basis of this thesis.

1.6.9 Vesicle post-processing

Where removal of unentrapped agent is desirable this can be achieved by several techniques, although this adds a costly step to the final method and may cause loss of or changes to the vesicular formulation. Column chromatography with sephadex columns may lead to loss of lipids and vesicles due to retention on the column, and permeability changes and leakage may occur, whilst centrifugation, although useful and effective, is an expensive process which does not lend itself to industrial scale-up [88].

For a commercial product it is also important to consider sterility, often considered only to be possible with liposomes via filtration, which does not remove most viruses, necessitating costly and time consuming microbiological control [67]. However, Kikuchi *et al.* (1991) have reported a method by which liposomes can be heat sterilised (121^0C, 20min) whilst retaining structural integrity [89]. This is not applicable where a heat sensitive active agent, such as a protein, is entrapped, making it unsuitable in the case of most vaccines.

The use of vesicles is of particular interest in diseases where frequent, widespread immunisation is required, as it can reduce the need for trained personnel and allow administration without injection. One such disease is influenza, currently prevented via IM injection, which has a high rate of mutation and therefore requires annual vaccination, and can give rise to highly virulent pandemic outbreaks. For these reasons, offering treatment orally instead of by IM injection would facilitate cheaper and simpler vaccination programmes, as well as potentially allowing front-line defence against the virus at the site of infection, the airway mucosae.

1.7 Influenza

The influenza virus is an infective agent of the respiratory tract which, since the 1970s, has been attributed with an annual average of between 25,000 and 45,000 deaths in the USA, most of these in vulnerable populations such as the elderly [90, 91], resulting in an annual average of 610,660 life years lost [92]. The estimated cost of treating influenza epidemics in the USA is $10.4 billion annually, however, when indirect costs such as loss of productivity due to absenteeism are factored in, the total annual cost to the US economy is estimated to be $87.1 billion [92], around 15% of the US defence budget in 2009 [93].

1.7.1 The Virus

Influenza viruses are between 80 to 170 nm in size, and belong to the *Orthomyxoviridae* family, which consist of pleomorphic, enveloped viruses with segmented genomes of negative sense single-strand RNA [94]. In Influenza A, 8 RNA segments encode 10 different proteins; these form an envelope containing tetrameric neuraminidase, trimeric hemagglutinin and M2 glycoproteins, which is lined with multiple ribonucleoprotein complexes enclosed in a continuous layer of matrix protein [94, 95] (Figure. 3). The neuraminidase (N1-N9) and hemagglutinin (H1-H15) genes are used to classify the virus, with types N1, N2, H1, H2 and H3 epidemic in humans, for example "swine flu", is classified as H1N1 [96]. The route of infection for influenza has long been considered to be by the airborne route, however, recently Brankston *et al.*, (2007) suggested this may be of less importance than traditionally thought, with close contact with an infected individual more likely to cause infection via aerosol droplet secretions or direct contact [97].

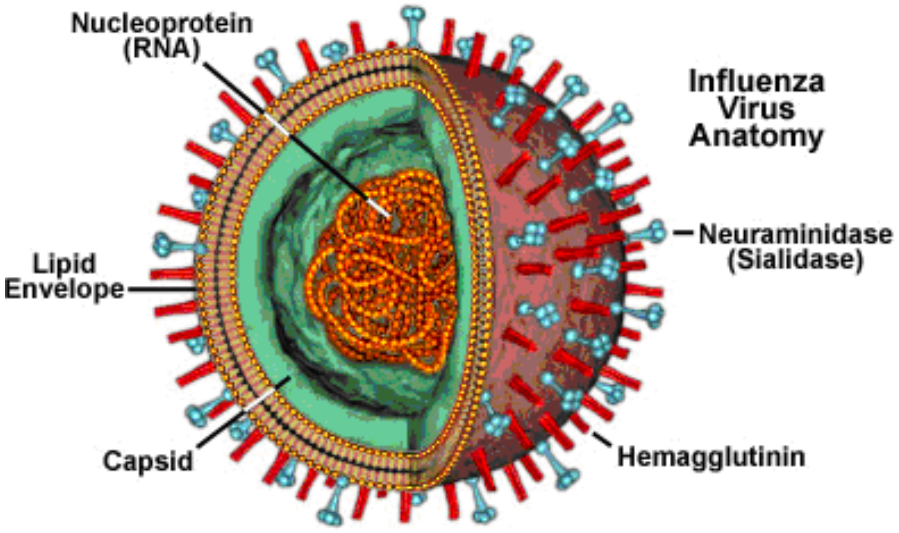


Figure 3. The anatomy of the influenza virus.

http://micro.magnet.fsu.edu/cells/viruses/influenzavirus.html. Accessed 25/3/2011.

Once transmission has occurred, the target for infection by the virus is the respiratory tract. Here many cell types are targeted including those of the immune system such as monocytes, macrophages and other leukocytes. Sialic acid residues on the columnar epithelial cells of the respiratory tract allow attachment of hemagglutinin (HA) projections from the virus, neuraminidases from which then hydrolyse the protective mucus of the cell [98]. If the virus is not cleared by respiratory tract cilia, clathrin-dependent endocytosis of the virus then occurs, bringing it into the acidic conditions found in the endosome, which allow fusion with the endosomal membrane, expelling the viral nucleocapsid into the cytoplasm of the host cell [96]. This is then transferred to the nucleus, followed by synthesis of viral mRNA, which is transferred to the cytoplasm for translation and production of new polymerase proteins. These are translocated back to the nucleus, regulating secondary mRNA synthesis and new virion production, which then bud from the cell and repeat the cycle (see Figure 4) [96]. RNA viruses have no capability for proof-reading or mismatch repair, hence there is a high

mutation rate within the antigenic sites proximal to the membrane distal regions of the hemagglutinin due to pressure from the host's immune response, which is termed antigenic drift [96, 99]. Mutations can also occur due to co-infection of a cell with two differing virion subtypes, which can lead to one acquiring a complete gene from the other, called antigenic shift [96]. These processes are the reason for the seasonal epidemics and pandemics, which occur due to the high number of new strains of the virus being constantly created.

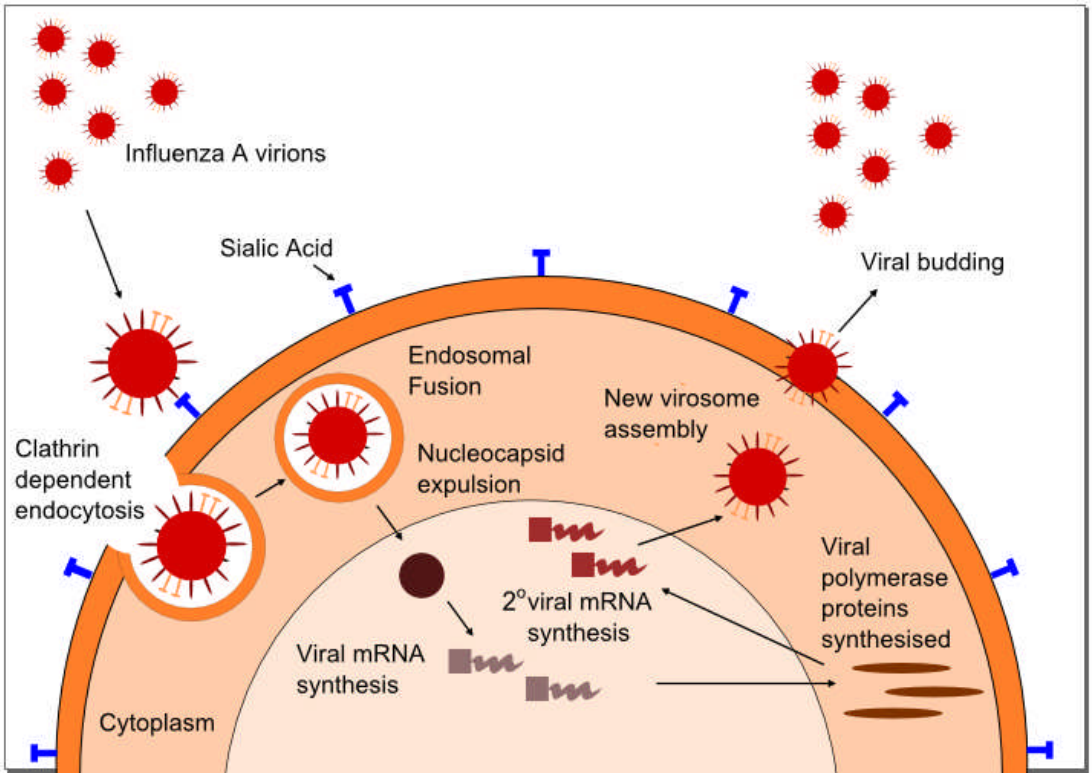


Figure 4 – The replication cycle of the influenza virus. After infection of an individual, virions are localised to the respiratory tract, where the hemagglutinin and neuraminidase projections allow attachment to the cell via sialic acid residues. Clathrin-dependent endocytosis then occurs, allowing the virions into the cytoplasm, where the nucleocapsid is expelled and transferred to the nucleus. Viral mRNA is then synthesised and transferred to the cytoplasm, where viral polymerases are formed and transferred back into the nucleus, allowing secondary viral mRNA synthesis, new virosome formation and viral budding, after which the new virions repeat the cycle [96, 98, 99].

1.8 Immune response to influenza infection

The majority of influenza viruses are detected and cleared from the body within a few hours of infection by the non-specific innate immune system. When a virus manages to evade these defences the adaptive immune system comes into play, also inducing antigen specific memory cells which prevent against further infection [100, 101].

1.8.1 The innate immune response

Defences against infection exist from the earliest point of entry of the virus into the body, the nasal and pulmonary mucosal surfaces, where inhibitory factors which are similar to the N-acetylneuraminic acid-containing receptors on the HA molecules reduce the viruses' ability to infect cells. If the virus successfully causes infection, then infected cells and APC (macrophages and DC) interact with healthy cells via secretion of interferon α and β (IFN), making them resistant to infection, with the levels of each directly correlating to the amount of viral replication [100, 101]. Activated macrophages then help lyse infected cells and release IL-1, IL-6, TNF-α and IL-12, activating natural killer cells (NK) and inducing fever, which can be directly related to the amount of viral shedding. NK cells can be detected 48h post-infection in pulmonary lymphocytes, and these produce IFN-γ and inhibit the spread of the virus. Infected cells are lysed by NK cells via pore formation in the cell membrane by perforin, leading to their disruption [101]. The complement system also mediates protection, as demonstrated by a rise in mortality of C-5 deficient mice infected with a lethal influenza virus dose [102, 103].

1.8.2 The adaptive immune response

As well as their involvement in the innate immune response, APC are crucial components in the adaptive immune response, stimulating the cellular response from effector cells such as cytotoxic T cells and NK, which remove infected cells before viral budding occurs. The viral antigens taken up by the APC bind to the major histocompatability complex (MHC) class I or II molecules within the cells. There they are recognised by virus-specific $CD4^+$ helper T cell precursors (which secrete Th1- and Th2-type cells) or $CD8^+$ precursor T cells (which secrete cytotoxic T lymphocytes, CTL), respectively, which are then activated by cytokines produced by APC. Th2 cells secrete IL-4 and IL-5, causing antibody-forming cells (AFC) to produce antibodies (Ab) such as IgA, IgG1 and IgE, which can bind to viral antigens and neutralise them. Th1 cells secrete IL-2, stimulating production of $CD8^+$ CTLs, which recognise the viral proteins expressed on the surface of infected cells via MHC class I complexes [100, 101]. The processes for both the innate and adaptive immune response are summarised in Figure 5.

1.8.3 Pre-treated vs. naïve immune responses.

The main components in fighting off a murine-adapted influenza infection in naïve mice are secretory IgA (S-IgA) and CTL. S-IgA antibodies develop at around 5 days post-infection, and increase to a plateau at approximately 11 days post-infection, with CTLs having a more transient appearance, peaking around day 7. In general, for a sub-lethal dose of influenza the virus will be cleared from the upper respiratory tract within 10 days [101]. In pre-immunised animals two responses can occur, either with the S-IgA and IgG induced by the vaccination present in the mucosa of the upper respiratory tract, or one where they are absent. When the antibodies are present, they

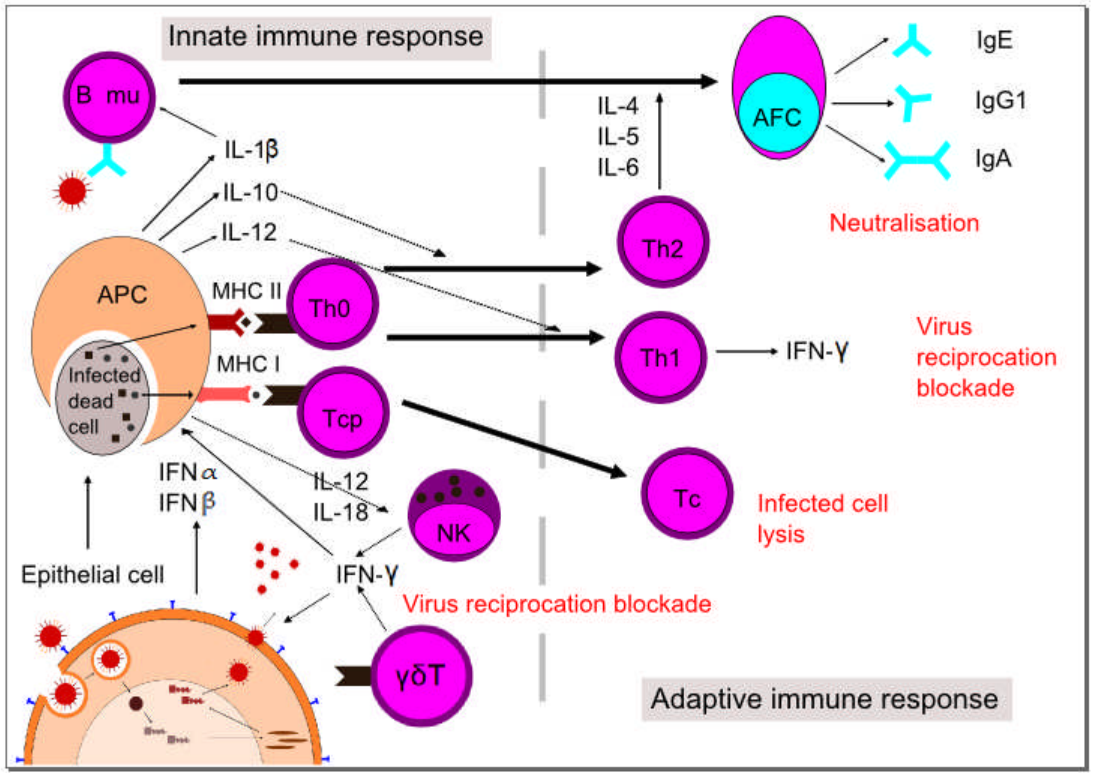


Figure 5 – Immune response to influenza. Adapted from [101]

form virus-Ig complexes, inactivating the virus quickly after infection. In their absence 3 days can pass before B memory cells induce accelerated Ab production, which then clears the infection. After the same time span for both situations, CTL memory cells increase CTL production, lysing infected epithelial cells. Memory Th1 cells mediating delayed-type hypersensitivity (DTH) will also secrete IFN-γ, helping block viral reproduction. The processes are summarised in Figure 6 [101].

It is clear then that S-IgA and IgG can play a major role in quickly eliminating the virus from the body. Current IM vaccinations, however, only induce IgG, which must migrate from the serum to the mucosal surfaces, and will only give protection against the homologous strain of the virus. S-IgA, on the other hand, is directly secreted at the point of infection, and due to its

polymeric nature it can neutralise other variants from the same subtype [101]. Induction of S-IgA via mucosal immunisation is therefore highly desirable, as current IM vaccinations provide protection only against the three strains used in any given year, whereas a mucosal immunisation could give protection against these three and potentially many more.

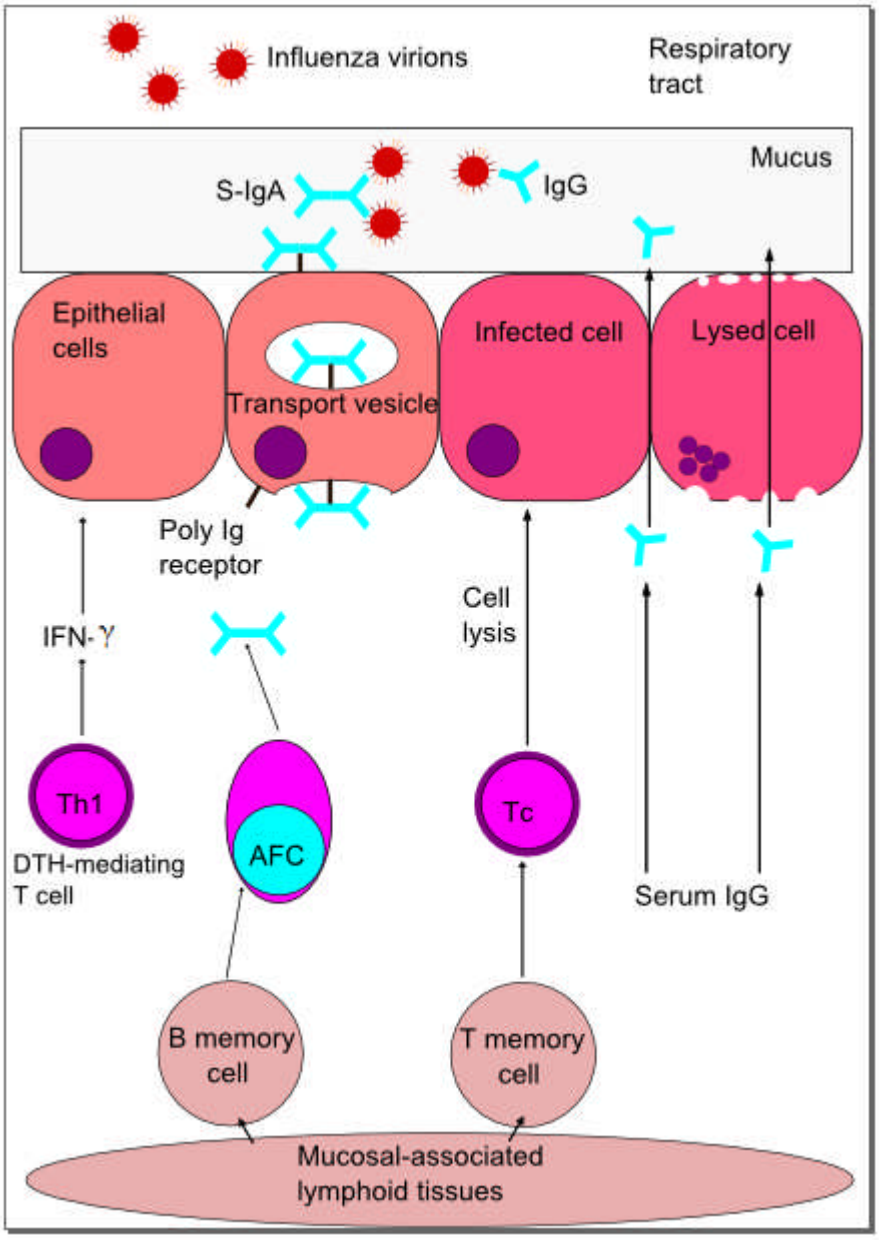


Figure 6 – The immune response to influenza in a vaccinated animal. Adapted from [101].

1.9 Influenza treatment

1.9.1 Vaccination

The most effective targets for vaccination against influenza are the HA proteins, however, due to the frequency with which antigenic shift and drift occur these proteins are also the most variable part of the virus. This means that circulating viruses change from year to year, and vaccines against influenza can therefore only be synthesised once the strains have been identified. The annual vaccination consists of 15μg of the three most common strains expected to be circulating in a given year, which were H1N1, H3N2 (both influenza A) and an influenza B virus in 2010 [104, 105]. At present the most commonly used vaccines are either whole or subunit inactivated antigen preparations, generally grown in embryonated chicken eggs then inactivated with formaldehyde. These have been shown to have an efficacy of approximately 70% for those between 14-60 years old, with a reduction in efficacy for the elderly and infants [105, 106]. Whilst this is useful for seasonal epidemics where the major viral strains can be predicted, the virus can undergo zoonosis, as recently occurred with the H1N1 "swine flu". In such a case this form of vaccination provides little cross-protection against the new strain, and it can be months before an effective vaccine can be safely produced against it. To provide protection against any new strains emerging it is therefore desirable to generate a cell-mediated immune response, as well as the local immune neutralising antibody generated with inactivated vaccines. In theory, this would target those parts of the virus which are most conserved, the neuraminidases (NA) [105]. The process used to achieve this is the nasal administration of live attenuated influenza vaccines (LAIVs), which incorporate both the HA and NA into their backbones, and therefore have both the target strain's antigenic phenotype as well as the sub-type's

attenuated phenotype [107]. Despite this, clinical trials of both inactivated vaccines and LAIVs have shown that the inactivated vaccines are more efficacious against influenza [108, 109].

Vaccine efficacy can also be enhanced through the use of adjuvants, although there is clear need for novel adjuvants, with the most widely accepted, alum, providing only marginal improvements [105, 110]. This has been addressed by the development of new oil-in-water based adjuvants such as AS09 and MF59, which have reduced the amount of antigen required and are capable of inducing immunity after only one immunisation, and MF59 is now licensed for use in seasonal influenza vaccines in Europe [105, 111].

1.9.2 Drug treatments

Antiviral drugs which act by blocking the M2 channel, such as amantadine and rimantadine, have been available for decades, however they are not widely used due to side effects in the central nervous system, especially in the elderly, and growing drug resistance [112]. An alternative drug target is the viral neuraminidase, and inhibitors of all nine subtypes have been developed by both GlaxoSmithKline (zanamavir, Relenza©) and Roche Ltd. (oseltamavir, Tamiflu©) which bind to the sialic acid residues on the host cell, preventing their cleavage by the viral neuraminidase enzyme and subsequent infection of new host cells [112]. After trials in which approximately 85% protection against influenza was conferred these were approved for clinical use, and oseltamavir is now considered a mainstay for control of any pandemic outbreaks [113]. Recently however, growing viral resistance to oseltamivir has been observed, and it is possible, though less likely due to possessing a closer structure to the natural neuraminidase

substrate, that resistance to zanamavir may also emerge [114]. Thus it seems likely that drug treatment of influenza will remain a back-up to vaccination, to be used mainly in pandemic outbreaks to slow the spread of resistance.

1.9.3 Alternative influenza vaccine delivery

Influenza vaccinations are one of the most commonly given, and must be repeated annually in order to provide protection against that year's circulating strains. Influenza also has a high risk of pandemic outbreak due to the high rate of mutation (see section 1.7.1), with three outbreaks occurring in the 20th century, the most severe of which, the 1918-1920 "Spanish flu", caused between 20-100 million deaths. It is estimated that if a similar pandemic occurred today, approximately 65 million could die [115]. Vaccination therefore forms an essential part of any plan for combating an influenza pandemic, though IM injection can only provide protection against homologous strains (see section 1.8.3). This means that the virus causing the outbreak must first be identified and a vaccine against it created [116]. Given the number of vaccinations which occur annually, and the vast number required for a pandemic outbreak, a method of vaccination which could allow self-administration without needles would therefore save time and money, and when delivered mucosally the vaccine could also provide cross-protection against heterologous strains [101].

1.9.4 Intranasal administration

The first licensed intranasal influenza vaccine (Nasalflu®) was introduced in Switzerland in 2000, produced by Berna Biotech, and was an inactivated virosomal subunit vaccine adjuvanted with *Eschericia coli* heat-labile toxin (*E.coli* HLT). The vaccine was approved after trials during four influenza

seasons in 1218 volunteers, which showed no serious adverse events. However, once the vaccine began widespread use, incidences of Bell's Palsy, paralysis of a facial nerve, were noted. After 46 cases had occurred, the vaccine was withdrawn from clinical use, although it was not determined which of the components was responsible [117].

At present, the only licensed mucosally delivered influenza vaccine is FluMist™, an intranasally delivered live cold adapted influenza vaccine (CAIV) which has been used since 2003 and can potentially be self-administered. FluMist™ is a trivalent vaccine that consists of two influenza A strains and one influenza B strain, as with the normal IM vaccine, however these strains have been attenuated by serial passage in tissue culture cells at 25°C. This creates a live vaccine which is well adapted to replication in the cooler environment of the nasal passages, but cannot replicate at higher temperatures, preventing reactogenicity in the body [13, 33]. FluMist™ has been shown to be a safe and effective vaccine against influenza, which can also induce cross-protection against heterologous strains, although it is somewhat less efficacious than the standard IM vaccine [108, 118]. At present FluMist™ is only licensed for use in people between 2 and 49 years; thus the age groups most vulnerable to influenza cannot be protected with this vaccine. The cost of FluMist™ [34], although it has been reduced in the years since its introduction, it is still around 1.5-2 times more expensive than the standard vaccine. Combined with its unsuitability for the elderly and infants, it is therefore unlikely to replace IM products.

1.9.5 Methods in development

Improvements to the influenza vaccine are highly desirable, given the problems discussed previously, and many different methods are being developed. These include those that build on the IM injection foundation, such as enhancing the immunogenicity of the IM vaccine with the adjuvant MF59 [119], and alternative delivery systems. One such method, which utilises virus-like particles (VLPs), uses a patch of microneedles to deliver the vaccine subcutaneously, potentially allowing self-administration. It has been shown to provide similar efficacy at low dose (0.3μg) compared to a higher dose via IM injection (1μg), enabling the amount of vaccine administered to be reduced [120]. Given that an intranasal vaccine has already been licensed, there is considerable interest in systems which use this site, and nanocarriers including liposomes, ISCOMs and polyester nanoparticles are presently in development [121]. While intranasal delivery remains the only mucosal site used for influenza vaccine delivery, immunisation via the gut-associated lymphoid tissue (GALT) is potentially of greater interest, as oral delivery of both vaccines and drugs is considered the gold standard. This method is the most attractive to patients as they are already familiar with it, though it is considerably more problematic given the harsh environment of the gastrointestinal (GI) tract. Early work via this route was done by Pang *et al.* in 1992, who used chicken red blood cell ghosts (CRBC) adsorbed with γ-irradiated influenza A virus to induce both IgG and IgA with protection afforded against heterologous strains, though this method would be difficult to scale up [122]. Further work by Moldoveanu *et al.* in 1993 showed protection against a virus challenge after oral immunisation with polymeric microspheres to primed BALB/c mice [123]. Scheepers *et al.* showed similar

results using ISCOMs, although this was thought to be caused by contact with the oro-pharyngeal cavity, not the GI tract [124].

1.10 The mucosal immune system

While nearly all vaccination targets the systemic immune system, which operates in a relatively sterile, enclosed area, the vast majority of infections occur via the mucosal surfaces, such as cholera in the GI tract, influenza in the respiratory system, and HIV in the reproductive tract [125]. The mucosal immune system comprises around 400m^2 in an average adult, and provides approximately 80% of the body's immune cells, covering the digestive tract, respiratory tract, nasal passages, reproductive tract, eye conjuctiva, inner ear and exocrine gland ducts, and is the largest mammalian lymphoid tissue [125, 126]. Operating in an environment rich in foreign matter, the mucosal immune system must perform a more complex task than the systemic immune system, which is free to react against any invaders, and performs functions such as prevention of microbe colonisation, prevention of uptake of undegraded antigens (foreign proteins from food, airborne material and microorganisms) and preventing these antigens from causing a harmful immune response should they reach the body's interior [125]. Immunisation via this system would therefore provide many benefits, as discussed previously. However, because of the greater complexity of the mucosal immune system, problems such as poor uptake of antigen, degradation of antigen in the GI system and induction of tolerance have meant that only five mucosal vaccines exist, against polio, cholera (x2), typhoid, rotavirus and influenza [126]. We therefore require improved methods of protection and delivery for antigens, as well as greater understanding of the nature of the immune system, in order to stimulate it to create the desired response.

1.10.1 Mucosal immune system anatamophysiology

Mucosal-associated lymphoid tissue (MALT) is a term which covers the whole of the mucosal immune system. This incorporates the mammary and salivary glands, urogenital organs, nasopharynx-associated lymphoid tissue (NALT), GALT and bronchus-associated lymphoid tissue (BALT), with each of these linked by the common mucosal immune system (CMIS), allowing communication [125, 126]. Only the GALT will be covered here, as this thesis is concerned solely with oral mucosal immunisation.

1.10.2 The gut-associated lymphoid tissue (GALT)

The GALT (Figure 7) is loosely organised in some areas, with clusters of lymphoid cells in the lamina propria of the intestinal villi, and highly organised in others specialised in immune sampling, such as the Peyer's patches (PP), found in the lower part of the small intestine in humans [126]. In the loosely organised areas, antigens may enter via the intestinal mucosal epithelium, where they encounter intraepithelial lymphocytes (IELs), mainly CD8+ T-lymphocytes, under which are loose clusters of B cells, plasma cells, T helper (T_H) cells and macrophages in the lamina propria. In the more highly organised areas, PP are found underneath the lamina propria, with macroscopic nodules or aggregates of around 40 lymphoid follicles, which in mature PP can develop into secondary follicles connected by follicular DC. Over the PP there is a diffuse area, the subepithelial dome (SED), on top of which is the follicle-associated epithelium (FAE), a mucous membrane characterised by the presence of M cells, flattened epithelial cells specialised in antigen sampling. M cells, identified by their lack of microvilli and mucous covering, contain a "pocket" enclosing multiple lymphocytes. They are thought to play an important part in any mucosal infection or

immunisation as they are easily entered by antigens or particulates in comparison with the rest of the intestine, as microorganisms are more adherent to the apical M cell membrane [126].

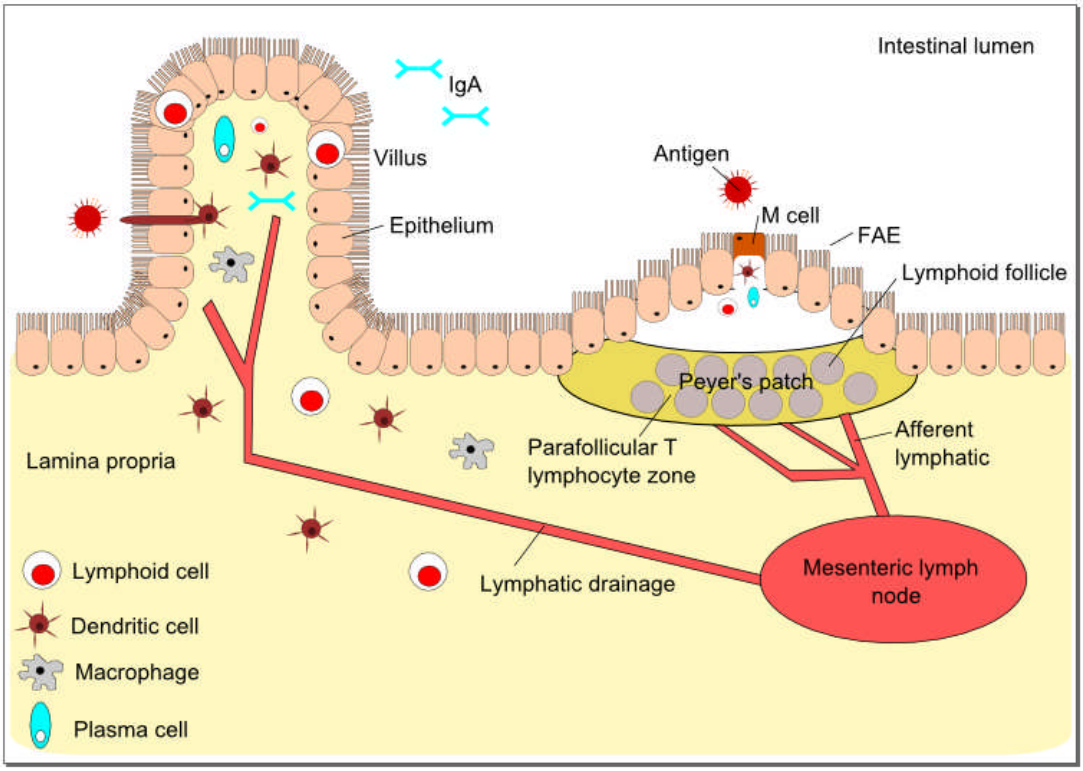


Figure 7 – Cells and structure of the GALT, adapted from [126].

1.10.3 The M cell immune response

On encountering an antigen in the intestinal lumen, the M cell will initiate endocytosis, transporting the antigen from the luminal membrane to the M cell pocket. It is not known whether M cells themselves are involved in processing of the antigen, or if they merely provide transport [127], but it is thought that professional APCs such as macrophages and DC are involved, either in the epithelium or the underlying dome of the M cell [126]. DC are attracted to M cells by chemokines secreted by the FAE, with phenotypically immature cells attracted to T-cell regions allowing upregulation of MHC

molecules and maturation marker expression [128]. DC also provide signals to the follicle of the PP, which along with local factors such as IL-10 and transforming growth factor (TGF)-β, switches them from expression of IgM to IgA, which can then be secreted to block or hinder microorganisms present in the lumen. Furthermore, the mobile nature of DC allows them to drain to the mesenteric lymph node (MLN), possibly transporting antigens as far as the spleen, where a systemic immune response can also be generated [126].

The structures present in the PP, i.e. the B cell follicle, interfollicular T cell area, dendritic cell network and germinal areas, mean that IgA producing B cells can readily be produced at the inductive site. These will then migrate via the MLN to diffuse effector sites such as the lamina propria of the respiratory or intestinal tract, where they can be brought to full maturation by local cytokines such as IL-5, IL-6 and IL-10, creating Ig-secreting plasma or blast cells [129].

1.10.4 Oral tolerance

One of the most significant barriers to oral vaccines is the fact that the GI tract is replete with foreign material, so the MALT must constantly adapt to microorganisms. This can result in a vaccine which is highly immunogenic when given parenterally having no effect when applied to a mucosal surface, i.e. tolerance [126]. Despite having had knowledge of oral tolerance for nearly a century, how the mucosal immune system differentiates between normal commensal bacteria and pathogens is still not fully understood and the subject of much debate. Various mechanisms for tolerance have been proposed, and may involve antigen being picked up by DC intercalated

between epithelial cells, then transported to the MLN without being processed, with increased dendritic cell populations associated with tolerance [130]. It is also possible that antigen may pass through epithelial cells without being phagocytosed in the lamina-propria, followed by transportation via the blood stream to the liver. Tolerance can furthermore be induced by a single high dose of antigen, or repeated exposure to low doses [131]. One theory that most agree with is that T cells play a major role in the induction of tolerance, by clonal anergy, active suppression by T regulatory cells, and clonal deletion of T cells [126]. While there is still much debate surrounding how mucosal tolerance arises, the general consensus is now that PPs do not play a role [132, 133], and this could mean that targeting of M cells is therefore one way to bypass oral tolerance, which may selectively take up microparticles [134, 135].

1.10.5 Microparticles for mucosal uptake

When attempting to stimulate an immune response via the oral mucosal route, the intended antigen faces a number of problems. The first of these is passing through the gastrointestinal tract, where pH can range from pH 1.2 in the stomach to pH 7.5 in the intestines, and digestive enzymes such as pepsin and pancreatin are present [136]. Should the antigen survive these conditions, there is still no guarantee of uptake occurring, due to epithelial barriers and sequestration in mucus [128], and if a large dose of antigen is given to make sure some reaches the target, it may only induce tolerance, as previously discussed. In order to effectively induce an immune response, it is therefore necessary to formulate the vaccine in such a way that it will be presented as a typical pathogen would, so particles in the nano- or micro-particle size range are particularly useful. Particles in this size range are

likely to be preferentially taken up by M cells, and will therefore target the professional APCs in the underlying PP, which most regard as the main target for mucosal vaccination [126].

1.11 Project rationale

From the evidence so far presented, it can be seen that IM vaccines are a potent weapon against disease; however, there are limitations and problems associated with their use. As previously discussed, IM vaccines require trained personnel, give poorer patient compliance (compared with non-needle based treatments), and raise only systemic antibodies without mucosal protection. This is of particular concern in a disease such as influenza, where the rapidly mutating nature of the virus results in annual vaccination programmes, with ease of administration and patient compliance of high importance. Influenza also infects primarily via the respiratory tract, and the absence of any mucosal immunity (via IgA) means that the virus can gain a foothold in the body, before any clearance from systemic immunity. Inducing mucosal immunity, as well as systemic, affords an additional advantage, as IgA is capable of cross-protection against strains other than those immunised against. It is therefore clear that a product, which does not require needles for vaccination, and could also induce a mucosal antibody response, would offer significant advantages over IM immunisation. The available routes of administration for such a product are via the mucosal surfaces of the body, with this thesis concerned with immunisation via the GALT. Vaccination at this surface, via oral administration, would be simpler, and more patient-friendly, than other mucosal surfaces, and has therefore been the route studied for the bilosome, a liposome-based vaccine delivery system.

Oral vaccination via the mucosal surfaces of the intestine is seen as the "Holy Grail" of vaccine administration, allowing induction of both systemic and mucosal antibodies, combined with a method of delivery, which is simple, patient-friendly and safer than traditional IM injection. As discussed in the previous section, this route of administration is problematic for protein antigens, and a delivery system is therefore required. The bilosome (section 1.4.4) has previously been used as a delivery system for a range of antigens such as influenza, cholera and tetanus [63, 64, 66], and this thesis is concerned primarily with its further development. The overall aims of this work were to refine the formulation method of the bilosome into a simpler, more-efficient, cost-effective and easily scalable product, which would therefore have increased viability as a commercial product. In addition, the bilosomes mechanism of action was studied, in order to provide further information, which may allow greater understanding of how future adaptations and refinements could enhance the immune response. Three areas form the basis for this thesis: adaptation and refinement of the original Mann *et al* homogenisation method [66] (Chapter 2), development of new surfactants and formulation processes (Chapter 3), and assessment of the mechanism of action of the bilosome (Chapter 4). A comprehensive list of the aims and objectives for each of these is given at the beginning of each chapter.

Chapter 2 – Refinement and adaptation of original Mann et al. method

2.1 Introduction

This thesis has built on earlier work on the bilosome by Mann *et al*. [63, 66, 137] and Conacher *et al.* [61]. The product of this was a formulation process similar to the homogenisation melt (HM) method described in section 2.2.1.1, which had been shown to successfully induce systemic and mucosal antibody titres against influenza in a murine model [66, 137]. The initial issues to be addressed were therefore:

(1) Bile salt protects the bilosome against leakage in the stomach, but can also disrupt bilosomes [61]. Therefore, what is the optimum bile salt concentration?

(2) The current process is a 3-step protocol taking approximately 3 ½ h, and produces a wet product. Can the formulation process be simplified?

(3) Lyophilisation, giving a dry powder product, is often used to enhance the shelf-life of wet products. What effect does lyophilisation have on the bilosome?

(4) How long could the bilosome be stored for, and under which conditions?

2.1.1 Chapter aims

1: Examine the physical characteristics of the bilosome with various bile salt concentrations, in terms of size, zeta potential and antigen entrapment.

2: Examine the immunogenicity of each of these formulations, and determine any correlation between the physical characteristics and the immune response.

3: Determine the optimum bile salt concentration.

4: Examine the physical characteristics of a simplified 1-step formulation.

5: Compare the immune response of the 1-step process to the original.

6: Examine and compare the physical characteristics of rehydrated lyophilised bilosomes with the original wet formulation.

7: Determine the effect of lyophilisation on the immune response to the bilosome.

8: Assess the size and antigen entrapment of lyophilised (stored at room temperature) and wet bilosomes (stored at 4°C) over a period of 9 months.

9: Compare the immunogenicity of these formulations at the end of this period with their fresh counterparts.

2.2 Materials and methods

2.2.1 Vesicle preparation

2.2.1.1 Homogenisation melt method (HM)

1-Monopalmitoyl glycerol (MPG, 150μmol, Larodan AG, Sweden), cholesterol (CHO, Sigma-Aldrich, UK) and dicetyl phosphate (DCP, Sigma-Aldrich, UK) (5:4:1 molar ratio) were melted in an oil bath at 120°C for 2min, and then hydrated with 3.78ml of 0.025M carbonate buffer pH 9.7 The lipid mixture was homogenised for 2min at 8000rpm (Silverson Machines Ltd., UK), followed by addition of 1ml 100mM sodium deoxycholate in 0.025M carbonate buffer pH 9.7 and homogenised for 8min at 8000rpm. This mixture was incubated at 30°C for 2h in a water bath, then 5.22 ml carbonate buffer (0.025M, pH 9.7) containing 1.2mg antigen was added and the formulation homogenised for 3 min at 8000rpm.

2.2.1.2 HM Adaptations

Effect of bile salt concentration - Initial hydration for the groups with varying bile salt concentrations were either 4.78ml, 4.58ml, 4.28ml, 3.78ml, 2.78ml or 0.78ml of 0.025 M carbonate buffer pH 9.7 preheated to 60°C followed by addition of 0ml (final concentration = 0mM), 0.2ml (final concentration = 2mM), 0.5ml (final concentration = 5mM), 1ml (final concentration = 10mM), 2ml (final concentration = 20mM) or 4ml (final concentration = 40mM) 100mM sodium deoxycholate in 0.025M carbonate buffer pH 9.7, respectively.

1-step formulation – 1ml of 100mM bile salt dissolved in 0.025M carbonate buffer pH 9.7 and 9ml antigen solution (1.2mg antigen in 9ml of 0.025M carbonate buffer pH 9.7, preheated to 60°C), followed by homogenisation for

13min at 8000rpm, then cooled to 30°C over 2h in a water bath with occasional agitation.

2.2.2 Vesicle storage

Bilosomes formulated by the 3-step method were stored for 9 months either as a non-lyophilised wet formulation, refrigerated at 4°C, or lyophilised, stored at room temperature.

2.2.3 Lyophilisation of vesicles

Lyophilisation was achieved using an Edwards Modulyo freeze drier at -45°C for 24h, then stored sealed at 4°C.

2.2.4 Vesicle characterisation

2.2.4.1 Estimation of size by dynamic light scattering

Particle size measurements were made on a Malvern Instruments Zetasizer Nano ZS at 25°C. A solution of 20µl of sample in 980µl 0.025M carbonate buffer pH 9.7 was added to a cuvette (Sigma-Aldrich Ltd, UK) and allowed to equilibrate inside the machine for 2min, after which the intensity of the scattered laser light was measured by a photomultiplier, with each sample measured three times.

2.2.4.2 Freeze-fracture electron microscopy (FFEM)

Samples were sandwiched between two copper support plates (Bal-tec, Lichtenstein) and frozen in a cryogenic mixture of propane/isopentane (3:1 v/v) at -190°C. These were then stored at -150°C. Fracturing was carried out at -100°C at 10^{-6} torr, with the fracture face replicated with platinum/carbon (Pt/C, Agar Scientific, UK) at 45°C, and carbon coated at 90° to the surface.

Acetone was used to clean the replicates and they were collected onto a mesh (100 grid bars/in.), dried and viewed on an LEO 912 electron microscope at 80kV.

2.2.4.3 Zeta potential

Zeta potential measurements were made using a solution of 20µl of sample dispersed in 980µl 0.025M carbonate buffer pH 9.7, which was added to a zeta potential cell (Malvern Instruments Ltd., UK). The mobility of the sample in respect to an applied electric field was then measured in triplicate at 25°C.

2.2.4.4 Protein entrapment by Ninhydrin assay

Entrapment of protein was quantified using a modified ninhydrin assay, a method reported to be unaffected by lipid interference and previously described by Brewer *et al.,* (1995) [138]. In order to separate entrapped antigen from free antigen a 0.11 ml sample of vesicles, diluted in 4ml 0.025M carbonate buffer pH 9.7, was spun in a Beckman tube in a Beckman XL-90 ultracentrifuge (Beckman RIIC, UK) at 35,000rpm for 2h. The supernatant was then discarded and the pellet resuspended in 0.11 ml 0.025M carbonate buffer pH 9.7, then transferred to 1.5ml microfuge tubes (Elkay, UK). The samples, along with standards (0, 0.25, 0.5, 0.75, 1, 2.5, 5, 7.5, 10, 15 and 20µl of antigen), were aliquoted into 1.5ml microfuge tubes, and placed in an oven at 90°C overnight. Protein degradation was then achieved by adding 150µl of 13.5 M NaOH to each tube, with a pinhole made in the lid before autoclaving at 121°C/131kPa for 20min. After removal from the autoclave, the holes were sealed with autoclave tape and the NaOH neutralised with 250µl glacial acetic acid, followed by vortexing for 5s. 500µl of ninhydrin reagent

(Sigma-Aldrich, UK) was then added to each tube, each sample vortexed and placed in a water bath at 90°C for 20min. An aliquot of 250µl of the resultant mixture was transferred to a fresh tube containing 750µl 50% (v/v) propan-2-ol and vortexed, and 200µl of each sample transferred to a flat-bottomed 96 well plate (Iwaki, Japan). Absorbance was read at 540nm on a SpectraMax 190 plate reader (Molecular Devices, USA), with test sample levels determined by linear regression from the standard calibration curve. The quantity of protein in the supernatant was subtracted from the total protein added to calculate the entrapment efficiency (EE). Experimental conditions were the same for standards and samples and a correlation coefficient of >0.99 was obtained.

2.2.5 Immunisation

2.2.5.1 Animals and schedule

In-house bred male BALB/c mice, 8-10 weeks old, housed in a fully climatised room were randomised and placed into groups of 5. All mice were starved, but allowed access to water, for 2 h pre-immunisation, with food and water available *ad libitum* between immunisations. Each oral dose consisted of 0.4ml bilosome, containing approximately 50µg N/Cal hemagglutinin, administered by intragastric gavage on days 1, 4, 14, 17, and retention of the total volume in the stomach could be inferred from the absence of any reflux or nasal discharge. The control group was administered 0.05ml 230µg/ml N/Cal in each of the hind legs on the same days by IM injection. Tail bleeds were collected in heparinised capillary tubes on days 7, 20, and 33 post-immunisation and centrifuged at 13,000rpm in 1.5ml microfuge tubes for 20min. Plasma was transferred into fresh 0.5 ml micro-centrifuge tubes (Fischer, UK), and stored at -20°C until IgG1/IgG2a levels

were determined by enzyme linked immunosorbant assay (ELISA). The study was terminated on day 33. IgA levels were determined by ELISA of lung lavages obtained by perfusing the lungs post-mortem with 0.5ml 1x PBS.

2.2.5.2 Study details

A summary of the various experimental and control groups for each study is given in Table 4. All animals were treated as detailed in section 2.2.7.1.

Study	Bile salt concentration (HM method)	1 step HM vs. HM, lyophilisation	Storage (HM method)
Aims	1. Examine immunogenicity 2. Determine optimum concentration	1. Compare immunogenicity 1s vs. 3s 2. Examine effect of lyophilisation on immunogenicity	1. Compare immunogenicity of fresh vs. stored bilosomes
Group: 1	Control, IM injection	Bilosomes	Bilosomes, fresh
2	NISV, 0mM bile salt	Bilosomes, lyophilised	Bilosomes, stored
3	Bilosomes, 2mM bile salt	1-step bilosomes	Lyophilised bilosomes, fresh
4	Bilosomes, 5mM bile salt	1-step bilosomes, lyophilised	Lyophilised bilosomes, stored
5	Bilosomes, 10mM bile salt	Control, empty 1-step bilosomes	Control, IM injection
6	Bilosomes, 20mM bile salt	Control, IM injection	Control, no treatment
7	Bilosomes, 40mM bile salt	-	-
8	Control, no treatment	-	-

Table 4: Summary of groups for each animal study and the relevant sections detailing bilosome formulation for each. See sections 2.2.1.1, 2.2.1.2, and 2.2.2 for formulation details.

2.2.6 Enzyme linked immunosorbant assay (ELISA)

Unless otherwise stated, all incubations were for 1h at 37°C, and washes were performed three times with 1x phosphate buffered saline (PBS), pH7.4 containing 0.01% (v/v) Tween 20 (PBS-Tween). Tissue culture plates (96-well, Iwaki, Japan) were coated with 0.2µg N/Cal (60µl/well) and incubated, then washed and blocked with 200µl/well of 3% (w/v) Marvel™ (Premier Foods UK Ltd.) in PBS. The plates were washed and 150µl/well of a 1:100 dilution of sera or lung lavage fluid added to the first column, then 100µl/well of PBS added to the rest of the plate, with a 1:3 serial dilution created by transferring 50ul from the first column to the next, across the plate. The plates were incubated and washed, followed by addition of 100µl/well of a 1:3000 dilution of IgG1, IgG2 or IgA goat anti-mouse (Southern Biotech, UK), followed by incubation for 45 min at 37°C. The plates were washed and incubated with 150µl/well of tetramethylbenzidine (TMB) substrate (250µl of stock 3,3',5,5'-teramethyl benzidine 6µg/ml dissolved in dimethylsulphoxide (DMSO), added to 25ml of 0.1M sodium acetate citrate buffer, pH5.5 with 4µl of 30% (v/v) H_2O_2). After 15 min the reaction was stopped with 50µl/well of 10% (v/v) H_2SO_4, and the absorbance read at 450nm on a SpectraMax 190 plate reader.

2.2.7 Statistical Analysis

Statistical analysis was performed either using a two-tailed unpaired t-test where applicable, or one-way ANOVA with *post-hoc* analysis by Tukey test where significant differences were indicated. All analysis was performed at the 95% confidence level with Minitab v15.

2.3 Results

2.3.1 Effect of varying bile salt concentration on the bilosome

All previous work with the bilosome has used a bile salt concentration of 10mM. In order to assess whether this concentration was optimum, a range of bilosomes containing varying concentrations were tested.

2.3.1.1 Effect of bile salt concentration on size and entrapment efficiency

There was no significant difference ($p > 0.05$) in size from 0mM to 20mM of bile salt incorporated into the formulation, values for which were; NISV = 182 ± 36nm, B2 = 159 ± 71nm, B5 = 181 ± 23nm, B10 = 199 ± 44nm, B20 = 125 ± 30nm (Figure 8). For B40 however, the size was 374 ± 47nm, a significant increase over all the other formulations with $p < 0.05$ in all cases. The entrapment efficiencies were: NISV = 25.4 ± 2.3%, B2 = 17.5 ± 1.8%, B5 = 21.1 ± 1.8%, B10 = 21.3 ± 1.7%, B20 = 18.9 ± 1.4% and B4 = 15.9 ± 1.7%. Significant differences were observed for the NISV formulation, which had higher EE than the B2 ($p = 0.011$), B20 ($p = 0.020$) and B40 ($p = 0.006$) formulations, with the B40 formulation also having a lower EE than the B5 ($p = 0.022$) and B10 ($p = 0.017$) formulations (Figure 8).

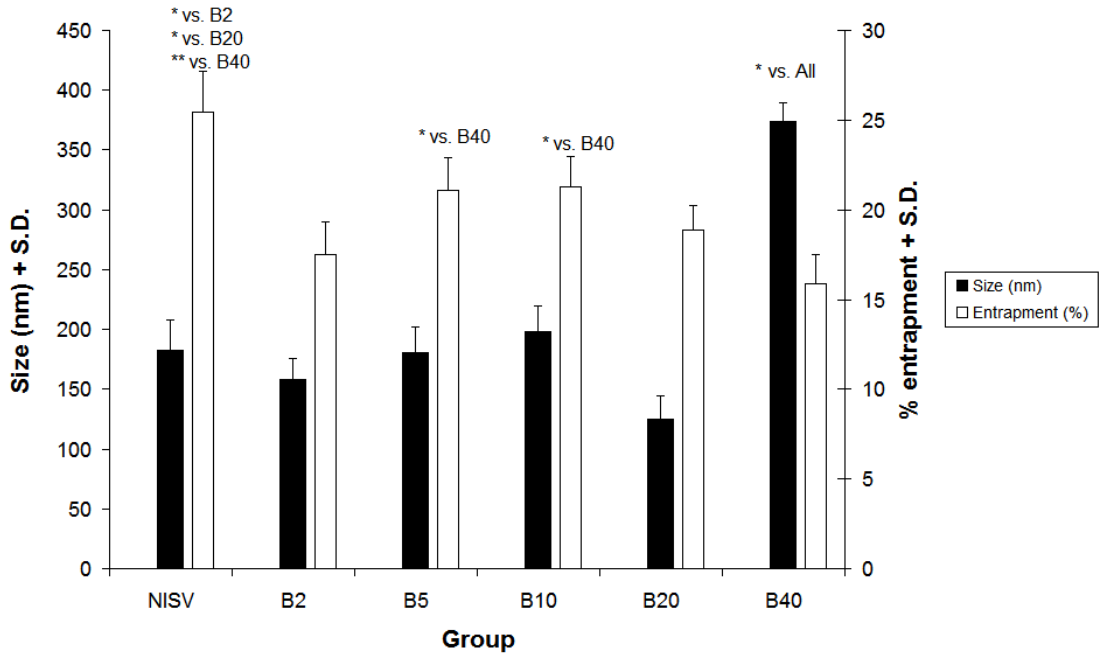


*Figure 8 – Bilosome size in nm and entrapment in % + S.D. for bilosomes with a range of bile salt concentrations. Size measured by triplicate reading of 20µl sample in 1ml 0.025M carbonate buffer pH 9.7 in a Malvern ZetaSizer, entrapment efficiency determined by reading absorbance at 540nm for samples (n = 3) versus a protein standard curve estimated using a ninhydrin assay. NISV = 0mM bile salt, B2-B40 indicates bile salt concentration in mM. * indicates significantly higher value with p value < 0.05, ** indicates p value < 0.01*

2.3.1.2 Effect of varying bile salt concentrations on bilosome generated immune response

All formulations induced significant antibody responses versus no treatment control (NT) for IgG1and IgG2a. Significant IgA titres were observed for all formulations other than NISV and B2. Antibody titres and relevant *p* values are summarised in Table 5. Bile salt concentrations ranged from 2-40mM, and are indicated for each formulation by B2, B5, etc. NISV = Non-ionic surfactant vesicles, i.e. 0mM bile salt.

There was no significant difference between oral groups for IgG1 or IgG2a ($p > 0.05$) (Figure 9). All oral formulations induced significantly lower IgG1 and IgG2a titres than IM injection. For IgA there was greater variability, with B10 inducing the highest titres, significantly greater than NISV ($p = 0.0083$) and B2 ($p = 0.0015$). B2 also induced significantly lower titres than B5 ($p = 0.0352$) and failed to induce a significant titre versus NT control.

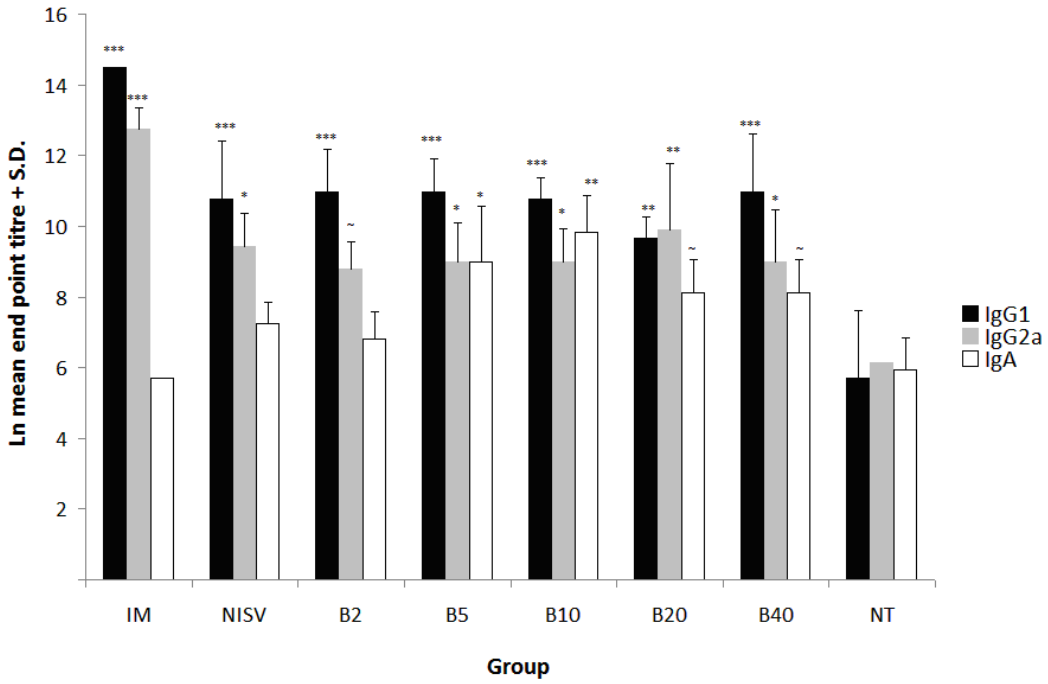


*Figure 9 – Ln mean end point titres ± S.D determined by ELISA for IgG1 and IgG2a by serum sample and IgA by lung lavage. Significantly higher titres than the NT control are indicated by p values: ~ < 0.05, * < 0.01, ** < 0.001 *** < 0.0001. IM = intramuscular injection, NISV = non-ionic surfactant vesicles (no bile salt), B2-40 = bilosomes formulated with 2-40mM bile salt, NT = no treatment.*

	IgG1	*p* vs NT	IgG2a	p vs NT	IgA	p vs NT	p vs IM
IM	14.49 ± 0.00	< 0.0001	12.73 ± 0.60	< 0.0001	5.70 ± 0.00	1.0000	NA
NISV	10.76 ± 1.67	< 0.0001	9.44 ± 0.60	0.0013	7.24 ± 0.60	0.3907	0.5452
B2	10.98 ± 1.20	< 0.0001	8.78 ± 0.92	0.0153	6.80 ± 0.78	0.8225	0.8597
B5	10.98 ± 0.92	< 0.0001	9.00 ± 0.78	0.0068	9.00 ± 1.55	0.0012	0.0093
B10	10.76 ± 0.60	< 0.0001	9.00 ± 1.10	0.0068	9.82 ± 1.05	0.0001	0.0007
B20	9.66 ± 0.60	0.0004	9.89 ± 0.92	0.0002	8.12 ± 0.92	0.0217	0.0861
B40	10.98 ± 1.63	< 0.0001	9.00 ± 1.90	0.0068	8.12 ± 0.92	0.0217	0.0861

Table 5 – Summary of Ln mean end point titres ± S.D. for bilosomes with varying bile salt concentrations determined by ELISA of serum (IgG1 and IgG2a) or lung lavage (IgA).

2.3.2 Streamlining of the HM method and storage adaptations

With 10mM found to give the highest entrapment efficiency, and greatest overall titres, this concentration was used in all future bilosome formulations. The next stages in the formulation adaptation were to attempt simplification of the process, from 3-step (HM), to a 1-step protocol. In order to enhance the storage properties of the bilosome, a dry powder was created by lyophilisation. The 1-step HM, HM, and lyophilised powder variants were therefore tested in order to assess their physical characteristics and immunogenicity.

2.3.2.1 Physical characteristics of 1-step HM. HM, and lyophilised bilosomes.

Size analysis using a Malvern Instruments Zetasizer Nano ZS indicated that the HM protocol resulted in bilosomes between 150-200 nm (Figure 10) with a small population around 1000 nm, with mean sizes ± S.D. of: 1-step HM = 165.9 ± 27.3 nm, HM = 194.7 ± 83.7 nm, and these differences were not found to be significant. Lyophilisation of the bilosomes caused a significant ($p < 0.05$) increase in size on rehydration, giving a mean size ± S.D. of 793.9 ± 39.8 nm for 1-step HM and 976.7 ± 94.5 nm for HM. There was no significant difference between the lyophilised groups. In all cases the zeta potential of the bilosomes was around -100 mV, with no significant differences ($p > 0.05$) between the groups (Table 6). Analysis of the 3-step vesicles using a LEO912 energy filtering microscope at 80 kV showed spherical structures without lipid sheets or crystalline bodies, with two populations of approximately 150-250 nm and 800-1000 nm, confirming the dynamic light scattering results (Figure 11).

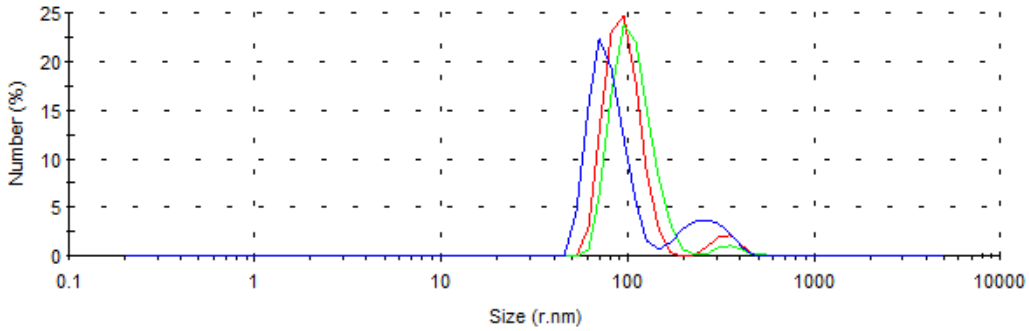


Figure 10: Typical size distribution curves for triplicate readings of bilosome samples generated using a Malvern Instruments Zetasizer Nano ZS with 20µl of sample in 1ml of 0.025M carbonate buffer pH 9.7 at 25°C, using the percentage by number.

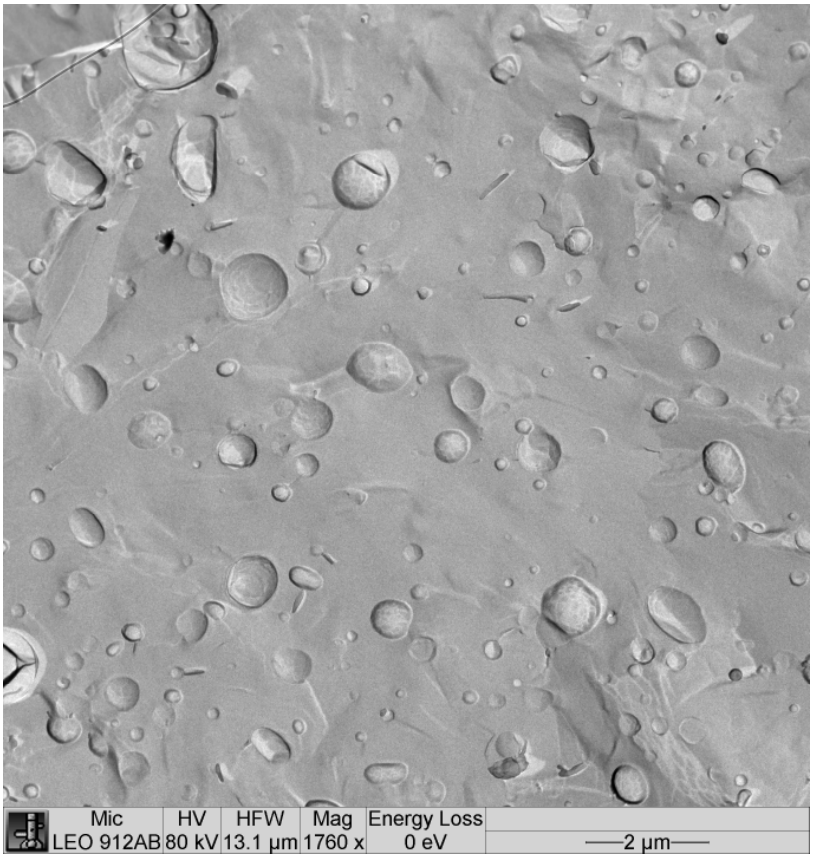


Figure 11: FFEM of bilosomes produced by the HM method before lyophilisation. Imaging was carried out using a LEO912 energy filtering electron microscope at 80 kV.

Formulation	Size (nm) ± S.D.	Zeta Potential (mV) ± S.D.
1-step HM	165.9 ± 27.3	-102.7 ± 11.9
HM	194.7 ± 83.7	-104.5 ± 13.9
1-step HM lyophilised	793.9 ± 39.8	-102.5 ± 13.2
HM lyophilised	976.7 ± 94.5	-113.5 ± 6.4
Empty	211.8 ± 45.0	-90.1 ± 11.5

Table 6: Size / zeta potential of bilosomes formulations determined using triplicate readings of 20 µl sample in 1 ml filtered 0.025M pH 9.7 carbonate buffer in a Malvern Zetasizer Nano ZS.

For both the HM protocols, antigen EE was not significantly different ($p > 0.05$), giving values of: 1-step HM 21.3 ± 14.3%, and HM = 29.0 ± 7.9%. Lyophilisation appeared to enhance EE: 1-step HM lyophilised 65.9 ± 4.8% and HM lyophilised 53.3 ± 1.1%, which were both significantly (1-step HM $p = 0.0157$, HM $p = 0.0022$) higher than their respective wet formulations, though there was no significant difference ($p > 0.05$) between the two lyophilised groups.

2.3.2.2 Immunogenicity of 1-step HM, HM, and lyophilised bilosomes

For IgG1 and IgG2a significantly higher end-point titres were observed for the lyophilised and un-lyophilised HM groups (equivalent to B10 in section 4.1.1), compared with empty bilosomes (Figure 12); Ln titres ± S.E and p value versus empty bilosomes - **HM** IgG1 = 13.17 ± 1.81, $p = 0.001$, IgG2a = 9.00 ± 0.78, $p = 0.002$, and **HM lyophilised** IgG1 = 11.86 ± 2.00, $p = 0.035$, IgG2a = 8.56 ± 1.67, $p = 0.012$. There was no significant difference between

either of the 3 step groups ($p > 0.05$). No significant titres for any antibody were seen with either of the 1 step HM groups, with no significant difference in titres between the two groups. Mucosal IgA titres were significantly higher than with empty bilosomes for only the 3-step lyophilised variant (7.24 ± 0.99, $p = 0.006$)(Figure 12).

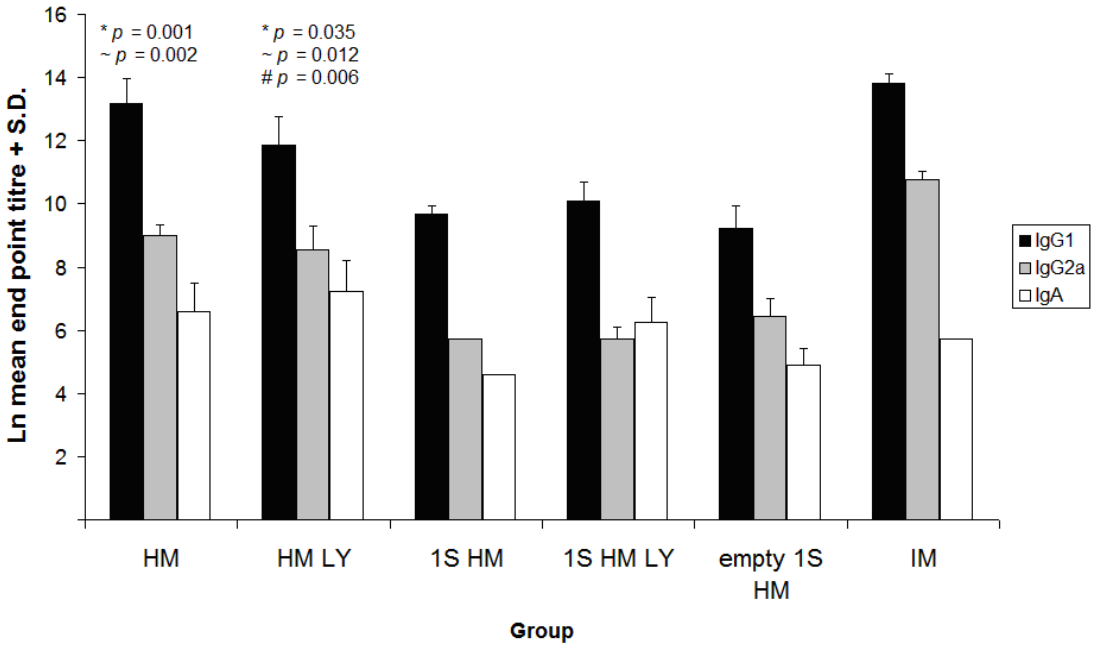


*Figure 12 – Ln mean end point titres + S.D determined by ELISA for IgG1and IgG2a by serum sample and IgA by lung lavage. * indicates p value for IgG1 versus empty control, ~ indicates p value for IgG2a versus empty control, # indicates p value for IgA versus empty control. HM = homogenisation melt method bilosomes, 1S = 1-step HM bilosomes, LY = lyophilised, IM = intramuscular injection.*

2.3.3 Effect of storage for 9 months on lyophilised and unlyophilised bilosomes

Once the immunogenicity of the lyophilised HM bilosomes had been confirmed, a comparative study of physical characteristics with respect to storage time was made between stored HM and lyophilised HM bilosomes. After 9 months the immunogenicity of the stored variants was compared with fresh formulations.

2.3.3.1 Size and entrapment of stored formulations over time

Both HM and lyophilised HM bilosomes were monitored over 9 months to observe any changes in size or entrapment with time, whilst stored either at 4°C (HM) or room temperature (lyophilised HM). The size of the HM bilosomes remained stable up to 4 weeks after formulation, and then increased in size from around 200 nm at 0 weeks to approximately 350 nm during weeks 14 to 25, with a final size of 560 nm at the 9-month point (Figure 13). These changes were not found to be statistically significant ($p > 0.05$). As size increased for the HM bilosomes there was a concurrent decrease in the antigen entrapped, falling from 30% initially to 20% after 9 months (Figure 14), although the changes were not found to be statistically significant ($p > 0.05$). The lyophilised HM bilosomes (after rehydration) showed no significant change ($p > 0.05$) in size up to 9 weeks, remaining at approximately 800nm, after which a reduction in size occurred, with those at 12 weeks and later significantly ($p < 0.05$) smaller than those at 9 weeks and earlier. Entrapment appeared slightly erratic, beginning at around 50%, then falling to around 30% for weeks 2 and 4, though it was again at around 45% for weeks 8 and 9. At the end of the study entrapped antigen had fallen to around 30%, although none of the changes were found to be statistically significant ($p > 0.05$).

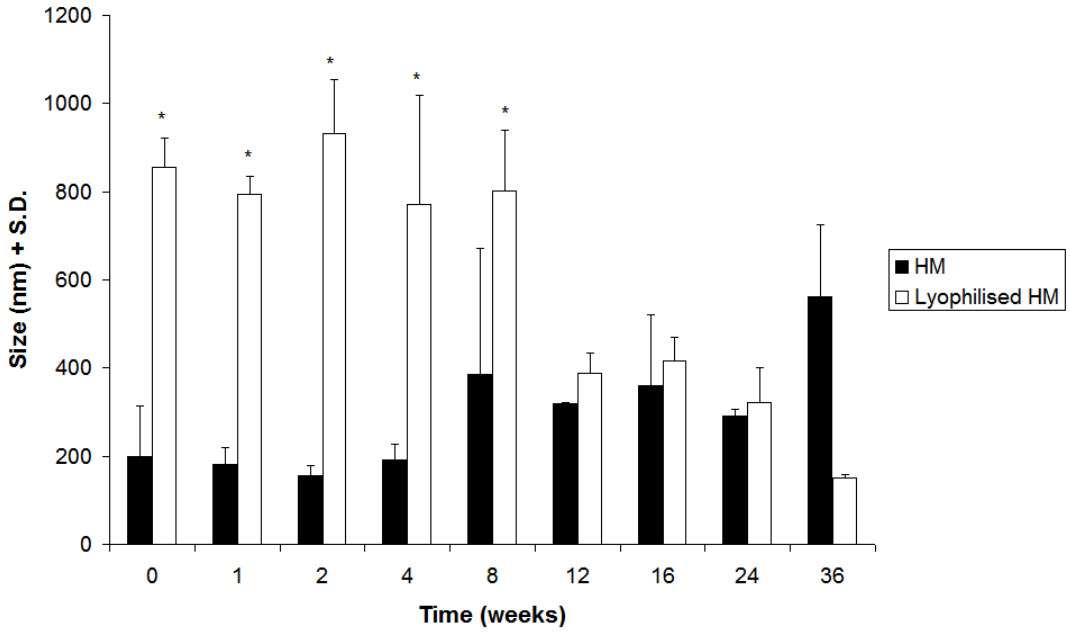


*Figure 13: Size (nm) vs. time for HM or lyophilised HM bilosomes. Size was measured using triplicate readings of 20 µl of sample in 1 ml 0.025M pH 9.7 carbonate buffer in a Malvern Zetasizer Nano ZS. * indicates a significantly greater size ($p < 0.05$) than bilosomes stored for more than 8 weeks.*

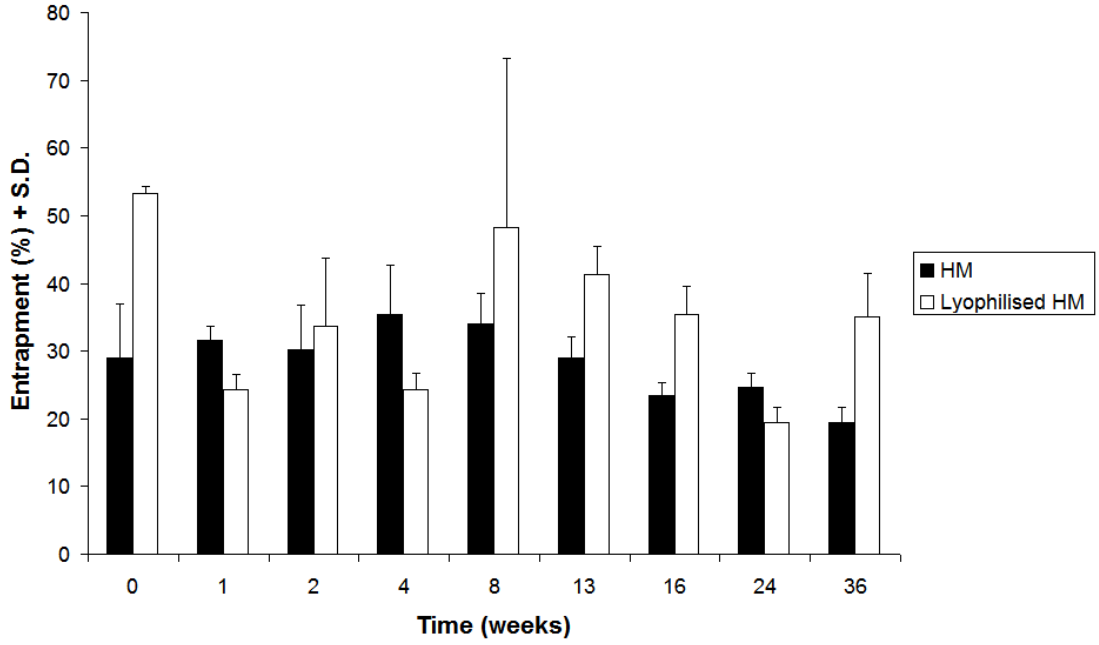


Figure 14: % entrapment vs. time for HM or lyophilised HM bilosomes. Antigen entrapment was determined by ninhydrin assay, n = 3 for each sample.

2.3.3.2 Effect of storage on immunogenicity

At the studies conclusion significantly higher antibody titres compared to the no treatment control group were observed in all cases for IgG1 and IgG2a. The Ln mean end-point titre values ± S.D. and *p* vs. no treatment control are summarised in Table 7. There were significant differences in IgG1 titres between the fresh, wet bilosome group, and the lyophilised stored group ($p = 0.0231$). Higher levels of IgG2a were observed in the stored, lyophilised group versus the stored, wet group ($p = 0.0079$). The results for IgA showed poor immunogenicity for both the non-lyophilised formulations, with no significant difference observed when compared with either IM or no treatment groups. Significant titres were observed versus the NT control for the lyophilised fresh bilosomes, with an Ln mean end point titre ± S.D. of 8.56 ± 0.60, $p = 0.0160$ (Figure 15).

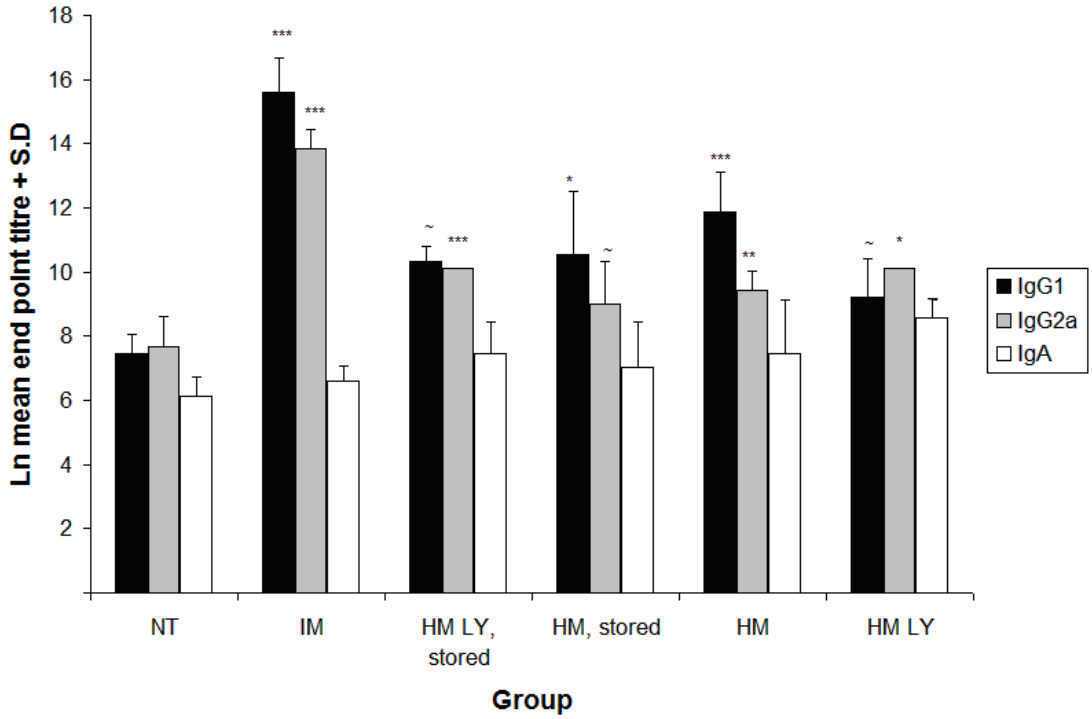


*Figure 15 – Ln mean end point titres + S.D determined by ELISA for IgG1and IgG2a by serum sample and IgA by lung lavage for bilosomes produced by the HM method either stored for 9 months at room temperature (lyophilised) or 4°C (wet), compared with fresh bilosomes. Significantly higher titres compared with the NT control are indicated by p values: ~ < 0.05, * < 0.01, ** < 0.001, *** < 0.0001.HM = homogenisation melt method, NT = no treatment, IM = intramuscular injection, LY = lyophilised.*

	IgG1	*p* vs. NT	IgG2a	*p* vs. NT
Stored HM, lyophilised	10.32 ± 0.49	0.012	10.10 ± 0.00	< 0.0001
Stored HM	10.54 ± 2.00	0.006	9.00 ± 1.35	0.0219
HM	11.86 ± 1.25	0.0001	9.44 ± 0.60	0.001
HM, lyophilised	9.22 ± 1.20	0.2352	10.10 ± 0.00	0.0003

Table 7 – Summary of Ln mean end point titres ± S.D for bilosomes produced by the HM method either stored for 9 months at room temperature (lyophilised) or 4°C (wet), compared with fresh bilosomes determined by serum ELISA. NT = no treatment control.

2.4 Discussion

2.4.1 Effect of bile salt concentration

All previous work with the bilosome [61, 63, 66, 137] has used the standard 10mM bile salt, as described by Conacher *et al.* in their initial work with the system [61]. In order to examine whether bile salt concentrations had an effect on size and entrapment a range of bile salt concentrations were compared.

Bilosomes formed with varying amounts of bile salt showed a range of sizes, approximately 110-450nm, although there was no significant difference between those formulated with 0-20mM bile salt, with only the 40mM bile salt bilosomes having a significantly greater size of around 450nm. The entrapment efficiency of these bilosomes had a trend towards lower entrapment at the highest (B40) and lowest (B2) concentrations of bile salt. Those formulated without bile salt (NISV) had the highest EE, around 25%, significantly ($p < 0.05$) greater than B2, B20 and B40. This was not significantly higher than those with 10mM bile salt, which were also significantly higher than B40. Bile salt (sodium deoxycholate) is a charged molecule; therefore, as the concentration is increased, the degree of electrostatic repulsion also increases. This leads to larger bilosomes, such as the B40 formulation, which are likely to be "leakier" than their smaller counterparts, resulting in lower antigen entrapment. Confirmation of this could have been made through zeta potential measurements, however, in this case they were not performed, and it would be useful in future research to perform such measurements. Previous work with the bilosome has used a freeze-thaw cycle to enhance entrapment, and has given EE's between 40-50% [63, 66]. While this improves EE, it makes the formulation process more

time-consuming and complex, and it was decided not to use it in this case. EE is also known to vary with different antigens [40, 43, 44], and the values obtained here are therefore within the expected range given the absence of the freeze-thaw step and the use of a different antigen (N/Cal vs. A/Panama). It would be desirable in the future to test a range of different antigens in the bilosome, and attempt to establish any correlation between the nature of the antigen and the bilosomes formed.

Formulations with a range of bile salt concentrations induced significant IgG1 and IgG2a titres ($p < 0.05$) in all cases (see Figure 9). For IgA, however, B2 and NISV failed to induce a significant response versus NT and IM controls, and B20 and B40 failed to induce a significant response versus the IM control. There was no significant difference between groups for IgG1 and IgG2a, however, for IgA, B10 induced significantly higher titres than NISV ($p = 0.008$) and B2 ($p = 0.0015$). Thus from this data a bile salt concentration of 10mM was considered best, as this gave the best antibody response over all of IgG1, IgG2a and IgA. The antibody responses in both studies are consistent with those found in previous work [61, 137], although one previous study found no IgG2a titres were induced with similar sized bilosomes [66], with a follow up study showing that larger bilosomes (~1000nm) induced significantly higher IgG2a titres [137].

Bile salts, such as sodium-deoxycholate, -taurocholate, and –glycocholate, are naturally present in the GI tract, and will damage liposomes due to their detergent activity [61, 139, 140]. It has previously found that pre-treatment with bile salt stabilises liposomes against any future exposure, which led to the creation of the bilosome by Conacher *et al.* (2001) [61]. This work tested

three different bile salts, and found that sodium deoxycholate gave the greatest protection, with over 80% of the entrapped protein retained after exposure to a 20mM bile salt solution [61]. Conacher *et al.* also demonstrated an immune response to the entrapped ovalbumin after oral vaccination, and since then other groups have demonstrated the capability of the bilosome to generate immune responses against a range of antigens [61, 64, 66, 141]. The effect of bile salt concentration on the formulation has, however, not previously been studied, and this was therefore chosen as the starting point in this work. The data obtained in these studies showed that a 10mM concentration of bile salt induced the highest antibody titres across all of IgG1, IgG2a and IgA. The next steps are to assess the effect of formulation and storage methods in bilosomes using 10mM bile salt, to investigate whether the method can be simplified and the need for cold chain storage removed.

2.4.2 1-step HM vs. HM and lyophilisation effects

The bilosome has now been shown to effectively induce an immune response with N/Cal antigen, and an optimum concentration of 10mM bile salt has been established. Following this, the next issues to be addressed were the complexity of the HM formulation process, currently a 3 ½h 3-step protocol, and the production of a dry powder formulation, which could potentially improve ease of storage. Two manufacturing protocols, HM and 1-step HM, as well as the respective lyophilised formulations, were therefore assessed in terms of their physical characteristics and their immunogenicity. With both the methods of formulation, the size range determined by dynamic light scattering was between 150-220nm, although a small population (~2-4%) was observed at around 1000nm, a result that was confirmed by FFEM, with no

lipid sheets or crystalline structures observed. Lipid vesicles have highly variable sizes, dependant mainly on formulation method and lipid constituents, and can range from 50 nm to several microns in size [138, 141, 142]. However, the size range obtained with the modifications described is consistent with bilosomes formed by earlier studies [66]. Lyophilisation of liposomes has previously been shown to produce anywhere between a 2-7 fold increase in size on rehydration [143, 144], therefore the 4-fold increase observed in this study is within the reported range. Antigen entrapment in the 3-step wet and 3-step lyophilised formulations of N/Cal was found to be approximately 30% and 50% respectively, a result which is comparable with previous work with the A/Panama flu vaccine (~50%, Mann *et al.*, 2004), although entrapment has been shown to be highly variable and dependent on the antigen used [145].

No significant responses were observed for either of the 1-step HM methods. Both of the HM formulations induced similar immune responses to HA, indicated by the significantly raised IgG1 titres compared with the empty bilosome group, with a modest IgG2a response. IgA levels in the 3-step lyophilised group were also significantly higher than those treated with empty bilosomes. These results are similar to those of previous work on bilosomes manufactured according to the Mann *et al.*, (2005) [66] method with entrapped A/Panama antigen, although no IgG2a response was observed in this case.

The size of vesicles has previously been shown to be an important factor in the immune response by Brewer *et al.* (1998), where it was found that vesicles smaller than 100nm induced a T_H2 response, while those larger than 200nm

induced a T_H1 response [60]. This was shown to be caused by differences in how each were processed, with the smaller vesicles trafficked to APC late endosomes, and larger vesicles to early endosomes [146]. While this is likely to be of importance in understanding the results with the bilosome, it is important to note that the Brewer *et al.* studies were obtained with subcutaneous immunisation, rather than oral [60, 146]. A more relevant comparison can be made with the work of Mann *et al.* (2009), where the effects of bilosomes of approximately 1000nm and 200nm on the immune response, after oral administration, were examined [137]. Here it was found that, while both formulations induced comparable IgG1 titres (T_H1), the larger bilosomes induced significantly greater IgG2a titres (T_H2) [137]. This is likely due to some difference in the underlying mechanisms involved in processing the bilosome in the GI tract, however, the specifics of these are still to be established. It has been shown that smaller particles have higher absorption in the GI tract, with the optimum size between 100-200nm [147, 148]. Despite this, exactly why this difference exists has not yet been established, and mechanisms for vesicle and antigen uptake may include transcytosis through M cells, binding to intestinal enterocytes, and luminal sampling by dendritic cells [64, 128, 149-151]. The dose loaded within the vesicle, and delivered to the APC, has also been shown to be of significance, with smaller doses producing an up-regulation of the T_H2 response, and larger doses up-regulating the T_H1 response [137, 152, 153]. Up-regulation of the T_H2 response is also caused by dendritic cells located within Peyer's patches [154], which have been shown to be targeted by particles in the size range used here [149-151]. The strong IgG1 titres observed with both the HM formulations may therefore be caused by any one, or a combination, of these factors. Given that these were observed with both the lyophilised, and

unlyophilised, formulations, it seems likely that their size is not the most significant factor in regulating the T_H2 response. The size of these formulations (~200nm for unlyophilised, ~1000nm for lyophilised) is comparable to those used in the Mann *et al.* (2009), which also found no difference in the T_H2 response, however, a significant difference was observed in the T_H1 response [137]. This study also used an influenza vaccine (A/Panama), at a similar dose (45μg vs. 50μg), however, the entrapment efficiency was found to be 50% for both the small and large vesicles [137]. Entrapment efficiency with the HM formulations was found to be approximately 30% for the unlyophilised (small) bilosomes, and 50% for the lyophilised (large) bilosomes. Despite the apparently lower amount of antigen used with the small bilosomes in the HM study, the ratio of T_H2 vs. T_H1 response was similar, and it is in comparing the larger formulations that there is an apparent discrepancy. With the larger bilosomes used in the HM study, the ratio of T_H2 vs. T_H1 response was similar to that with the smaller vesicles, however, Mann *et al.* (2009) observed a significant up-regulation of the T_H1 response [137]. While the difference in these studies is significant, it should be noted that the large vesicles used by Mann *et al.* were formulated using a lipid film cast method with chloroform, and were not subject to homogenisation. The size range of these vesicles, while having a mean of 980nm, also had two significant populations, between 60-350nm, and 400-2500nm, whereas the lyophilised formulation had one distinct size range, between 900-1100nm [137]. It is apparent then, that while these vesicles appear to be comparable in terms of entrapment and mean size, there is a significant difference in the range of sizes present, and this may explain the difference in the T_H1 responses observed. As a T_H1 response has been shown to afford increased protection against influenza infection [155], it will

therefore be of great importance to attempt to enhance the response observed with the HM formulations. It would be useful in any future work to assess the ratio of T_H2 vs. T_H1 responses with a mixed population of sizes, possibly by combining unlyophilised bilosomes with lyophilised bilosomes. While the T_H1 response is lower than the T_H2 response with the HM bilosomes, it is important to note that significant IgG2a titres were achieved, and it may be possible to enhance these titres in future, as attempted in section 3.3.4

The data here therefore indicates that the 1-step HM protocol is not viable, however, lyophilisation of bilosomes has been shown to be feasible without detriment to immunogenicity. Lyophilisation has previously been described with a number of liposomal systems [144, 156-159]. It is reported that lyophilisation results in damage to the liposomes, resulting in expansion and changes in lamellarity on rehydration, as observed with the bilosome formulations. With the bilosome, however, this does not appear to result in any detrimental effect on immunogenicity, with the lyophilised formulation proving no different from the fresh formulation. Despite this, the EE of the lyophilised formulation was significantly higher than the fresh formulation, and it could reasonably be expected that this could lead to increased immunogenicity. It has been shown previously that the design of the lyophilisation protocol [144, 156], and the use of cryoprotectants such as trehalose or mannitol [144, 159], can significantly impact upon the properties of liposomes on rehydration. It is therefore possible that consideration of these factors would result in an improved immune response, and it would be highly desirable to establish their effects in any future formulation design.

The results of this study have indicated that the HM method cannot be simplified to a 1-step procedure, and that lyophilisation of the bilosome, without detriment to immunogenicity, is possible. Subsequently, the next step would be to test the lyophilised HM formulation, using the 10mM bile salt concentration previously established, to determine its stability over a period of time.

2.4.3 The effect of storage under different conditions on the bilosome

Monitoring the size of the bilosomes with respect to time showed that the wet formulation appears to be stable for up to a month (stored at 4^{O}C), and the lyophilised formulation for up to 2 months (stored at room temperature). After this time the wet formulation appeared to have doubled in size, however, this was not found to be a significant increase. The lyophilised formulation decreased significantly in size ($p < 0.05$) after two months, to around 300nm, which was not significantly different ($p > 0.05$), to the wet formulation. Over time the entrapment remained above 30% for up to 12 weeks for the wet formulation, and up to 14 weeks for the lyophilised formulation, although it should be noted that the lyophilised variant decreased from an initial value of around 50%, whereas the wet formulation remained constant. Statistical analysis, however, indicated that there was no significant difference between any time points for either formulation.

It is possible that the reduction in size of the lyophilised formulation may indicate that an equilibrium state had been reached, however, it seems more likely that this may be due to hygroscopic effects during storage. A relatively simple protocol was used, and in future it will be necessary to determine the moisture content of the bilosome after lyophilisation, in order to find the

optimum process to minimize this. Other means of minimizing moisture uptake would include sealing the vials under vacuum, or storing under an inert gas, and establishing a truly airtight seal around the vial. Cryoprotectants can also limit any change in size, and liposomes stabilised with trehalose and sucrose have been shown to retain their size for up to a year [158]. It is also possible that any changes in the formulations may be due to a lack of sterility, or degradation of the lipid components, which are more likely to affect the wet formulation. This may explain the apparent reduction in EE and increase in size of the wet formulation at the studies conclusion, however it should be noted that these were not found to be statistically different from any other time point using ANOVA with post-hoc Tukey analysis. Another point to be considered in any future study is the regulation of the temperature of the lyophilised formulation, as these were kept at room temperature, which could have varied between 10-30°C.

Size and EE have been shown to be stable for between 1-3 months for lyophilised and wet liposomal formulations with various constituents at various temperatures without cryoprotectants [160-162]. Other studies showed an increase in size over this time [163], although lyophilisation was not performed in this case. It appears then that both the wet and lyophilised formulations have similar stability to those studies performed previously without cryoprotectants [160-164].

In the stability study all formulations induced significant IgG1 and IgG2a antibody titres, with only the fresh, lyophilised formulation inducing significant IgA levels, similar to data in the study comparing 1-step, 3-step and lyophilised variants, where the lyophilised variant appeared to induce

slightly higher IgA levels. Influenza vaccine has been shown to be stable for up to a year at room temperature [165], therefore it is the stability of the bilosome formulation which is the key issue. Given the apparent (although not statistically significant) reduction in entrapment efficiency over time, it would be expected that the formulations immunogenicity would similarly decrease, however, there was no significant difference between the two wet formulations or the two lyophilised formulations This then indicates that these bilosomes can be stored at either 4^{O}C (wet) or room temperature (lyophilised) for up to 9 months without significantly affecting their immunogenicity. Previous work by Mohammed *et al*. (2010) [158] has shown a similar result, with retention of the immunogenicity of lyophilised liposomal formulations after 1 year of storage. In this case, however, the formulations were lyophilised with cryoprotectants (sucrose, trehalose or lysine), and γ-irradiated to ensure sterility. This study indicated little difference in IgG1 and IgG2a for fresh or lyophilised formulations, as observed with the bilosome. However, examination of spleen cell proliferation and cytokine production revealed some differences between fresh and stored formulations. It will therefore be necessary to examine the effects of cryoprotectants and γ-irradiation on the bilosome in future, as these will be necessary to give a product which conforms to the regulatory requirement for a stable and sterile product [144]. The animal studies should also be extended to include other assays than antibody production, as these may not always reveal differences between the formulations. Other issues exist which would be useful for a future study to address, such as the immunogenicity of a wet formulation stored at room temperature, extending the study time to 1-2 years, and performing animal studies at each time point, as opposed to solely at the end of the study. The shelf-life of an

influenza vaccine is highly important in creating a viable commercial product, as it is estimated that in the case of a highly virulent pandemic occurring, close to 6 billion people could require immunisation [113, 116, 166]. To combat this eventuality, governments therefore stockpile vaccines, and the stability and immunogenicity of a product after 1-2 years will therefore be highly relevant in the decision on which products to purchase.

Several facts about bilosome formulation have now been established; a bile salt concentration of 10mM is optimum, lyophilisation is possible without degradation of the immune response, and this allows storage at room temperature for up to 9 months. This forms a basis then for the next chapter, which will address some of the issues with the HM formulation, such as cost, ease of production, and enhancing immunogenicity.

Chapter 3 – Adaptation of the formulation process and constituents

3.1 Introduction

Adaptation of the bilosome to allow storage at room temperature has proved successful, providing comparable immunogenicity to a fresh formulation. However, the attempt to simplify the HM formulation from a 3½ h, 3-step process proved unsuccessful. In addition, MPG is an expensive surfactant (~50 euros / gram, Larodan fine chemicals; price correct 12/4/11), which must be purchased from an outside supplier. The use of less expensive surfactants, which can be synthesised in-house, is therefore desirable. The surfactants developed for this proved unsuitable for use with the original HM method, and in combination with the desire to simplify the formulation process, new methods were developed. This resulted in the water bath / homogenisation (WB) method, which, although immunogenic with the novel surfactants, and quicker than the HM method, did not consistently produce a regular bilosome formulation. The WB method was therefore developed into the microwave (MW) method, a 2-step process taking approximately 45min. While all of these methods retained their immunogenicity, IM injection consistently produced significantly higher systemic antibody titres, as has previously been found [61, 63, 66, 137]. Therefore, in an attempt to enhance the immunogenicity squalene, an adjuvant used in the MF59 influenza vaccine [167-169], was incorporated into the MW formulation. Subsequently, the main issues for this chapter were:

(1) Novel surfactants, which can be synthesised in-house are required to provide a cheaper alternative to MPG.

(2) These surfactants are unsuitable for use with the HM method. Would an adapted method, using a water bath and homogenisation (WB),

produce bilosomes with comparable physical characteristics and immunogenicity to the HM method?

(3) As the WB method gives poor formulation reproducibility, can an alternative method using a microwave reactor offer consistent formulations and immunogenicity?

(4) Squalene is used as an adjuvant in MF59. Can its incorporation into the MW formulation afford enhanced immunogenicity?

3.1.1 Chapter aims

1: Synthesise and characterise a range of novel alkylgluconamide surfactants by IR, ^{1}H and ^{13}C NMR spectral analysis and differential scanning calorimetry.

2: Assess the physical characteristics (size, zeta potential and antigen entrapment) of WB bilosomes containing the novel surfactants, and compare these with HM bilosomes.

3: Determine the immunogenicity of bilosomes containing the novel surfactants, formulated by the WB method.

4: Assess the physical characteristics of MW bilosomes containing the novel surfactants, and compare these with HM bilosomes.

5: Determine the immunogenicity of MW bilosomes containing those novel surfactants identified as suitable in the WB study (hexylgluconamide and hexadecylgluconamide).

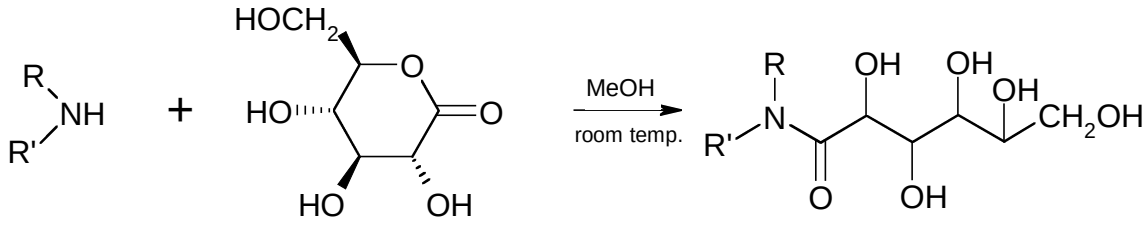
6: Evaluate the effect of squalene incorporation on the physical characteristics of MW bilosomes containing the hexadecylgluconamide (HXD) surfactant.

7: Compare the immunogenicity of HXD MW bilosomes incorporating two different squalene concentrations with HXD MW bilosomes without squalene.

3.2 Materials and Methods

3.2.1 Synthesis of novel alkylgluconamides

In order to examine surfactants other than MPG, a range of novel surfactants were synthesised. In a fume hood 50mM of alkylamine (hexylamine, dihexylamine, hexadecylamine, dodecylamine, dioctylamine or octadecylamine from Sigma-Aldrich UK) was added to a 1L round-bottomed flask with 500ml methanol, then 50mM of D-(+) gluconic acid δ lactone (Sigma-Aldrich UK) added, the flasks sealed, and the mixture stirred with a magnetic flea for 24h at room temperature. Products were isolated by rotary evaporation of the solvent, followed by drying for 12h in a 40°C oven. The general scheme for the reaction is shown in Figure 16.



Alkylamine D-(+) gluconic acid δ lactone Alkylgluconamide

Figure 16: General reaction of alkylamine with D-(+) gluconic acid δ lactone. Hexylamine R: H, R': $CH_3(CH_2)_5$; hexadecylamine R: H, R': $CH_3(CH_2)_{15}$; dodecylamine R: H, R': $CH_3(CH_2)_{12}$; octadecylamine R: H, R': $CH_3(CH_2)_{17}$.

3.2.2 Spectral and thermal analysis of surfactants

Spectral analysis was performed in order to assess the nature of the chemical bonds within the alkylgluconamides synthesised by 2.1.2, and therefore the presence of the desired product. Thermal analysis provided information on the melting points of the compounds.

3.2.2.1 Infra-Red (IR) spectral analysis

Samples were prepared for IR spectral analysis by lightly grinding 1mg in a mortar and pestle with 250mg potassium bromide (stored at 40°C), then added to the press assembly (KBr Die 13mm, Crystal Laboratories, USA) with a single die inserted. The top die was then inserted and the assembly transferred into the press (30 ton press C30, Research and Industrial Instrument Company, UK), with air removed from the system via a vacuum pump, and a pressure of 10bar applied for 2min, after which the pump was disconnected. After a further 3min the assembly was removed from the press and the resultant disc checked for flaws. A background reading was then measured on a Genesis Series FTIR from ATI Mattson at the wavelength range of 4000-500cm^{-1} using WinFIRST software, after which the IR spectra of the disc was read.

3.2.2.2 NMR spectral analysis

^{1}H and ^{13}C NMR spectra of the alkylgluconamide surfactants (2.1.2) were obtained in deuterated DMSO (Sigma-Aldrich UK Ltd.) at 400MHz and 100MHz respectively in a JEOL FTNMR.

3.2.2.3 Differential scanning calorimetry (DSC)

DSC thermograms were obtained with 3mg of sample using a Mettler Toledo® DSC 822^{e} with a heating rate of 5°C/min. Data was plotted using WinFIRST software. Samples were heated from 30°C to 160°C, then cooled to 30°C and again heated to 160°C.

3.2.3 Water bath / Homogenisation method for novel alkylgluconamides (WB) for bilosomes

Alkylgluconamides could not be formulated into bilosomes by the HM method, due to their high melting points. Bilosomes were thus formulated by dispersion of a 5:4:1 molar ratio of alkylgluconamide (209mg hexylgluconamide, 273mg dodecylgluconamide, 315mg hexadecylgluconamide and 336mg octadecylgluconamide), cholesterol (234mg) and DCP (82mg) in 3.78 ml 0.025M carbonate buffer, pH 9.7, with 1 ml of 10mM bile salt, by heating the solution to 70°C in a water bath, followed by homogenisation at 8000rpm for 10 min. This formulation was then allowed to cool to 30 °C over 3 h and 5.22 ml carbonate buffer containing 1.2 mg antigen added.

3.2.4 Microwave Method (MW) for bilosomes

Lipids (as for the HM method) were weighed and transferred to a 20ml microwave vessel along with 1ml 100mg/ml deoxycholic acid (Sigma-Aldrich, UK) in 0.025M carbonate buffer pH 9.7, 3.78ml 0.025M carbonate buffer pH 9.7 and a magnetic stirring bar, taking care to wash all solids from the vessel walls. The vessel was sealed and inserted into a microwave reactor (Biotage Initiator) with 15s pre-stirring, then 1min at 140°C with stirring.

After cooling to 50°C, 5.22ml carbonate buffer (0.025M, pH 9.7) containing 1.2mg antigen was added and the mixture stirred for 30min.

3.2.5 Squalene-containing bilosomes

Bilosomes were formulated using the MW method using hexadecylgluconamide (HXD) instead of MPG. The groups are summarised in Table 8. All chemicals were purchased from Sigma-Aldrich UK Ltd., with HXD synthesised in house by the method laid out in section 2.1.3.

Group	Molar ratio of HXD:CHO:DCP	Molar ratio of squalene (vs. DCP)	N/Cal antigen
Empty	5:4:1	None	None
Empty/SQ2	5:4:1	1	None
Std	5:4:1	None	120μg/ml
SQ2	5:4:1	2	120μg/ml
SQ1	5:4:1	1	120μg/ml

Table 8: Summary of lipid components, squalene and antigen present in the formulations for the study on the effect of squalene on bilosome immunogenicity.

3.2.6 Vesicle characterisation

3.2.6.1 Estimation of size by dynamic light scattering

Particle size measurements were performed as per section 2.2.4.1

3.2.6.2 Freeze-fracture electron microscopy (FFEM)

FFEM images were captured as per section 2.2.4.2

3.2.6.3 Zeta potential

Zeta potential measurements were made as per section 2.2.4.3

3.2.6.4 Lowry-Peterson assay

Unentrapped antigen was separated from entrapped antigen and lipid components by ultracentrifugation of a 0.1ml sample, in 2ml 0.025M carbonate buffer pH 9.7 in a Beckman tube, at 40,000 rpm for 2h. The pellet was discarded and to 0.5ml of the supernatant was added 0.05ml 0.15% (w/v) sodium deoxycholate, followed by vortexing and incubation at room temperature for 10min. This was followed by addition of 0.05ml 72% (w/v) trichloroacetic acid to the samples, then vortexing and spinning at 13,000rpm for 30min in a centrifuge (MSE Micro Centaur). During this time a standard curve was prepared of the relevant protein in 0.2ml dH_2O. The supernatant was carefully removed from the samples and the pellets resuspended in 0.2ml dH_2O, then 0.2ml 2M NaOH was added to both samples and standards, followed by incubation at 100°C in a water bath for 10min. To each tube, 1ml of a 100:1:1 mixture of 2% (w/v) Na_2CO_3: 1% (w/v) $CuSO_4.5(H_2O)$: 2% (w/v) Na_2Tartrate.$2(H_2O)$ was added, then incubated at room temperature for 10min and 0.1ml of 1N Folin and Ciocalteu's phenol reagent (Sigma-Aldrich, UK) added. Aliquots of 200µl of each sample and standard were added in triplicate to a flat-bottomed 96 well plate (Iwaki, Japan) and absorbance read at 540nm on a SpectraMax 190 plate reader (Molecular Devices, USA), with test sample levels determined by linear regression from the standard calibration curve. The quantity in the supernatant was subtracted from the total protein added to calculate the entrapment efficiency. Experimental conditions were the same for standards and samples and a correlation coefficient of >0.99 was obtained.

3.2.7 Immunisation

3.2.7.1 Animals and schedule

In-house bred male BALB/c mice, 8-10 weeks old, housed in a fully climatised room were randomised and placed into groups of 5. All mice were starved, but allowed access to water, for 2 h pre-immunisation, with food and water available *ad libitum* between immunisations. Each oral dose consisted of 0.4ml bilosome, containing approximately 50μg N/Cal hemagglutinin, administered by intragastric gavage on days 1, 4, 14, 17, and retention of the total volume in the stomach could be inferred from the absence of any reflux or nasal discharge. The control group was administered 0.05ml 230μg/ml N/Cal in each of the hind legs on the same days by IM injection. Tail bleeds were collected in heparinised capillary tubes on days 7, 20, and 33 post-immunisation and centrifuged at 13,000rpm in 1.5ml microfuge tubes for 20min. Plasma was transferred into fresh 0.5 ml micro-centrifuge tubes (Fischer, UK), and stored at -20°C until IgG1/IgG2a levels were determined by enzyme linked immunosorbant assay (ELISA). The study was terminated on day 33. IgA levels were determined by ELISA of lung lavages obtained by perfusing the lungs post-mortem with 0.5ml 1x PBS.

3.2.7.2 Study details

A summary of the various experimental and control groups for each study is given in Table 9. All animals were treated as detailed in section 3.2.7.1.

Study	WB method	MW method	Squalene incorporation (MW method)
Aims	(1) Examine immunogenicity vs. HM	(1) Examine immunogenicity vs. HM	(1) Examine immunogenicity with various squalene concentrations
Group number: 1	control, empty HM bilosomes	control, empty MW bilosomes	control, empty
2	WB HX bilosomes	HM bilosomes	control, IM
3	WB DOD bilosomes	MW MPG bilosomes	empty/SQ2
4	WB HXD bilosomes	MW MPG bilosomes, lyophilised	HX bilosomes
5	WB OCT bilosomes	MW HX bilosomes	HX bilosomes, SQ2
6	Control, IM injection	MW HX bilosomes, lyophilised	HX bilosomes, SQ1
7	-	MW HXD bilosomes	-
8	-	MW HXD bilosomes, lyophilised	-
9	-	control, IM injection	-

Table 9: Summary of groups for each animal study and the relevant sections detailing bilosome formulation for each. IM = intramuscular, HM = homogenisation method, WB = water bath / homogenisation method, MW = microwave method, HX = hexylgluconamide surfactant, DOD = dodecylgluconamide surfactant, HXD = hexadecylgluconamide surfactant, OCT = octadecylgluconamide surfactant, MPG = 1-monopalmitoyl glycerol surfactant, SQ = squalene. See sections 2.2.1.1, 3.2.3-3.2.5 for formulation details.

3.2.8 Enzyme linked immunosorbant assay (ELISA)

ELISA antibody assays were performed as per section 2.2.6.

3.2.9 Statistical Analysis

Statistical analysis was performed either using a two-tailed unpaired t-test where applicable, or one-way ANOVA with *post-hoc* analysis by Tukey test where significant differences were indicated. All analysis was performed at the 95% confidence level with Minitab v15.

3.3 Results

3.3.1 Synthesis and characterisation of alkylgluconamides

Yields obtained for the novel alkylgluconamide surfactants were: hexylgluconamide = 68.2%, dodecylgluconamide = 90.6%, hexadecylgluconamide = 96.4% and octadecylgluconamide = 95.7%. DSC thermograms allow analysis of the melting curve; the method used here was a heating from 20-160°C, followed by cooling to 30°C and a re-heating to 160°C. This indicated onset of melting and complete melting at: hexylgluconamide = 147.3°C, 156.6°C, dodecylgluconamide = 151.6°C, 156.6°C, hexadecylgluconamide = 144.8°C, 156.6°C and octadecylgluconamide = 141.7°C, 151.5°C. An example thermogram for hexylgluconamide is shown in Figure 17.

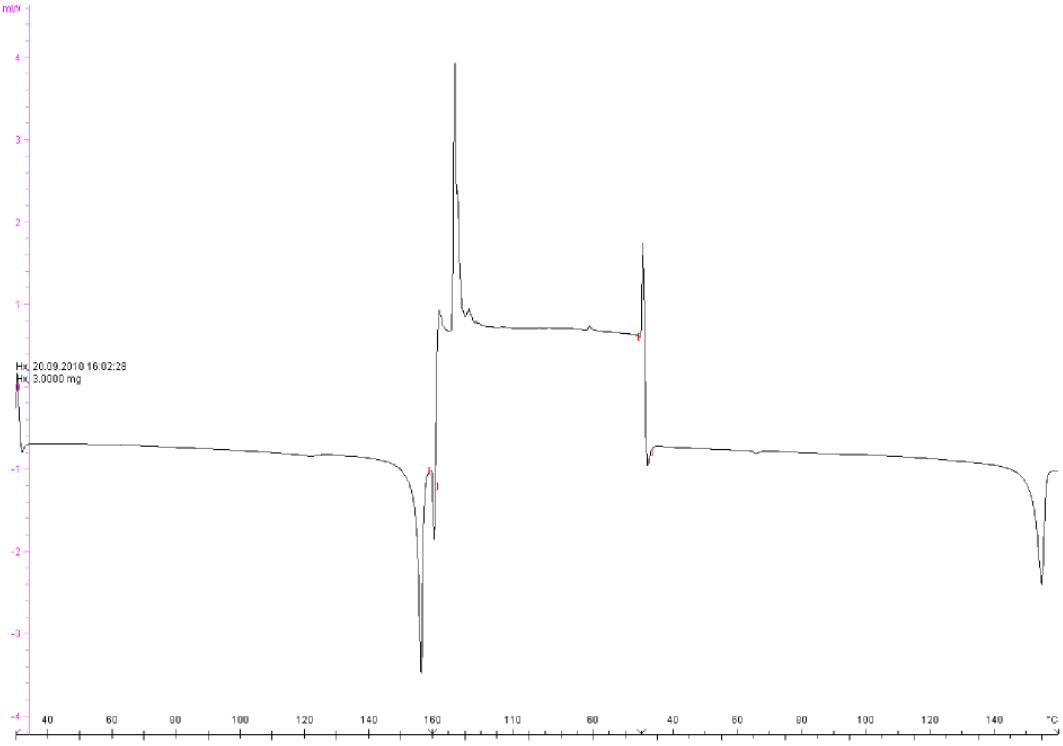


Figure 17 – Example DSC thermogram for hexylgluconamide obtained using 3mg of sample in a Mettler Toledo® DSC 822^e heating from 30-160°C, cooling to 30°C and again heating to 160°C.

3.3.1.1 IR analysis

IR spectra (Nujol mull) for hexylgluconamide: 1651.8 cm^{-1} (C=O), 2856.5 cm^{-1} (C-H), 2925.8 cm^{-1} (C-H), 2938.7 cm^{-1} (C-H), 3300-3500 cm^{-1} (O-H), 3527.1 cm^{-1} (N-H).

Dodecylgluconamide: 1647.2 cm^{-1} (C=O), 2840.5 cm^{-1} (C-H), 2920.5 cm^{-1} (C-H), 2943.2 cm^{-1} (C-H), 3300-3500 cm^{-1} (O-H), 3533.7 cm^{-1} (N-H).

Hexadecylgluconamide: 1647.3 cm^{-1} (C=O), 2848.2 cm^{-1} (C-H), 2919.8 cm^{-1} (C-H), 3300-3500 cm^{-1} (O-H), 3534.3 cm^{-1} (N-H).

Octadecylgluconamide: 1622.4 cm^{-1} (C=O), 2849.1 cm^{-1} (C-H), 2917.7 cm^{-1} (C-H), 3300-3500 cm^{-1} (O-H), 3560.9 cm^{-1} (N-H).

For each of the alkylgluconamides ring opening of the lactone, and hence reaction, could be inferred by the absence of any lactone ring peaks in the IR spectra at approximately 1740cm^{-1}. Example spectra are shown in Figure 18.

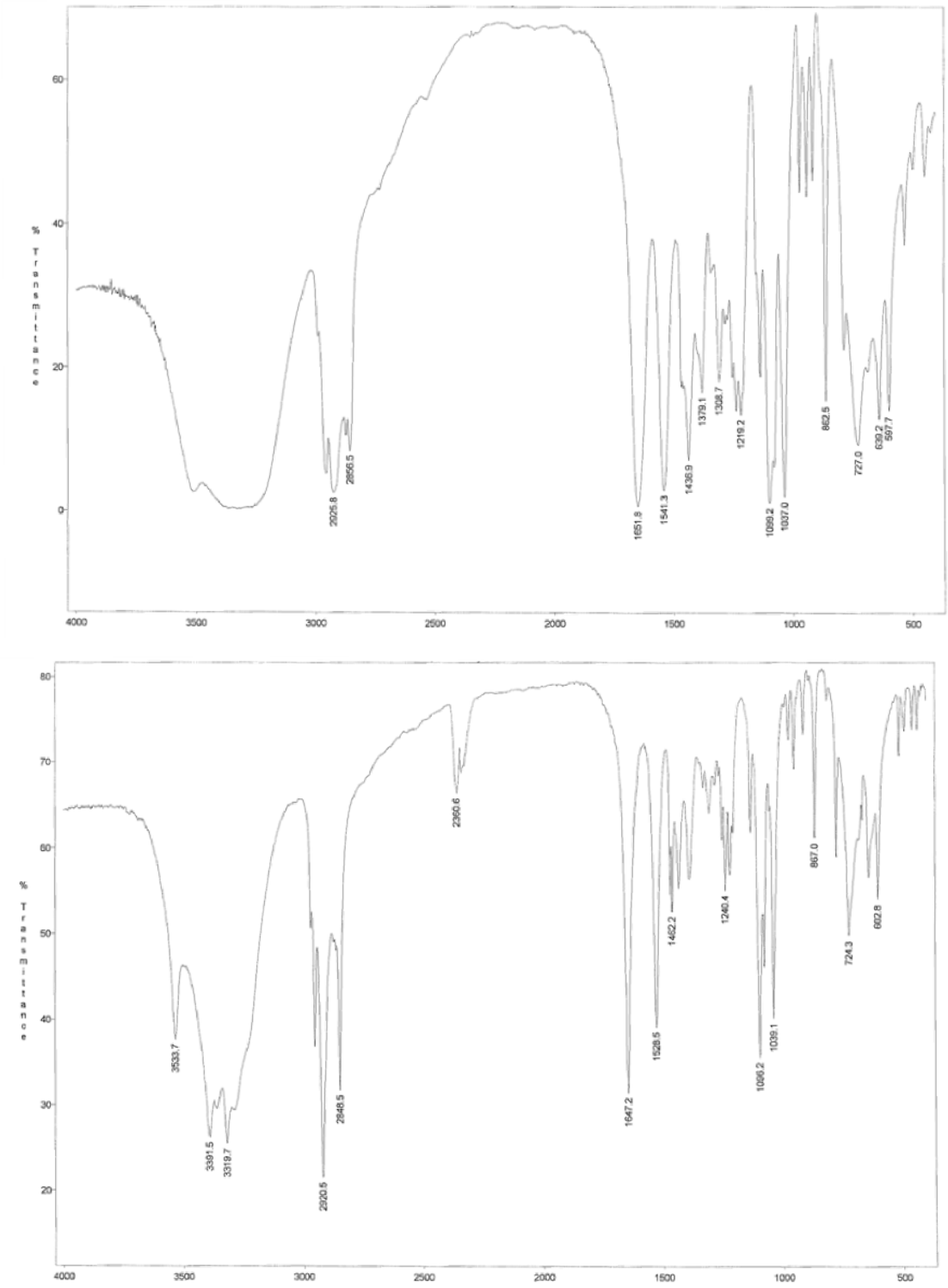


Figure 18 – IR spectra for hexylgluconamide (top) and octadecylgluconamide (bottom) obtained using a KBr disc containing 1mg of sample with a Genesis Series FTIR from ATI Mattson at the wavelength range of 4000-500cm^{-1} using WinFIRST software.

3.3.1.2 NMR analysis

^{1}H and ^{13}C NMR analysis of the alkylgluconamides was performed in order to confirm that the expected structures had been formed. Chemical shifts, J-coupling values and peak types (s = singlet, d = doublet, t = triplet, q = quartet, qi = quintet, sx = sextuplet, dd = doublet of doublets, m = multiplet) are given below.

Hexylgluconamide

^{1}H NMR (DMSO): δ (ppm) = 0.87 (t, J = 5.27 Hz, -$CH_2CH_2\underline{CH_3}$), 1.25 (s, -$CH\underline{HCH_2CH_2}CH_3$), 1.4 (d, J = 6.59 Hz, -$\underline{CH}HCH_2CH_2CH_3$), 3.07 (m, J = 6.15 Hz –NH-$\underline{CH_2}CH_2$-), 3.33 (m, J = 4.83 Hz, -NH-$CH_2\underline{CH_2}$-), 3.39 (s, -CHOH-$\underline{CH}$OH-CH_2OH), 3.47 (s, CHOH-$\underline{CH_2}$OH), 3.57 (d, J = 8.79 Hz, $\underline{CH}$OH-CHOH-CH_2OH), 3.89 (s, CHOH-$CH_2\underline{OH}$), 3.97 (d, J = 3.52 Hz NH-CO-$\underline{CH}$OH-), 4.33 (s, -CO-CHOH-$\underline{CH}$OH-), 4.39 (d, J = 7.03 Hz, -$CH\underline{OH}$-CH_2OH), 4.46 (s, -CHOH-$CH\underline{OH}$-CHOH-), 4.52 (d, J = 4.83, -$CH\underline{OH}$-CHOH-CH_2OH), 5.35 (s, NH-CO-$CH\underline{OH}$-), 7.59 (s, $\underline{NH}$).

^{13}C NMR (DMSO): δ (ppm) = 14.52 (s, -CH_3), 22.64 (s, -CH_2CH_3), 26.6 (s, -$\underline{C}H_2$-CH_2-CH_3), 29.7 (s, NH-CH_2-$\underline{C}H_2$-), 31.6 (s, $\underline{C}H_2$-CH_2-CH_2-CH_3), 38.8 (s, NH-$\underline{C}H_2$-CH_2-), 63.95 (s, CHOH-$\underline{C}H_2$OH), 70.69 (s, $\underline{C}$HOH-CHOH-CH_2OH), 72.05 (s, C=O-CHOH –$\underline{C}$HOH-), 72.98, (s, C=O-$\underline{C}$HOH-), 74.21, (s, $\underline{C}$HOH-CH_2OH), 172.8, (s, -$\underline{C}$=O).

Dodecylgluconamide

^{1}H NMR (DMSO): δ = 0.86 (t, J = 7.03 Hz, -$CH_2CH_2\underline{CH_3}$), 1.28, (t, J = 7.03, -$CH_2(\underline{CH_2CH_2})_9$CH3), 1.4 (t, J = 6.59, NH-$CH_2\underline{CH_2}$), 3.07 (m, J = 6.15, NH-$\underline{CH_2}CH_2$-), 3.37 (m, J = 5.27 Hz, -NH-$CH_2\underline{CH_2}$-), 3.39 (s, -CHOH-$\underline{CH}$OH-

CH_2OH), 3.47 (m, J = 2.64 Hz, CHOH-$\underline{CH_2}OH$), 3.57 (t, J = 2.64 Hz, $\underline{CH}OH$-CHOH-CH_2OH), 3.90 (sx, J = 2.20 Hz, CHOH-$CH_2\underline{OH}$), 3.97 (dd, J = 4.39 Hz NH-CO-$\underline{CH}OH$-), 4.32, (t, J = 5.71 Hz -CO-CHOH-$\underline{CH}OH$-), 4.38 (t, J = 7.03 Hz, -$CH\underline{OH}$-CH_2OH), 4.46 (d, J = 5.27 Hz, -CHOH-$CH\underline{OH}$-CHOH-), 4.53 (d, J = 4.83, -$CH\underline{OH}$-CHOH-CH_2OH), 5.34 (d, J = 4.83 Hz, NH-CO-$CH\underline{OH}$-), 7.58 (t, J = 6.15 Hz, $\underline{NH}$).

^{13}C NMR (DMSO): δ (ppm) = 14.53 (s, -CH_3), 22.67 (s, -CH_2CH_3), 26.96 (s, -$\underline{C}H_2$-CH_2-CH_3), 29.61 (m, NH-CH_2-CH_2-($\underline{C}H_2$)$_7$-), 31.88 (s, NH-CH_2-$\underline{C}H_2$-), 38.82 (s, NH-$\underline{C}H_2$-CH_2-), 63.95 (s, CHOH-$\underline{C}H_2OH$), 70.67 (s, $\underline{C}$HOH-CHOH-CH_2OH), 72.06 (s, C=O-CHOH –$\underline{C}$HOH-), 72.98, (s, C=O-$\underline{C}$HOH-), 74.21, (s, $\underline{C}$HOH-CH_2OH), 172.8, (s, -$\underline{C}$=O).

Hexadecylgluconamide

^{1}H NMR (DMSO): δ = 0.86 (t, J = 6.59 Hz, -$CH_2CH_2\underline{CH_3}$), 1.22, (d, J = 17.14, -CH_2($\underline{CH_2CH_2}$)$_{13}$CH3), 1.4 (t, J = 6.59, NH-$CH_2\underline{CH_2}$), 3.07 (qi, J = 7.03, NH-$\underline{CH_2}CH_2$-), 3.37 (m, J = 2.86 Hz, -NH-$CH_2\underline{CH_2}$-), 3.39 (s, -CHOH-$\underline{CH}$OH-CH_2OH), 3.47 (sx, J = 5.71 Hz, CHOH-$\underline{CH_2}OH$), 3.57 (t, J = 7.91 Hz, $\underline{CH}$OH-CHOH-CH_2OH), 3.90 (d, J = 4.83 Hz, CHOH-$CH_2\underline{OH}$), 3.97 (t, J = 3.95 Hz NH-CO-$\underline{CH}OH$-), 4.32, (t, J = 5.27 Hz -CO-CHOH-$\underline{CH}OH$-), 4.38 (t, J = 7.47 Hz, -$CH\underline{OH}$-CH_2OH), 4.47 (d, J = 4.83 Hz, -CHOH-$CH\underline{OH}$-CHOH-), 4.53 (d, J = 4.39, -$CH\underline{OH}$-CHOH-CH_2OH), 5.34 (d, J = 5.27 Hz, NH-CO-$CH\underline{OH}$-), 7.59 (t, J = 5.71 Hz, $\underline{NH}$).

^{13}C NMR (DMSO): δ (ppm) = 14.54 (s, -CH_3), 22.67 (s, -CH_2CH_3), 26.96 (s, -$\underline{C}H_2$-CH_2-CH_3), 29.63 (m, NH-CH_2-CH_2-($\underline{C}H_2$)$_{11}$-), 31.87 (s, NH-CH_2-$\underline{C}H_2$-), 38.82 (s, NH-$\underline{C}H_2$-CH_2-), 63.95 (s, CHOH-$\underline{C}H_2OH$), 70.67 (s, $\underline{C}$HOH-CHOH-

CH_2OH), 72.06 (s, C=O-CHOH –CHOH-), 72.98, (s, C=O-CHOH-), 74.21, (s, CHOH-CH_2OH), 172.8, (s, -C=O).

Octadecylgluconamide

^1H NMR (DMSO): δ = 0.85 (t, J = 6.59 Hz, -$CH_2CH_2CH_3$), 1.24, (d, J = 9.45, -$CH_2(CH_2CH_2)_{15}CH3$), 1.4 (s, NH-CH_2CH_2), 3.06 (m, J = 6.09, NH-CH_2CH_2-), 3.37 (m, J = 18.02 Hz, -NH-CH_2CH_2-), 3.39 (s, -CHOH-CHOH-CH_2OH), 3.47 (s, CHOH-CH_2OH), 3.56 (s, C*H*OH-CHOH-CH_2OH), 3.89 (s, CHOH-CH_2OH), 3.96 (t, J = 4.39 Hz NH-CO-CHOH-), 4.32, (s, -CO-CHOH-CHOH-), 4.38 (d, J = 7.03 Hz, -CHOH-CH_2OH), 4.47 (d, J = 4.39 Hz, -CHOH-CHOH-CHOH-), 4.53 (d, J = 4.39, -CHOH-CHOH-CH_2OH), 5.34 (d, J = 5.27 Hz, NH-CO-CHOH-), 7.59 (s, NH).

^{13}C NMR (DMSO): δ (ppm) = 14.53 (s, -CH_3), 22.67 (s, -CH_2CH_3), 26.96 (s, -CH_2-CH_2-CH_3), 29.63 (m, NH-CH_2-CH_2-(CH_2)$_{13}$-), 31.86 (s, NH-CH_2-CH_2-), 38.82 (s, NH-CH_2-CH_2-), 63.94 (s, CHOH-CH_2OH), 70.67 (s, CHOH-CHOH-CH_2OH), 72.06 (s, C=O-CHOH –CHOH-), 72.97, (s, C=O-CHOH-), 74.21, (s, CHOH-CH_2OH), 172.8, (s, -C=O).

3.3.2 Bilosomes incorporating novel surfactants, formulated by the WB method

As the novel alkylgluconamide surfactants were unsuitable for use with the HM method, a simpler formulation process, which would allow their incorporation, was designed. The water bath / homogenisation method (WB) was a 1-step process which was considerably faster than the HM method, and also allowed alkylgluconamide incorporation.

3.3.2.1 Physical characteristics of bilosomes formulated using the WB method

The WB method gave bilosomes, which were significantly ($p < 0.05$) smaller than HM bilosomes in two cases, and were of similar zeta potential, all around -100 mV (see Table 10 for values). Those significantly smaller than the HM bilosomes were; hexylgluconamide = 114.0 ± 15.0nm ($p = 0.004$ vs. HM) and octadecylgluconamide = 104.0 ± 1.8 nm ($p = 0.013$ vs. HM). There was no significant difference in size between WB bilosomes incorporating the novel surfactants. The sizes and zeta potentials for the WB method bilosomes are tabulated in Table 10.

Formulation	Size (nm) ± S.D. by DLS	EE (%) ± S.D. by ninhydrin assay	Zeta Potential (mV) ± S.D.
HM	203.3 ± 19.9	29.0 ± 7.9	-104.5 ± 13.9
WB MPG	203.0 ± 19.9	37.2 ± 1.3	-113.6 ± 11.9
WB HX	114.0 ± 15.0	35.5 ± 0.3	-116.8 ± 16.0
WB DOD	102.5 ± 59.5	20.3 ± 0.9	-100.0 ± 17.5
WB HXD	104.0 ± 1.8	43.7 ± 0.6	-113.7 ± 17.5
WB OCT	126.7 ± 65.5	35.2 ± 0.6	-88.2 ± 9.4

Table 10. – Summary of sizes and zeta potentials. HM = standard homogenisation method, WB = homogenisation/water method, MPG = 1-monopalmitoyl glycerol, HX = hexylgluconamide, DOD = dodecylgluconamide, HXD = hexadecylgluconamide, OCT = octadecylgluconamide.

Entrapment efficiencies ± S.D. for the WB bilosomes were hexylgluconamide = 35.5 ± 0.28%, dodecylgluconamide = 20.3 ± 0.86%, hexadecylgluconamide = 43.7 ± 0.62, octadecylgluconamide = 35.2 ± 0.62% and MPG = 37.2 ± 1.3%. There was no significant difference ($p > 0.05$) between hexylgluconamide, octadecylgluconamide and HM bilosomes, however those containing

dodecylgluconamide had a significantly lower ($p < 0.05$) EE than all others, and the hexadecylgluconamide containing bilosomes had a significantly ($p < 0.05$) higher EE than all others.

3.3.2.2 Antibody response to WB bilosomes

Bilosomes incorporating hexadecylgluconamide induced significantly higher titres compared with empty bilosomes for both systemic (IgG1 and IgG2a) and mucosal (IgA) antibodies, which were (Ln titres ± S.D.) IgG1 = 12.08 ± 0.49 $p < 0.0001$, IgG2a = 10.54 ± 0.98, $p = 0.006$, IgA = 6.88 ± 0.92, $p = 0.004$.

The dodecylgluconamide formulation induced significant IgG2a and IgA titres (Ln titres ± S.D. IgG2a = 10.32 ± 1.43, $p = 0.012$, IgA = 7.65 ± 0.63, $p = 0.0003$), while the hexylgluconamide formulation induced significant IgG1 and IgA titres (Ln titres ± S.D. IgG1 = 11.20 ± 1.10, $p = 0.0003$, IgA = 6.66 ± 0.60, $p = 0.01$). No significant levels for any of the antibodies were observed for the octadecylgluconamide formulation (Figure 19). There was no significant difference between those formulations, which induced a significant response versus the control group.

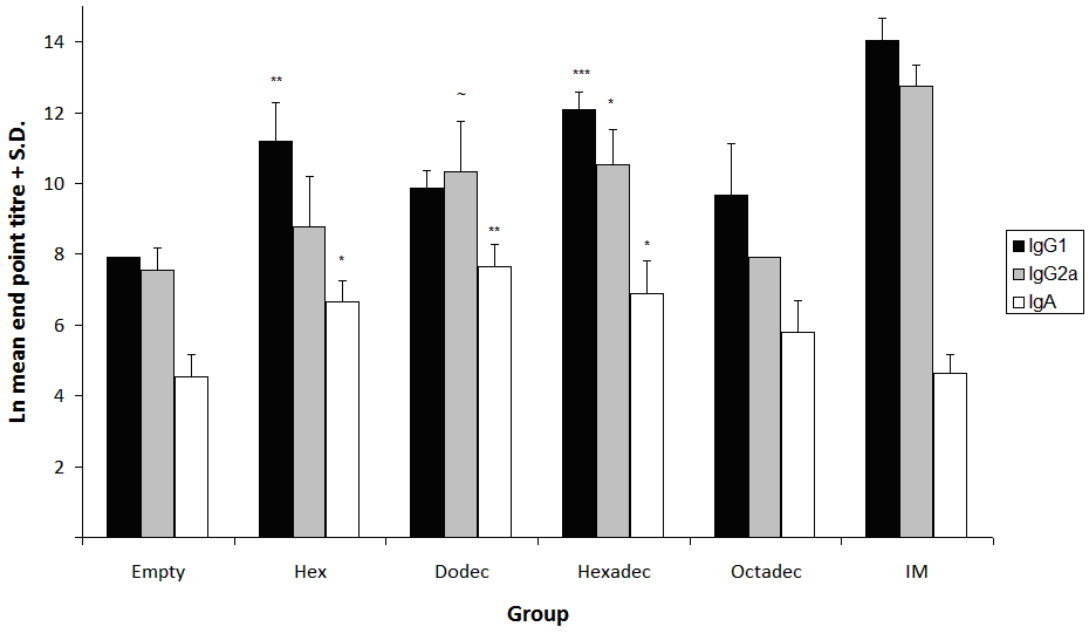


*Figure 19 – Ln mean end point titres ± S.D determined by ELISA for IgG1and IgG2a by serum sample and IgA by lung lavage for HW bilosomes formulated with novel alkylgluconamide surfactants. Significantly higher titres vs. the empty control group are indicated by p values: ~ < 0.05, * < 0.01, ** < 0.001 *** < 0.0001.*

3.3.3 Bilosomes formed by the MW method

As the novel surfactants had been successfully incorporated into WB bilosomes, it was decided to continue with their use. However, the WB method frequently produced formulations, which were poorly dispersed and granular. It was therefore necessary to develop the formulation process to produce well-dispersed, regular, and reproducible bilosomes, which resulted in the microwave (MW) method.

3.3.3.1 Physical characteristics of MW bilosomes

There was no significant difference in zeta potentials for any of the formulations, nor difference in size between MPG, hexyl- and dodecylgluconamide MW bilosomes and HM bilosomes.

Hexadecylgluconamide MW bilosomes were found to be significantly (p = 0.0003) larger than HM bilosomes, and octadecylgluconamide MW bilosomes were larger than MPG (p = 0.03), dodecyl- (p = 0.02) and hexadecylgluconamide (p = 0.02) MW bilosomes and HM bilosomes (p = 0.002).

The analysis of the MW vesicles using a LEO912 energy filtering microscope at 80 kV showed spherical structures without lipid sheets or crystalline bodies (Figure 20). These were analysed using ImageJ software (five images per set, various magnification levels)(National Institute of Health), and sizes were; MPG = 480.5 ± 323.9nm, hexylgluconamide = 389.6 ± 278.2nm, dodecylgluconamide = 261.3 ± 171.4nm, hexadecylgluconamide = 297.1 ± 193.4nm, and octadecylgluconamide = 295.4 ± 132.3nm. These results were not found to be significantly ($p > 0.05$) different from the values obtained via the ZetaSizer, and there was no significant ($p > 0.05$) difference between groups. The results were similar to previous data for HM bilosomes, and a summary of sizes and zeta potentials is given in Table 11.

Formulation	Size (nm) ± S.D. by DLS	Size (nm) ± S.D. by FFEM	Zeta Potential (mV) ± S.D.
HM	203.3 ± 19.9	280.0 ± 150.0	-104.5 ± 13.9
MW MPG	247.6 ± 108.1	480.5 ± 323.9	-108.9 ± 12.9
MW HX	421.2 ± 332.6	389.6 ± 278.2	-93.2 ± 5.8
MW DOD	177.3 ± 119.9	261.3 ± 171.4	-80.9 ± 15.3
MW HXD	400.7 ± 14.4	297.1 ± 193.4	-63.9 ± 15.4
MW OCT	545.0 ± 46.1	295.4 ± 132.2	-66.2 6.3

Table 11. – Summary of sizes and zeta potentials. HM = standard homogenisation method, MW = microwave method, MPG = 1-monopalmitoyl glycerol, HX = hexylgluconamide, DOD = dodecylgluconamide, HXD = hexadecylgluconamide, OCT = octadecylgluconamide.

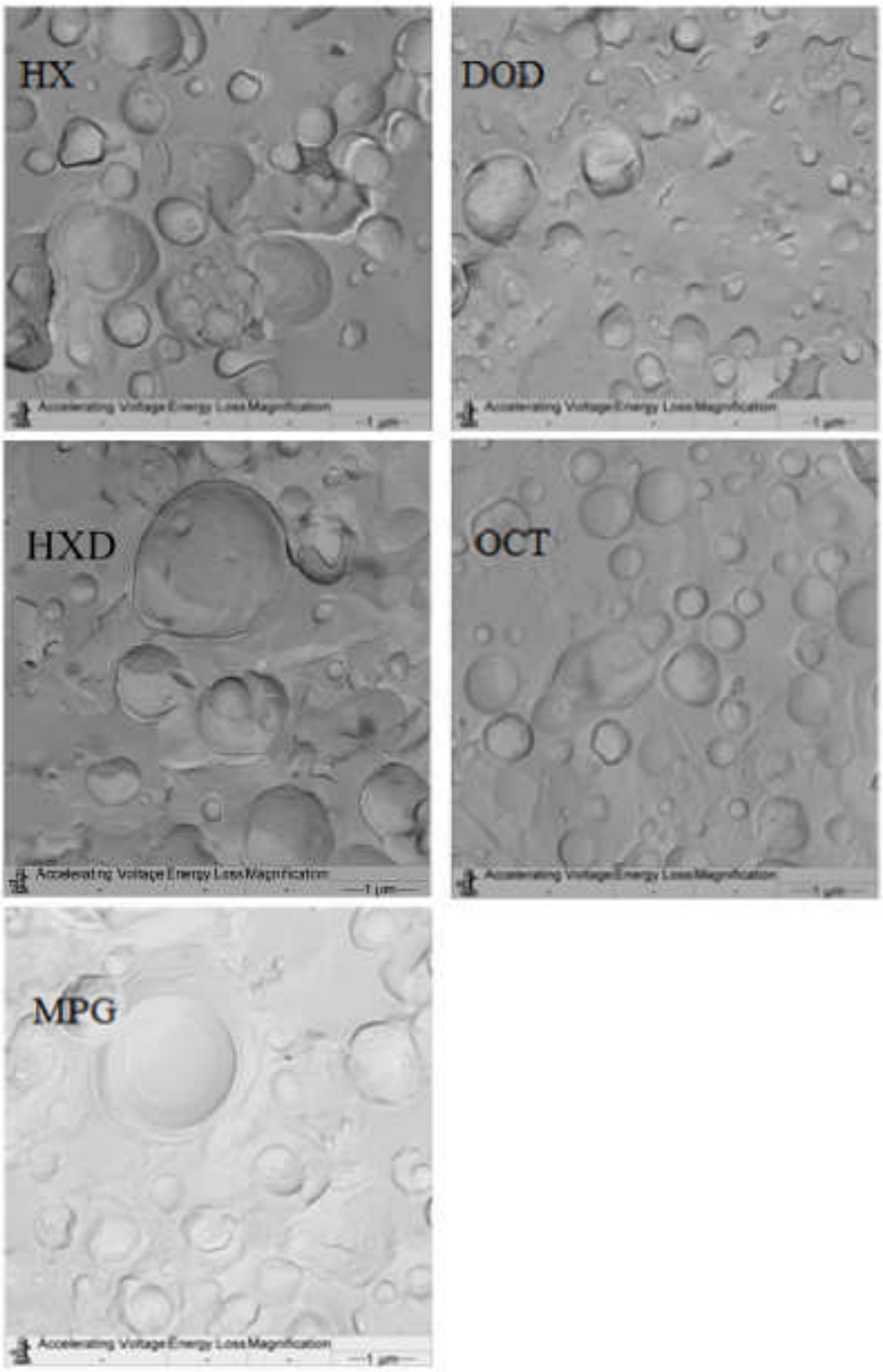


Figure 20 – FFEM images of MW bilosomes formulated with either HX = hexylgluconamide, DOD = dodecylgluconamide, HXD = hexadecylgluconamide, OCT = octadecylgluconamide and MPG = 1-monopalmitoyl-glycerol. Imaging was carried out using a LEO912 energy filtering electron microscope at 80 kV.

Analysis of the entrapment efficiency of the MW bilosomes showed that those formulated with MPG were not significantly different to those formulated by the HM method, with an efficiency of 37.1 ± 1.7% ($p > 0.05$). Those using the alkylgluconamide surfactants had entrapment efficiencies which increased linearly with increasing alkyl chain size, values for which were; hexylgluconamide = 32.7 ± 2.1% ($p = 0.052$ vs. MPG), dodecylgluconamide = 37.8 ± 0.8% ($p = 0.572$ vs. MPG), hexadecylgluconamide = 41.4 ± 1.1% ($p = 0.025$ vs. MPG), and octadecylgluconamide = 46.7 ± 0.8% ($p = 0.003$ vs. MPG). Each of these had significantly higher entrapment efficiency than those with smaller alkyl chains ($p < 0.05$).

3.3.3.2 Immune response to MW bilosomes

The HM, MW HX and MW HXD formulations induced significantly higher titres for IgG1, IgG2a and IgA compared with the empty bilosome control group, the titres and p values for which are in Table 12. There was no significant difference between groups for IgG1 and IgG2a titres, however, for IgA the hexylgluconamide MW formulation induced a significantly higher titre than the lyophilised MPG MW formulation ($p = 0.041$)(Figure 21).

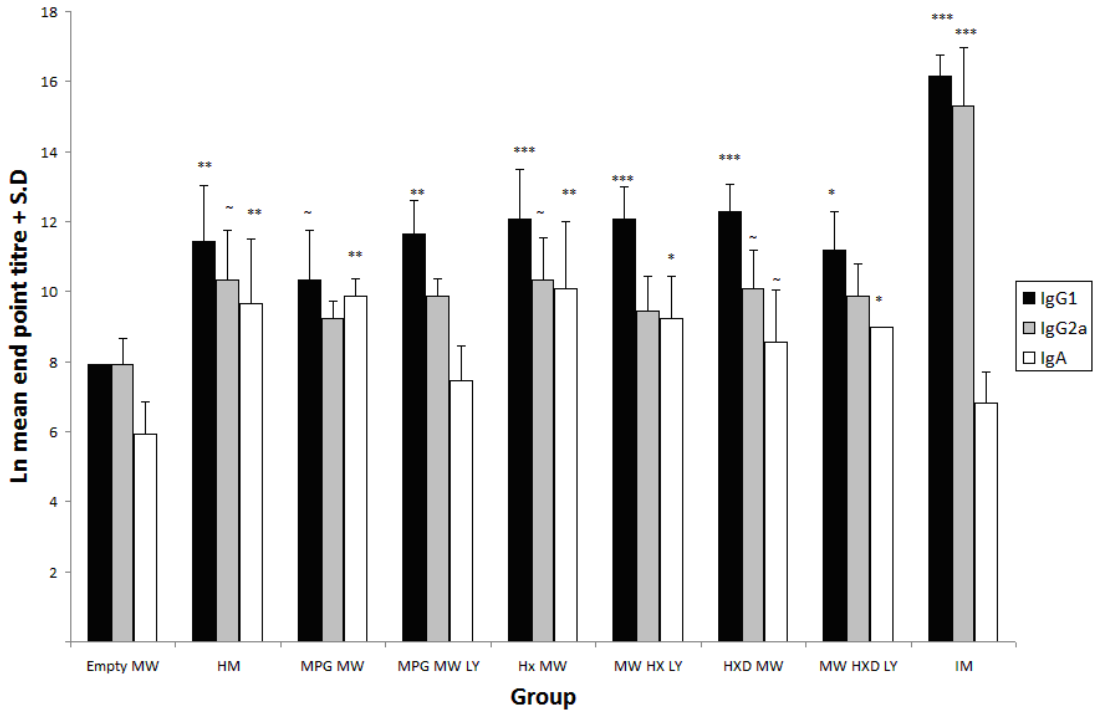


*Figure 21 – Ln mean end point titres ± S.D determined by ELISA for IgG1and IgG2a by serum sample and IgA by lung lavage for MW bilosomes formulated with novel alkylgluconamide surfactants. Surfactants used were, Hx = hexylgluconamide, Hxd = hexadecylgluconamide. FD = lyophilised, IM = intramuscular injection. p values ~ < 0.05, * < 0.01, ** < 0.001, *** < 0.0001 versus empty control.*

	IgG1			IgG2a			IgA			
	Ln titre	± S.D.	*p* vs. control	Ln titre	± S.D.	*p* vs. control	Ln titre	± S.D.	*p* vs. control	*p* vs. IM
Empty MW	7.90	0.00	NA	7.90	0.78	NA	5.92	0.92	NA	0.9758
HM	11.42	1.63	0.0005	10.32	1.43	0.0217	9.66	1.84	0.0009	0.0345
MPG MW	10.32	1.43	0.0343	9.22	0.49	0.5626	9.88	0.49	0.0004	0.0176
MPG MW LY	11.64	0.98	0.0002	9.88	0.49	0.1044	7.46	0.98	0.5702	0.9962
HX MW	12.08	1.43	< 0.0001	10.32	1.20	0.0217	10.10	1.90	0.0002	0.0087
HX MW LY	12.08	0.92	< 0.0001	9.44	0.98	0.3599	9.22	1.20	0.0045	0.1171
HXD MW	12.30	0.78	< 0.0001	10.10	1.10	0.0492	8.56	1.47	0.0411	0.4723
HXD MW LY	11.20	1.10	0.0011	9.88	0.92	0.1044	9.00	0.00	0.0097	0.1997
IM	16.14	0.63	< 0.0001	15.32	1.65	< 0.0001	6.80	0.90	0.9758	NA

Table 12. – Summary of Ln mean end point titres ± S.D. and the relevant p values for MW formulations. Surfactants used were: MPG = 1-Monopalmitoyl glycerol, HX = hexylgluconamide, HXD = hexadecylgluconamide. LY = lyophilised, IM = intramuscular injection.

3.3.4 Potential enhancement of immunogenicity using an adjuvant

As a simplified, quicker, and more versatile method of formulation had now been developed, an attempt was made to enhance immunogenicity using squalene, a known adjuvant.

3.3.4.1 Effect of squalene incorporation on physical characteristics

The formulations were all of similar zeta potential, around -70mV, whilst variations in size were observed, ranging from approximately 200nm to 2000nm, although these were not found to be significant ($p > 0.05$) due to an extremely wide range of sizes present in the larger formulations, indicated by the standard deviation. The sizes and zeta potentials are summarised in Table 13.

Bilosomes formulated with HXD were found to have an EE of 41.5 ± 0.7%, whilst SQ2 = 47.1 ± 0.7% and SQ1 = 41.0 ± 1.1%. There was no significant difference between SQ1 and HXD bilosomes, and SQ2 had a significantly higher EE than both ($p = 0.0014$, $p = 0.0020$, respectively).

Group	Size (nm) ± S.D.	Zeta Potential (mV) ± S.D.
Empty	2022.4 ± 1599.5	-65.1 ± 9.0
Empty / squalene (5:4:1:1)	252.5 ± 24.7	-64.9 ± 6.9
HXD	303.4 ± 103.9	-73.0 ± 11.6
HXD / SQ2 (5:4:2:1)	1040.8 ± 1273.5	-74.6 ± 9.3
HXD / SQ1 (5:4:1:1)	224.1 ± 81.4	-76.4 ± 9.9

Table 13 – Summary of size and zeta potential for bilosomes formulated with varying amounts of squalene and antigen. For each group n = 3.

3.3.4.2 Effect of squalene incorporation on immunogenicity

All three of the oral formulations containing antigen (N/Cal) gave significant antibody titres for IgG1, IgG2a and IgA versus both of the empty bilosome formulations (except SQ2 vs. empty SQ2), with no significant difference between the empty formulations. Ln mean end point titres ± S.D. are summarised in Table 14. There were no significant differences between any of these three groups (Figure 22).

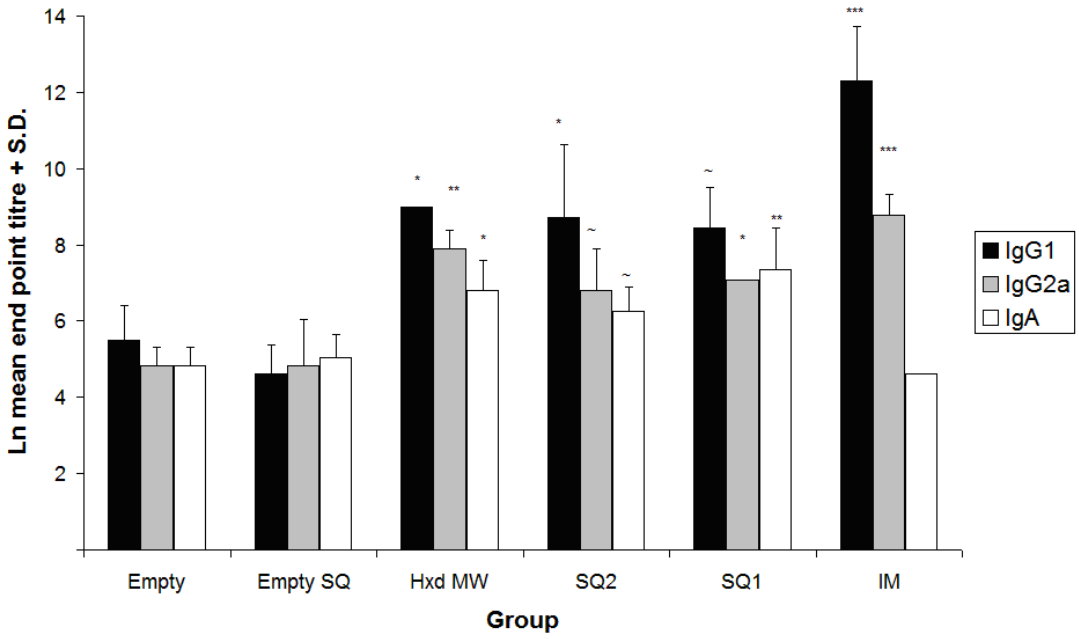


*Figure 22 – Ln mean end point titres ± S.D determined by ELISA for IgG1 and IgG2a by serum sample and IgA by lung lavage for squalene containing MW bilosomes. p values vs. empty bilosomes ~ < 0.05, * < 0.01, ** < 0.001, *** < 0.0001. SQ1 = 1M squalene, SQ2 = 2M squalene, IM = intramuscular injection.*

Ln mean end point titre ± S.D.

	IgG1			IgG2a			IgA			
Group	Titre	*p* empty	*p* empty SQ2		*p* empty	*p* empty SQ2		*p* empty	*p* empty SQ2	*p* IM
Hxd MW	9.00 ± 1.90	0.0011	0.0001	7.90 ± 1.10	0.0001	0.0001	6.80 ± 0.78	0.0013	0.004	0.0004
SQ2	8.72 ± 1.05	0.005	0.0003	6.80 ± 0.00	0.012	0.012	6.25 ± 0.63	0.041	0.111	0.014
SQ1	8.45 ± 1.42	0.011	0.0008	7.08 ± 0.55	0.004	0.004	7.35 ± 1.10	0.0001	0.0004	0.0001

Table 14 – Summary of Ln mean end point titres ± S.D. and the relevant p values for squalene-containing formulations. SQ2 = 2M squalene, Hxd MW, SQ1 = 1M squalene, Hxd MW.

3.4 Discussion

3.4.1 Synthesis of novel alkylgluconamides

The method described in section 2.1.2 has here been shown to produce four new alkylgluconamides in yields between 65-95%, similar to those previously formulated by Pilakowska-Pietras *et al* [170]. Lactone ring opening could be inferred from the absence of any bands in the IR spectra at 1740 cm^{-1} and both ^{1}H and ^{13}C NMR spectra for all four showed peaks in the expected regions based on the anticipated structures. Melting points appeared to decrease with increasing alkyl-chain length, with the exception of hexylgluconamide, which had a lower melting point than dodecylgluconamide. It was anticipated that melting point would increase linearly with alkyl-chain length; therefore the results here indicate that some other parameter is affecting the melting point. This may be due to certain chain-lengths providing greater stability in the solid phase, and any future work on these surfactants should attempt to address this issue.

In summary, the synthesis and characterisation of four novel alkylgluconamides has been described in a rapid and simple process from relatively inexpensive starting materials. Relatives of these compounds have been shown to have surfactant properties [170], and the intention was therefore to investigate the use of the novel compounds in bilosomes.

3.4.2 Bilosomes incorporating novel surfactants formed by the WB method

During testing of the alkylgluconamide surfactants in the standard HM method it was found that melting did not occur at a low enough temperature to prevent charring of the CHO and DCP components, and thus bilosomes incorporating these surfactants could not be formulated in this way. To

introduce energy into the system a water bath was used first to heat the lipid components with the buffer and bile salt before homogenisation, which was refined into a microwave method without homogenisation; a method which was substantially quicker, cleaner and simpler.

The bilosomes formed by the WB method were similar in size to those formed by the HM method in all but two cases, the hexyl- and octadecylgluconamide variants, which were significantly smaller, and all were in the expected size range compared to standard bilosome preparations [65, 66]. With the WB bilosomes there was no clear trend in EE, where dodecylgluconamide gave the lowest entrapment, at approximately 20%, and hexadecylgluconamide gave the highest, at ~ 44%. Those formulated with MPG had an EE of 37%, similar to the 35% obtained with those formulated by the HM method. As has been noted previously, changes in surfactant can lead to changes in EE, however, with varying alkyl chain lengths of 6, 12, 16, and 18-C one would expect a linear increase or decrease with respect to this, given equation 2 in section 1.5. No such trend was observed, which may be due to an unsuitable formulation method, as it was noted during the synthesis that incomplete mixing and dispersion of the constituents occurred. The formulations created by the HW method tended to be less well-dispersed and more granular than those formed by the HM method, which was a factor in the decision to switch to the MW method.

Zeta potentials in all cases were approximately -100mV, as has been previously found with the HM system [65], and it appears that the charge is mainly due to DCP, as there was no shift in the zeta measurements with a change in surfactant.

The animal study for the WB bilosomes showed significantly greater systemic (IgG1 and IgG2a) and mucosal (IgA) titres with the hexadecylgluconamide surfactant, with hexylgluconamide inducing significant IgA and IgG1 titres, and dodecylgluconamide inducing significant IgG2a and IgA titres. This therefore shows that bilosomes can be formulated with the novel surfactants, giving a similar result for hexadecylgluconamide as has been observed with the HM method and in previous work [66, 137]. However, the WB method does not simplify or improve the manufacturing process, or reduce the formulation time, and only achieves the aim of incorporating inexpensive in-house surfactants. It was therefore decided to move to a system, which would achieve these aims, whilst also allowing incorporation of the alkylgluconamide surfactants, which could not be used with the HM method.

3.4.3 Bilosomes formed by the MW method

Refinement and stream-lining of the formulation process were not achieved with the WB method; subsequently, the use of a microwave reactor, which allowed a high input of energy into a pressurised system in a short period of time, was investigated for bilosome formation. The incorporation of the novel surfactants was investigated by examining the physical characteristics, and two of the surfactants, both with and without lyophilisation, were tested in a mouse model versus the HM method. Those chosen were hexadecylgluconamide, which had the greatest overall titres in the WB study, and hexylgluconamide, which had failed to induce IgG2a, to see if significant titres could be achieved in all cases with the new system.

Those bilosomes formed by the MW method had a greater range of sizes, most likely due to the lack of homogenisation to reduce the polydispersity, and the hexadecyl- and octadecylgluconamide formulations were larger than the HM method, with the octadecylgluconamide also larger than the MPG, hexadecyl- and dodecylgluconamide variants. The range of sizes was within that expected from previous reports in the literature [141, 142, 146, 171], however, it could be of interest to examine the size of these bilosomes following homogenisation in future. The use of dynamic light scattering for these formulations may present a problem, as without homogenisation a range of sizes are likely to be present, and there may be more large particles which can skew the data leading to flawed results. Evidence of this can be seen in the larger standard deviations of the MW bilosome sizes, averaging approximately ±124nm, compared with the HM (averaging ±58nm) and WB (averaging ±32nm) bilosomes. Confirmation of these sizes was therefore done using FFEM, which showed spherical bilosomes without lipid sheets or crystalline structures, which were analysed using ImageJ. As FFEM uses a frozen sample, which is split along its length, the diameter of the observed structure may not represent the vesicles widest point. However, this method does give a good indication of the range of sizes present, and the mean sizes were not significantly different from those determined by DLS or previously reported sizes [65, 66]. Zeta potentials in all cases were approximately -100mV, as has been previously found with the HM system [65], and it appears that the charge is mainly due to DCP, as there was no shift in the zeta measurements with a change in surfactant. Bilosomes formed in this way showed a direct relationship between EE and carbon-chain length, with entrapment increasing from ~ 33% to 47%. A basis for this can possibly be

observed in Equation 2 (section 1.5), where it is shown that an increase in alkyl chain volume will lead to an increase in the critical packing parameter.

Significant antibody titres were induced in all cases for the MW method, which were the same as those seen with the HM method. The lyophilised variants were not as successful, with all three failing to induce significant IgG2a titres vs. the empty control group, although the lyophilised HX MW and HXD MW induced significant IgA titres vs. the empty control, group, but not the IM control.

Previous work using the bilosome with an influenza vaccine (A/Panama) has indicated that small HM bilosomes (~200nm) induced a Th1 biased response, with an absence of any IgG2a [137]. In further work it was discovered that this effect could be modulated by the size of the bilosome, with larger bilosomes (~1000nm, produced by a similar method to section 2.1.7) provoking a Th1 biased response as indicated by significantly higher levels of IgG2a [137]. A Th1 response has been shown to provide the greatest protection against influenza infection [155], a result which was confirmed in the Mann *et al.* study, where the larger bilosomes provided the greatest levels of protection in a ferret challenge model [137]. In light of these results it is apparent that the significant IgG2a titres observed with the MW method represent an improvement on the Mann method, although in previous studies using the HM method (adapted from the Mann method) these were also observed. This may be due to the difference in antigen (A/Panama vs. N/Caledonian), or the absence of the freeze-thaw cycle in the HM and MW methods. If size was the major determining factor then a significant skewing towards Th1 bias would be expected with lyophilised bilosomes, however,

this was not found to be the case, with the lyophilised versions of the HX and HXD MW bilosomes showing no significant difference in IgG2a to their wet counterparts. Further study using a range of antigens and freeze-thaw techniques will therefore be necessary to determine the effect these have on the skewing of the T_H2/T_H1 bias, and it would also be desirable to test a range of formulations in an animal viral challenge model.

Microwave reactors have previously been used to formulate surfactants which may be used in vesicles [172], and in the production of vaccines [173]. As far as I am aware, it has not, however, previously been used in the formulation of lipid vesicles. The process described here is therefore a truly novel method of vesicle formation, and as such, few direct comparisons exist. The effect of microwave energy on lipid vesicles has previously been examined, and it is accepted that this can lead to an increase in membrane permeability [174, 175]. As there is no exposure to microwave radiation after formulation, and given the low exposure time (1min), it is likely that this effect would be temporary. This could easily be determined in future work, by examining the relative release rates from HM and MW formulations of a fluorescent molecule such as carboxyfluorescein, which has previously been used in such studies [176, 177]. The increased permeability of the vesicle on exposure to microwave radiation may actually be beneficial in the formulation process, as it could reasonably be expected that this would work in both directions. This could mean that exposure of the formulation after the addition of antigen, at a certain power level, which would not damage the antigen, would allow an equilibration of antigen within and without the vesicle. If the volume of the vesicles within a formulation was 50%, then this could possibly allow an entrapment efficiency of 50%, assuming the vesicles

hardened after the removal of the radiation. It is possible that this could then be enhanced through lyophilisation, which produced an increase in entrapment from 30% to 50% in the HM bilosomes. Unfortunately, the entrapment efficiency of the lyophilised MW bilosomes was not determined here, an oversight which should be corrected in future work.

The MW method represents a significant improvement over the HM method, giving a reduction in manufacturing time from around 3h to approximately 35min, without the need for use of a homogeniser, and the possibility of contamination with metal particles and loss of volume this brings with it. Overall, the results are similar to those previously published [65, 66], and it is clear that the manufacturing process has been streamlined and improved, with no loss of *in-vivo* activity, whilst allowing incorporation of surfactants which were not possible with the previous HM method. Due to the capacity of the MW reactor used in this work the largest possible volume possible was 20ml, although 4 of these could be set up and run automatically. For a formulation to be viable at a commercial level it must be scalable to multi-litre levels; the next steps in the evolution of this process therefore lie in attempting to formulate the bilosomes on levels approaching these scales.

Immune responses following oral administration have previously been shown to be possible with liposomes and bilosomes, for a range of different antigens [61, 63, 141, 178, 179]. Work by Alves *et al.* (2008) has indicated that different degrees of immune response, from immunisation to oral tolerance, are induced depending on both the nature of the liposomes, and the breed of mouse [180]. This is of particular concern in work on the bilosome, as care must therefore be taken in comparing results generated in the BALB/c mice

used here with work by other groups, which may use different breeds. This also suggests that changing, or modifying, the lipid components may result in an alteration in the immune response. Such alterations have been studied by Channarong *et al.* (2010) for oral DNA vaccines, where liposomes consisting of a 5:4:1 molar ratio of phosphatidylcholine, cholesterol, and dicetyl phosphate were modified by surface coating with chitosan [181]. This resulted in greater internalization in the distal ileum (containing Peyer's patches, thought to be an essential target for mucosal immunization), and reduced the amount of damage to the DNA [181]. It should be noted, however, that while greater uptake was observed, no measurements of antibody titres were made, and this may not necessarily translate to enhanced immunogenicity. Despite this, these liposomes are exceptionally similar to the bilosome formulation incorporating sodium deoxycholate, and it could reasonably be expected that such a modification could give similar results. It would be useful to test this, and examine the effect upon the immune response, given work by Nakanishi *et al.* (1999), which determined that cationic liposomes induced a stronger cell-mediated immune response than anionic liposomes [182]. As the bilosome is strongly anionic, such modification could enhance the immune response already established with the MW bilosomes. An attempt to enhance the immune response would form the basis of the next stage of this work.

3.4.4 Effect of squalene incorporation on bilosome characteristics

The key aspect of any vaccine, regardless of delivery method, is induction of an antibody response, which will protect against infection by that disease. This is a major concern for vaccines such as sub-unit antigens, which, while safer than whole (live or killed) antigens, produce a weaker immune

response [36]. Throughout the work in this thesis the bilosome has induced significant antibody titres versus empty bilosome, or no treatment, control groups, however, these titres have consistently been lower than the IM control groups. While these responses have been shown to induce protection in an animal challenge [137], enhancement of them to comparable levels with IM injection would represent a significant step in the route to an oral influenza vaccine. This is particularly important for IgG2a, as a strong T_H1 response has been shown to correlate with enhanced protection against influenza infection [155]. Adjuvants can enhance immune response, and, while their use in influenza vaccines is limited, products, which utilise them exist. These include Inflexal V® (Berna Biotech), a liposome based adjuvant which incorporates viral constituents, and Fluad® (Novartis), which uses the oil-in-water emulsion MF59 [183, 184]. Inflexal V® has been shown to have higher efficacy than the non-adjuvanted trivalent vaccine Fluvarix® (GSK) in children [185], and vaccines adjuvanted with MF59 give enhanced immunogenicity in the elderly [184, 186, 187]. Adjuvants therefore have the potential to safely enhance the immune response to an influenza vaccine, which provides the rationale for attempting to incorporate such activity into the bilosome.

With a simplified, stream-lined formulation process now established with the MW method, incorporation of an adjuvant, and enhancement of the immune response, was the next objective. Squalene is used as an adjuvant in MF59, an oil-in-water based adjuvant for parenteral use produced by Novartis [167-169], and it was therefore decided to test if this effect could be replicated in an oral formulation.

Size analysis of the vesicles in this study indicated that three of the formulations were of the expected size in comparison with HM, WB, and MW method bilosomes. These were the empty squalene containing MW bilosomes (empty SQ), MW bilosomes, and 1M squalene containing (SQ1) MW bilosome groups, which were between 200-300nm in diameter. Whilst the other 2 groups were much larger, (empty MW ~2000nm, 2M squalene containing MW bilosomes (SQ2) ~1000nm) there was a wide range of sizes present in these samples, and statistical analysis showed no significant difference between them and the smaller groups (empty SQ, MW, SQ1). These measurements were repeated to verify results, which were outside the expected range, however, two repeated measurements (n = 3) showed no statistical difference from the original results (data not shown). As discussed in section 3.4.3, without homogenisation there can be a wide range of particle sizes present, and aggregates are also more likely to form. When using dynamic light scattering, the intensity of the scattered light is proportional to the square of the size, and this can therefore lead to a skewing of the average value towards the larger sizes present [188]. It would have been useful in this case to have confirmation of bilosome size via FFEM, however, due to time constraints, and due to the lack of enhanced immune responses with squalene (discussed later), these were not performed. Bilosomes formulated with hexadecylgluconamide surfactant had an EE of approximately 41%, which was not enhanced by incorporation of the lower concentration of squalene (SQ1). The higher concentration (SQ2) gave a significantly greater EE of approximately 47%. The zeta potential values in this study were all around -70mV, lower than the values obtained with HM bilosomes, which were largely around -100mV. They are similar to the HXD MW bilosomes in section 3.3.3.1 however, and given that there was no significant difference

between the zeta potentials for the groups, the change in zeta potential is likely due to some difference conferred by the HXD surfactant.

The EE, size and zeta potential of vesicles is known to be highly variable [40, 43, 44, 141, 142, 146], however, the bilosomes containing squalene had values consistent with those produced both in previous sections and with different antigens [63, 66]. As far as I am aware, squalene has not been previously incorporated into a vesicular vaccine delivery system as an adjuvant, and there are therefore no relevant comparisons in the literature for the physical characteristics of these bilosomes.

Significant antibody titres vs. relative controls were induced for all three of the oral formulations for both systemic (IgG1 and IgG2a) and mucosal (IgA) antibodies, a result previously observed using the MW formulation method and the hexadecylgluconamide surfactant. Given that squalene has been used as an adjuvant to successfully enhance vaccine efficacy, for example as the oil-in-water emulsion MF59 [167-169], it was thought that its incorporation into the bilosome formulation could potentially lead to higher antibody titres. This was not found to be the case, with each of the three antibody titres tested for showing no significant difference between the squalene containing formulations and the formulation without squalene. In light of work by Shahiwala and Amiji (2008) this appears to be an interesting result, as they have previously shown that co-administration of squalene and ovalbumin produces significantly higher systemic and mucosal antibody titres than a solution of ovalbumin itself [189]. However, squalene is also used orally as a drug delivery carrier system [169], therefore it is possible that in their work it acted as a carrier system for the ovalbumin, and with one

already present in this study (the bilosome) the presence of squalene had no effect. This could be due to several issues, such as oral tolerance of squalene, the interaction of squalene with the bilosome and the mechanism by which the antigen is presented to APCs in the GALT.

Squalene was selected as a potential adjuvant in this work as it is used as a commercial adjuvant (as MF59) with IM influenza vaccines, and is known to be safe [190]. Despite being used in over 20 countries, and in vaccines against diseases such as HIV, HSV, and influenza, the mechanism of action is still not fully understood. It has been shown that antigen uptake by dendritic cells (DCs), following IM injection, is promoted by the use of MF59, which initially (3h post-immunisation) formed extra-cellular droplets. After 48h these were internalised by DC, identified by expression of DEC-205 and MHC class II molecules, with enhanced localisation of antigen in the DC organelles versus IM antigen alone [190-192]. MF59 therefore enhances uptake by APC, similarly to alum, however, it does not affect the bio-distribution and clearance of antigen from the injection site as alum does, and thus would seem to have no depot effect. After administration, MF59 has been shown to enhance the recruitment of mononuclear cells expressing CD11b and F4/80 surface markers, driven by chemokine receptor 2. After 2 days MF59 was found in cells expressing CD80 and CD86 costimulatory molecules, localised in the subcapsular sinus of draining lymph nodes [190-192]. This suggests that the mechanism for MF59 involves recruitment of mature macrophages which engulf the antigen, and, while not directly activating DCs, these macrophages migrate to the draining lymph nodes where differentiation into DCs occurs [190-192]. While squalene is therefore useful as a parenteral adjuvant, little information on how it affects oral

vaccines exists. The data developed in this study would suggest that its use in oral vaccines may not be worth pursuing, and the use of other adjuvants should be investigated. One such adjuvant is *Escherichia coli* heat labile enterotoxin, which has been shown to be an effective mucosal adjuvant, although it is highly unlikely to achieve regulatory approval as it is also a potent enterotoxin [193-195]. However, recent work by Norton *et al.* (2011) has developed a detoxified variant, and it is possible that this could provide an effective mucosal adjuvant for the bilosome [195].

Refinement of the formulation process has now developed the 3 ½ h, 3-step HM process into a 2-step, 45min MW process, which has greater versatility as it allows incorporation of surfactants other than MPG. This represents a significant improvement, and it is to be hoped that future work can build on this, possibly to develop a continuous batch processing protocol, which would enable formulation on industrial scales. It has, however, been shown that squalene confers no adjuvant effect when incorporated in the bilosome, and it will therefore be necessary to develop other means, such as chitosan coating (section 3.4.3), to produce an enhanced immune response. It is likely that an increased understanding of the biological processes, which regulate the uptake and processing of the bilosome, will facilitate future developments in such an area. This forms the basis of the next chapter.

Chapter 4 - Biological assessment of changes in formulation

4.1 Introduction

A number of formulation refinements have now been made to the bilosome, simplifying the process, reducing the formulation time, and producing a lyophilised powder, which can be stored for up to 9 months. With these adaptations made, the other main area of interest for the bilosome is how it provokes an immune response, i.e. the mechanism of action. Four key areas were identified for study:

(1) It was postulated that the use of a mildly alkaline buffer (pH 9.7) could potentially neutralise stomach acid, thereby enhancing the immune response. Therefore, how would altering the pH of the buffer affect this response?

(2) The effect of stomach acid on the bilosome may play a key part in regulating uptake, and the immune response. How does gastric fluid affect the bilosome *in vitro*, and can suppression of gastric acid *in vivo* help induce a stronger immune response?

(3) Uptake of particulate antigens occurs in the intestine. Where exactly does this occur, in the villi or Peyer's patches of the small intestine?

(4) The immune response is regulated by cytokines; therefore if an immune response is provoked in the lungs, one would expect to see an up-regulation or down-regulation of cytokines. Can this be quantified?

4.1.1 Aims

1: Examine the effect of a neutral buffer pH on the physical characteristics of the bilosome.

2: Assess whether there is a difference in immune response between bilosomes with buffer pH 9.7 and 7.6.

3: Establish a method for analysing antigen integrity *in vitro*, and examine how antigen is affected by incubation under a variety of simulated gastric conditions.

4: Examine the immune response to bilosomes with antigen, empty bilosomes, and IM injection in mice with suppressed gastric acid production.

5. Examine bilosome uptake in the intestine using fluorescent proteins, *in vitro* and *in vivo*, using epifluorescent and confocal microscopy.

6. Examine the relative expression levels of cytokines in mouse lungs after oral immunisation.

4.2 Materials and Methods

4.2.1 Vesicle preparation

4.2.1.1 Bilosomes used for analysis of buffer pH effect

Bilosomes were formulated as per the HM method (section 2.2.1.1), with 0.025M carbonate buffer pH 7.6 or pH 9.7 used.

4.2.1.2 Bilosomes used for analysis of gastric acid suppression and fluorescent uptake

Bilosomes were formulated as per the MW method (section 3.2.4).

4.2.1.3 Texas-Red labelled ovalbumin containing bilosomes

Bilosomes were formulated by the MW method, using 1ml 100mM sodium deoxycholate, 4ml 0.025M carbonate buffer pH 9.7 and 5ml 100μg/ml Texas-Red labelled ovalbumin in 0.025M carbonate buffer pH 9.7. The formulation (0.4ml) was suspended in 4ml 0.025M carbonate buffer pH 9.7 and centrifuged at 35,000rpm for 2h to remove any unentrapped antigen, and the pellet resuspended in 0.4ml of 0.025M carbonate buffer pH 9.7.

4.2.1.4 Large (~1000nm) Texas-Red labelled ovalbumin containing bilosomes

1-Monopalmitoyl glycerol (150μmol, Larodan AG, Sweden), cholesterol (Sigma-Aldrich UK) and dicetyl phosphate (Sigma-Aldrich UK) (5:4:1 molar ratio) were weighed and dissolved in a 100ml round-bottomed flask with 40ml chloroform (Sigma-Aldrich, UK), then the solvent removed under vacuum by rotary evaporation to produce a thin lipid film. This was then hydrated with 1ml 100mM bile salt dissolved in 0.025M carbonate buffer, pH 9.7 and 9ml Texas-Red labelled ovalbumin solution (500μg Texas-Red labelled ovalbumin in 9ml of 0.025M carbonate buffer pH 9.7) overnight in an orbital shaker at 37°C. The formulation was centrifuged at 35,000rpm for 2h

to remove any unentrapped antigen, and the pellet resuspended in 0.025M carbonate buffer pH 9.7.

4.2.2 Immunisation

4.2.2.1 Animals and schedule

In-house bred male BALB/c mice, 8-10 weeks old, housed in a fully climatised room were randomised and placed into groups of 5. All mice were starved, but allowed access to water, for 2 h pre-immunisation, with food and water available *ad libitum* between immunisations. Each oral dose consisted of 0.4ml bilosome, containing approximately 50μg N/Cal hemagglutinin, administered by intragastric gavage on days 1, 4, 14, 17, and retention of the total volume in the stomach could be inferred from the absence of any reflux or nasal discharge. The control group was administered 0.05ml 230μg/ml N/Cal in each of the hind legs on the same days by IM injection. Tail bleeds were collected in heparinised capillary tubes on days 7, 20, and 33 post-immunisation and centrifuged at 13,000rpm in 1.5ml microfuge tubes for 20min. Plasma was transferred into fresh 0.5 ml micro-centrifuge tubes (Fischer, UK), and stored at -20°C until IgG1/IgG2a levels were determined by enzyme linked immunosorbant assay (ELISA). The study was terminated on day 33. IgA levels were determined by ELISA of lung lavages obtained by perfusing the lungs post-mortem with 0.5ml 1x PBS.

4.2.2.2 Study details

A summary of the various experimental and control groups for each study is given in Table 15. All animals were treated as detailed in section 4.2.2.1.

Study	Buffer pH (HM method)	Gastric acid suppression (MW method)	Fluorescent uptake
Aims	(1) Compare immunogenicity of each formulation	(1) Compare immunogenicity, with and without suppression	(1) Examine uptake in the small and large intestine
Group number: 1	Bilosomes, pH 9.7 buffer	Bilosomes	MW bilosomes (small), Texas-red ova
2	Bilosomes, pH 7.6 buffer	Bilosomes, ranitidine pre-treatment	Chloroform bilosomes (large), Texas-red
3	control, no treatment	Empty bilosomes, ranitidine pre-treatment	-
4	-	Empty bilosomes	-
5	-	control, IM injection	-
6	-	control, IM injection, ranitidine pre-treatment	-

Table 15: Summary of groups for each animal study and the relevant sections detailing bilosome formulation for each. IM = intramuscular, HM = homogenisation method, MW = microwave method. All MW bilosomes were formulated using hexadecylgluconamide surfactant. See sections 2.1.1.1, 2.1.4, 2.1.6, and 2.1.7 for formulation details.

4.2.3 Vesicle characterisation

4.2.3.1 Estimation of size by dynamic light scattering

Particle size measurements were made as per section 2.2.4.1.

4.2.3.2 Zeta potential

Zeta potential measurements were made as per section 2.2.4.3

4.2.3.3 Entrapment for HM bilosomes by ninhydrin assay

Antigen entrapment efficiency for the HM bilosomes (buffer pH study) was determined as per section 2.2.4.4.

4.2.3.4 Entrapment for MW bilosomes by Lowry-Peterson assay

Antigen entrapment efficiency for the MW bilosomes (gastric acid suppression and fluorescent uptake) was determined as per section 3.2.6.4.

4.2.4 Effect of simulated gastric conditions on protein recovery from bilosomes

To assess entrapped protein stability in the presence of low pH (such as that encountered in the stomach), evaluation of bilosome integrity after incubation in model gastric fluids was performed.

4.2.4.1 Incubation of bilosomes under simulated gastric conditions

Bilosomes (0.5ml), either centrifuged to remove unentrapped protein or as formulated, were incubated in 2ml of model gastric solution. This was either distilled water, pH 1.2 HCl or simulated gastric fluid (SGF) consisting of pH 1.2 HCl, 0.2% (w/v) NaCl and 0.32% (w/v) pepsin (enzymatic activity 800-2500units/mg, Sigma-Aldrich Ltd., UK) [196]. The entrapped protein was liberated from the bilosome by incubating 0.5ml of this fluid for 1h with 1ml DMSO, followed by centrifuging at 13,000 rpm for 10min to separate the lipid components from the dissolved protein. Samples of this solution were then run on a 16% sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) gel to determine integrity.

4.2.4.2 SDS-PAGE

The glass plates were assembled in the gel rig (Mini-PROTEAN 2, BioRad Ltd, UK) and the separating gel prepared by combining 3.4ml dH_2O, 4ml 30% Acrylamide (29.2% (w/v) acrylamide, 0.8% (w/v) *N*,*N*′-Methylenebisacrylamide (Bis)), 2.5ml 1.5M Tris buffer pH8.8, 50µl 10% (w/v) ammonium persulphate, 100µl 10% (w/v) sodium dodecyl sulphate (SDS) and 10µl TEMED, and mixed well. The separating gel was added to the assembled apparatus, and overlaid with a layer of isopropanol, then incubated at 37°C for 45min-1h, or until set. Prior to the end of this incubation period the stacking gel was prepared by combining 3.05ml dH_2O, 650µl 30% Acrylamide (29.2% w/v acrylamide, 0.8% w/v *N*,*N*′-Methylenebisacrylamide (Bis)), 1.25ml 0.5M Tris buffer pH6.8, 25µl 10% (w/v) ammonium persulphate, 50µl solution 10% (w/v) SDS, 7µl TEMED and mixed well. The isopropanol layer was then poured off, and the gel washed gently with dH_2O so that no isopropanol remained, followed by addition of the stacking gel and careful insertion of the gel comb, ensuring no air bubbles were created.

Samples were prepared for loading by combining equal volumes of sample with sample buffer and boiled in a water bath for 3min, then carefully added to the gel alongside molecular markers (molecular weight 30-200 kilodaltons). Electrophoresis buffer (12g tris, 57.6g glycine, 2g SDS, 2L dH_2O, pH8.3) was added and the gel ran at 200V for 30min, making sure the dye front did not overrun the bottom, followed by transfer of the separating gel to a container with fixing solution of 37.2ml methanol, 55.8ml dH_2O and 7ml glacial acetic acid for 1h. The fixing solution was replaced with a solution of

40ml Coomassie blue and 10ml methanol, followed by visualization of the gel under UV light.

4.2.5 Histological analysis

4.2.5.1 Dehydration of tissue for wax embedding

At terminal sacrifice, sections of intestine (duodenum, lower ileum and colon) were removed, split in half longitudinally and wound round a cocktail stick with the internal face outward, then fixed in neutral buffered formalin (4%, v/v, formaldehyde, 0.025M sodium phosphate monobasic monohydrate, 0.034M sodium phosphate dibasic anhydrous (Sigma Aldrich, UK)). The samples were then incubated in 50% (v/v) ethanol for 24h, fresh 50% (v/v) ethanol for a further 24h, and then 70% (v/v) ethanol for 24h. Sections were then placed in labelled histocassettes (Fisher Scientific, UK) and inserted into a ThermoShandon Citadel 1000 tissue processor using the programme shown in Table 16.

4.2.5.2 Embedding samples in paraffin wax

Intestine samples were removed from the tissue processor and submerged in wax in an embedder machine (Leica Microsystems, Germany). Metal cassettes were semi-filled with molten wax, and then a sample slid from its cocktail stick with forceps (retaining the coiled conformation) into the centre of the cassette with the coil vertical. The body of the histocassette was placed on top of the metal cassette, then topped up with wax and removed to a cooling board. After the wax had hardened, the metal cassette was removed.

4.2.5.3 Sample mounting on slides

Wax embedded samples were prepared for sectioning by placing on ice for 20min before cutting. Using a microtome (Finesse 315 version), 20µm sections were initially cut (35°/80 mm microtome blades, Thermo Electron Corporation, UK) until the sample was completely visible. 6µm sections were cut, placed in a 50°C electrothermal paraffin section mounting bath and layered carefully onto labelled slides (26x76x1.0mm microscope slides, Thermo Electron Corporation, Cheshire, UK) and cover slips fixed in place using xylene substitute mountant (ThermoFisher, UK). Slides were viewed using a Nikon Eclipse E600 with a FITC filter and a Photometrics Coolsnap FX camera.

Step	Reagent	Time (min)
1	70% Ethanol	45
2	90% Ethanol	45
3	100% Ethanol	45
4	100% Ethanol	45
5	50:50 Ethanol : Histoclear	60
6	Histoclear	60
7	Histoclear	60
8	Paraffin Wax	60

Table 16: Tissue processing protocol for dehydration of intestinal sections for immersion in paraffin wax.

4.2.5.4 Excision and mounting of Peyer's Patch (PP) tissue

Bilosome uptake in PP was examined using sections of duodenum excised from rats, as location and excision in mice was found to be problematic. Juvenile male Sprague Dawley rats (6-8 weeks) bred in house were starved for 2h prior, sacrificed and approximately 5cm long sections of small intestine removed proximal to the stomach, washed with 1x PBS to remove any intestinal contents, and split longitudinally to examine for PP. Once located, incisions were made 1cm proximal to the PP and the tissue excised, then fixed with Vetbond (3M Ltd., UK) on a 6cm petri dish attached to a microscope slide. RPMI medium (5ml) (Sigma-Aldrich Ltd., UK) containing 300μl of small bilosomes with Texas-Red labelled ovalbumin (see section 2.1.6) was then immediately added to the dish and allowed to incubate for 30min at 37°C. The medium was then poured off, the tissue gently washed with fresh medium to remove any loosely bound material, and fresh medium added. Images were taken using a Leica SP5 confocal microscope at excitation 514nm, emission 520-577nm using a Leica HCX/Apo/L/UVH dipping lens at 20x magnification.

4.2.6 Real-Time Polymerase Chain Reaction (RT-PCR)

Cytokine levels in mice lungs after oral administration of bilosomes were examined using RT-PCR.

4.2.6.1 RNA extraction

Upon study termination (effect of squalene, 3.2.5), lungs were harvested from the IM, empty and standard bilosome groups, rinsed in PBS to remove any blood and stored in 5 times volume RNAlater (Invitrogen UK Ltd.) at 4°C for later extraction of RNA with an E.Z.N.A.™ total RNA isolation kit (Omega

Bio-tek Inc., USA). All reagents and equipment are from the EZNA kit unless otherwise stated. The tissue (100mg) was placed in a flat bottomed 2ml tube containing 40µl β-mercaptoethanol (Sigma-Aldrich Ltd., UK) and 1.96ml lysis buffer. The tissue was disrupted using three 1min cycles at 30 cycles per second in a TissueLyser beadmill (Qiagen Ltd., UK). The solution was loaded in 800µl aliquots to a QIAshredder (Qiagen Ltd., UK) and placed in a centrifuge at 16,000 g for 5min to ensure complete disruption, retaining the flow-through each time. This was then centrifuged at 5000 g for 10min to pellet any solid material, with the supernatant removed and added to 2ml of 70% ethanol and thoroughly mixed, followed by application to a HiBind® RNA spin column. After incubating at room temperature for 3min the column was centrifuged at 4500 rpm for 5min at 25°C, then the flow-through discarded and 1.5ml wash buffer 1 added, followed by centrifugation under the same conditions. An on-column DNA digest was performed at this point by applying a solution of 4µl RNase-free DNAse I and 176µl E.Z.N.A.™ DNase I digestion buffer and incubating the column at room temperature for 30min. The column was then placed in a fresh collection tube and 1.5ml of buffer 1 applied and allowed to incubate at room temperature for 5min followed by centrifugation at 4500rpm for 5min, discarding the flow-through. In the same tube, 3ml of wash buffer 2 (20%, diluted with absolute ethanol) was applied to the column, followed by centrifugation as in the previous step, then the flow-through discarded and 3ml of wash buffer 2 again added and centrifugation as before. The HiBind® matrix was dried by centrifugation at 4500 rpm for 5min, after which it was transferred to a fresh collection tube and 250µl of DEPC-treated water, preheated to 70°C, added. This was incubated at room temperature for 3min, then centrifuged at 4500 rpm for 3min to elute the RNA, followed by quantification of the ratio and

RNA concentration. RNA integrity and quality was assessed using an Experion™ system (Bio-Rad laboratories Inc., UK), and RNA concentration determined using a NanoDrop 2000 (Thermo-Scientific Ltd., UK).

4.2.6.2 cDNA synthesis

After extraction 3µg of RNA from each sample was pooled into a master RNA sample for each group. From the master sample, 1µg was used for cDNA synthesis, to which 0.5µl oligo dT (MWG Biotech AG, Germany) and 0.5µl 20mM dNTP (Invitrogen UK Ltd.) were added and the total volume adjusted to 14µl with DNAse- and RNAse-free water (Sigma-Aldrich UK). Samples were incubated in a Perkin Elmer 480 thermal cycler at 65°C for 5min, and then placed on ice for 1min. To each group 4µl 5x 1st strand buffer, 1µl 0.1M DTT (Invitrogen Ltd., UK), 1µl DNAse- and RNAse-free water and 1µl reverse transcriptase (Invitrogen Ltd., UK) (RT+) was added, with a control replacing the reverse transcriptase with 1µl of water (RT-) also prepared. These were incubated at 50°C for 1h, then 70°C for 15min and stored at -20°C until required.

4.2.6.3 RT-PCR

20µl of each sample was diluted to a total volume of 100µl with 5x 1st strand buffer and 1µl of this added in triplicate to the wells of a white 0.2ml 8-tube PCR strip with clear flat 8-cap strips (Abgene Ltd., UK). To the relevant wells were added either: 2µl beta-2-microglobulin (B2M) primer and 2µl B2M probe (Eurofins Genetic Ltd., Germany), 1µl tumour necrosis factor alpha (TNF-α) TaqMan gene expression assay primer, or 1µl IL-10 TaqMan gene expression assay primer (Applied Biosystems, UK). Either 10µl TaqMan gene expression master mix (Applied Biosystems, UK) and 5µl DNAse and

RNAse free water (B2M), or 10µl TaqMan gene expression master mix and 8µl DNAse and RNAse free water (TNF-α, IL-10) was added to the relevant wells. These were placed in a Chromo4 RT-PCR (Bio-Rad laboratories Inc., UK) and heated for: 1) 95°C for 10min, 2) 95°C for 15s, 3) 60°C for 1min, 4) fluorescence read for FAM dye (excitation 492nm, emission 517nm) and the process repeated from step 2 40 times. Upon completion the data was analysed using Opticon Monitor 3 software (Bio-Rad laboratories Inc., UK) to determine the relevant C(t) values, with further analysis using REST 2009 software (Qiagen Ltd., UK). Confirmation of only one reaction product was given by running the reaction products on an agarose gel (section 2.7.4).

4.2.6.4 Agarose gel electrophoresis

Gels were made by dissolving 1g Agarose (Sigma-Aldrich Ltd., UK) in 50ml 1x TBE buffer (90mM Tris, 90mM Boric acid, 2mM EDTA, all Sigma-Aldrich Ltd., UK) and heating in a microwave. This was allowed to cool slightly and 2µl ethidium bromide (Sigma-Aldrich Ltd., UK) added. The solution was added to a MiniQ gel rig (Bioscience Services, UK) and allowed to set before removal of metal plates and the comb. 1x TBE buffer was added to cover the gel and 5µl of size markers (Invitrogen Ltd., UK) were placed in the first well, then 15µl of sample mixed with 2µl loading buffer (Invitrogen Ltd., UK) added to the relevant wells. The gels were run at 45V for 1h, followed by visualisation under UV light.

4.2.7 Statistical Analysis

Statistical analysis was performed either using a two-tailed unpaired t-test where applicable, or one-way ANOVA with *post-hoc* analysis by Tukey test

where significant differences were indicated. All analysis was performed at the 95% confidence level with Minitab v15.

4.3 Results

4.3.1 Effect of buffer pH

It was postulated that the mildly alkaline buffer (pH 9.7) used in the bilosome formulation could affect immunogenicity by neutralisation of gastric acid. To test this theory, comparison was made of the immunogenicity of formulations containing buffer pH 9.7 and buffer pH 7.6.

4.3.1.1 Effect of buffer pH on bilosome physical characteristics

There was no significant difference in size or entrapment efficiency for the two formulations rehydrated in either pH7.6 or 9.7, both being approximately 200nm with 30% antigen entrapment.

4.3.1.2 Immunogenicity of standard pH 9.7 buffer bilosomes versus neutral pH 7.6 bilosomes

In both cases significant antibody titres (vs. no treatment) were observed for IgG1 (Ln mean end point titre ± S.D., pH 9.7 = 11.42 ± 0.49, pH 7.6 = 9.66 ± 1.25), and IgG2a (pH 9.7 = 9.00 ±1.55, pH 7.6 = 9.22 ± 1.81). Only the pH 9.7 buffer induced significant IgA titres (6.15 ± 0.98), with no significant difference between the two buffers ($p > 0.05$) (Figure 23).

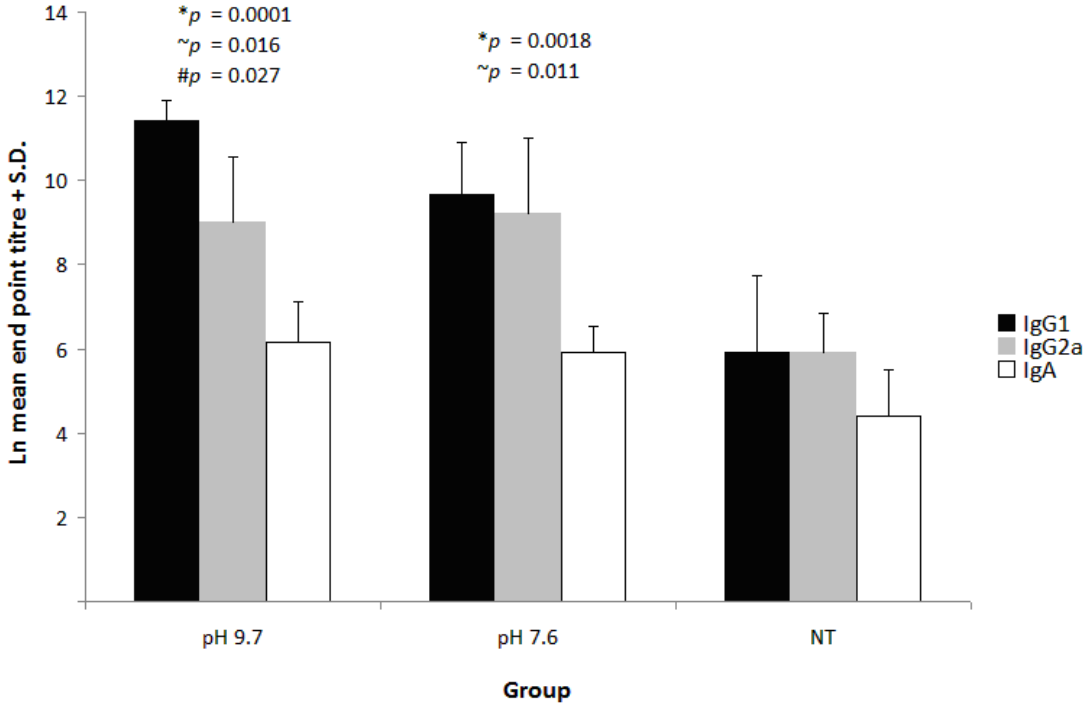


*Figure 23 – Ln mean end point titres ± S.D determined by ELISA for IgG1 and IgG2a by serum sample and IgA by lung lavage for bilosomes with varying buffer pH. * indicates p value for IgG1 versus no treatment control (significantly higher), ~ indicates p value for IgG2a versus no treatment control (significantly higher), # indicates p value for IgA versus no treatment control (significantly higher).*

4.3.2 In vitro and in vivo analysis of the effect of gastric acid on the bilosome

With section 4.3.1.2 showing that there was no significant difference in immunogenicity between the two buffers, neutralisation of stomach acid by the alkaline buffer was ruled out. However, stomach acid was still likely to be a factor in the mechanism by which the bilosome induced immunogenicity. This was analysed both *in vitro*, by assessment of antigen integrity after incubation of bilosomes with simulated gastric fluid, and *in vivo*, by suppression of gastric acid production prior to oral dosing.

4.3.2.1 SDS-PAGE gel assessment of antigen integrity

In order to assess the potential effects of stomach acid on the bilosome, SDS-PAGE was used to examine antigen integrity after exposure to simulated gastric fluid (SGF). After evaluating several methods of removing entrapped protein from the formulation including water, tween and acetone incubations and sonication, it was determined that incubation for 1h in DMSO liberated the highest amounts of ovalbumin from the bilosomes, and this was therefore used as a disruptant in all experiments. Ovalbumin bands (molecular weight of 43000 daltons) were clearly visible after incubation with either water or HCl pH 1.2 for up to 2h, however, when using SGF only a pepsin band is visible indicating an absence of intact ovalbumin in the sample (Figure 24).

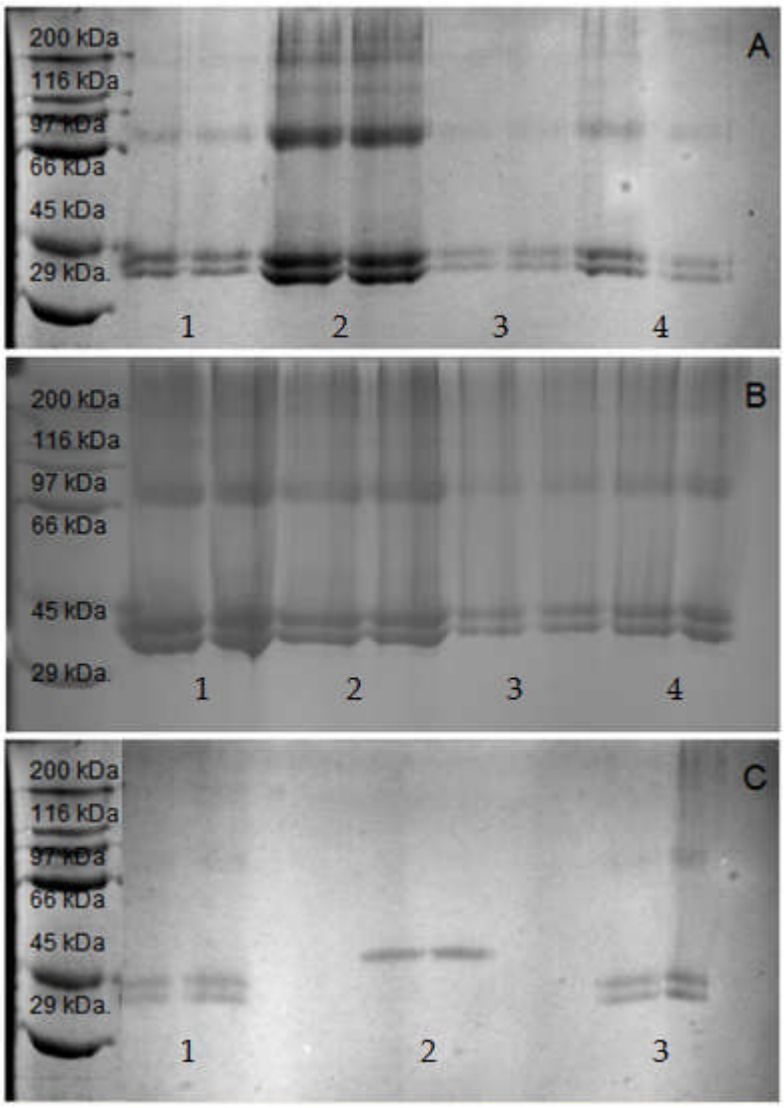


Figure 24 - SDS-PAGE gel of bilosomes loaded with 1mg/ml ovalbumin; (A) either centrifuged to remove unentrapped ovalbumin (track 3, 4) or as formulated (track 1, 2) incubated in either water (track 1, 3) or HCL pH 1.2 (track 2, 4) for 1h, (B) either centrifuged to remove unentrapped ovalbumin (3, 4) or as formulated (1, 2) incubated in HCL pH 1.2 for either 1h (1, 3) or 2h (2, 4), (C) centrifuged to remove unentrapped ovalbumin incubated in either 1) water, 2) HCL pH 1.2 with 0.2% (w/v) NaCl and 0.32% (w/v) pepsin or 3) HCl pH 1.2 for 1h All bilosome solutions were disrupted after acid incubation by incubation for 1h with DMSO.

4.3.2.2 Effect of gastric acid suppression on bilosome generated immune response

After examination of antigen integrity *in vitro*, showing potential degradation of the antigen, it was anticipated that suppressing stomach acid *in vivo* could lead to increased immunogenicity. This was achieved by pre-treatment of the animals with ranitidine hydrochloride for 24h prior to oral dosing. In this study no difference was found between those groups with and without ranitidine. Systemic antibody titres were significantly elevated versus the empty control groups, though only in one case for IgG2a, but significantly lower than the IM groups, as has previously been found. IgA titres were significantly higher than both IM controls and the empty control without ranitidine. Values were: **IgG1** Ln mean end point ± S.D **bilosome** = 9.22 ± 1.20 (p = 0.0008 vs. empty/ranitidine, p = 0.0048 vs. empty), **bilosome/ranitidine** = 9.66 ± 0.60 (p = 0.0001 vs. empty/ranitidine, p = 0.0008 vs. empty). **IgG2a** Ln mean end point ± S.D titre **bilosome** 7.24 ± 1.25, p = 0.034. **IgA** Ln mean end point ± S.D titres **bilosome** = 8.45 ± 0.63 (p = 0.045 vs. empty, p = 0.001 vs. IM and p = 0.0025 vs. IM/ranitidine), **bilosome/ranitidine** = 8.34 ± 0.98 (p = 0.048 vs. empty, p = 0.0008 vs. IM and p = 0.0023 vs. IM/ranitidine) (Figure 25).

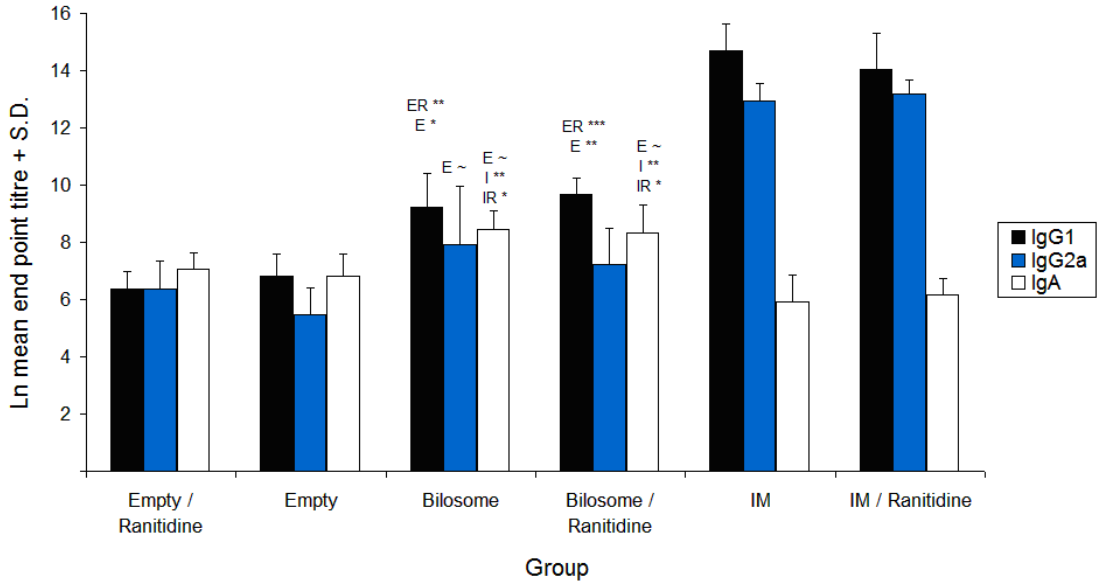


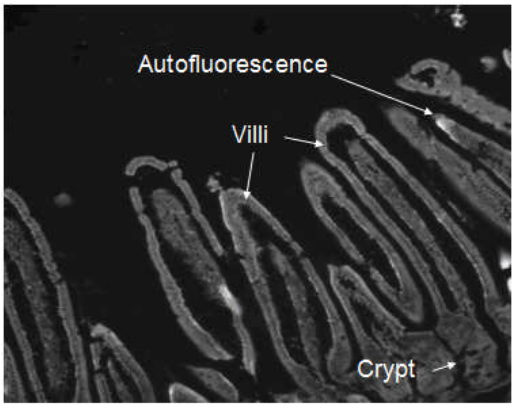
*Figure 25 – Ln mean end point titres ± S.D determined by ELISA for IgG1and IgG2a by serum sample and IgA by lung lavage for bilosomes administered both with and without pre-treatment with ranitidine. Significantly higher titres are indicated by p values: ~ < 0.05, * < 0.01, ** < 0.001, *** < 0.0001, ER = vs. empty/ranitidine, E = vs. empty, I = vs. IM, IR = vs. IM / Ranitidine, IM = intramuscular injection.*

4.3.3 Uptake of bilosomes in the intestine

After examination of the effects of buffer pH and gastric pH, showing no correlation with immunogenicity, further assessment of the mechanism of action of the bilosome was required. The next stage of the digestive tract which could affect immunogenicity is the intestine, and determination of how uptake occurs here is therefore of interest. This was examined by oral dosing of animals with bilosomes containing fluorescent antigen (Texas-Red labelled ovalbumin), followed by post-mortem histology of the intestine.

4.3.3.1 Fluorescent uptake in the intestine after oral dosing

Comparison of intestinal sections from animals dosed with fluorescent ovalbumin entrapped bilosomes, and dosed with empty bilosomes, showed that the majority of uptake was occurring in the small intestine, with little or no difference between large intestinal sections. Bright areas could be observed in small intestinal sections from 1 to 5h post-immunisation, with a slight reduction towards 24h. Uptake appeared to be mainly villous, with little ovalbumin observed in crypts or closer to the submucosae. There was no apparent difference between small bilosomes and large bilosomes in the location of the ovalbumin in the small intestine (Figure 26-29).

A 

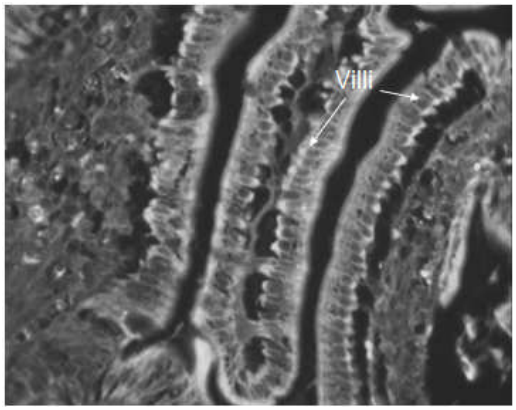
B 

Figure 26 – Background fluorescence visible in sections of the small intestine removed and embedded in wax at time points after administration of empty bilosomes at (A) 10x magnification, (B) 40x magnification. Slides were viewed using a Nikon Eclipse E600 with a FITC filter and a Photometrics Coolsnap FX camera.

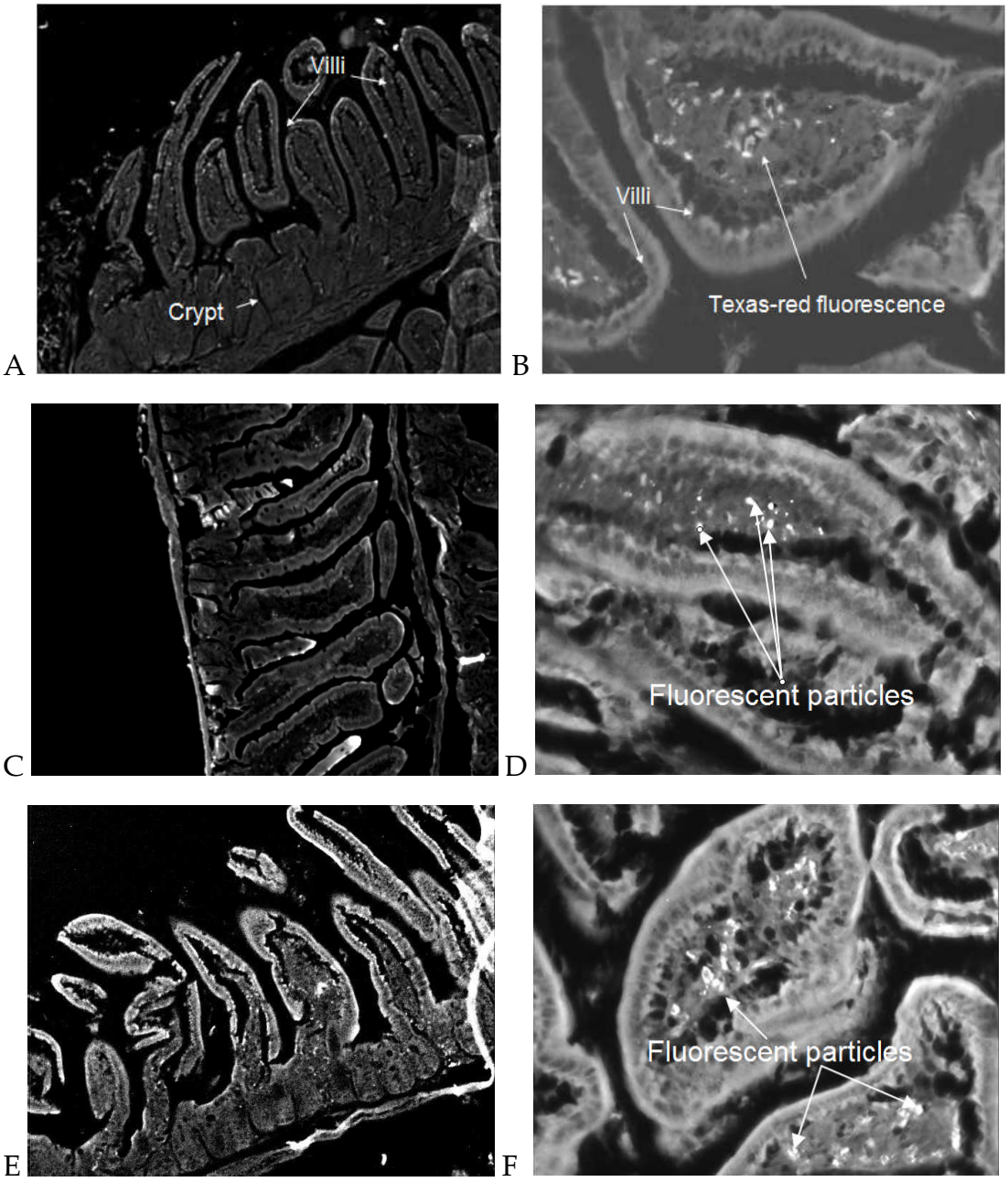


Figure 27 – Fluorescence (bright spots) visible in sections of the small intestine removed and embedded in wax at time points after administration of 200nm bilosomes loaded with Texas-Red labelled ovalbumin. A) 1h, 10x magnification, B) 1h, 40x magnification, C) 3h, 10x magnification, D) 3h, 40x magnification, E) 5h, 10x magnification, F) 5h, 40x magnification. Slides were viewed using a Nikon Eclipse E600 with a FITC filter and a Photometrics Coolsnap FX camera.

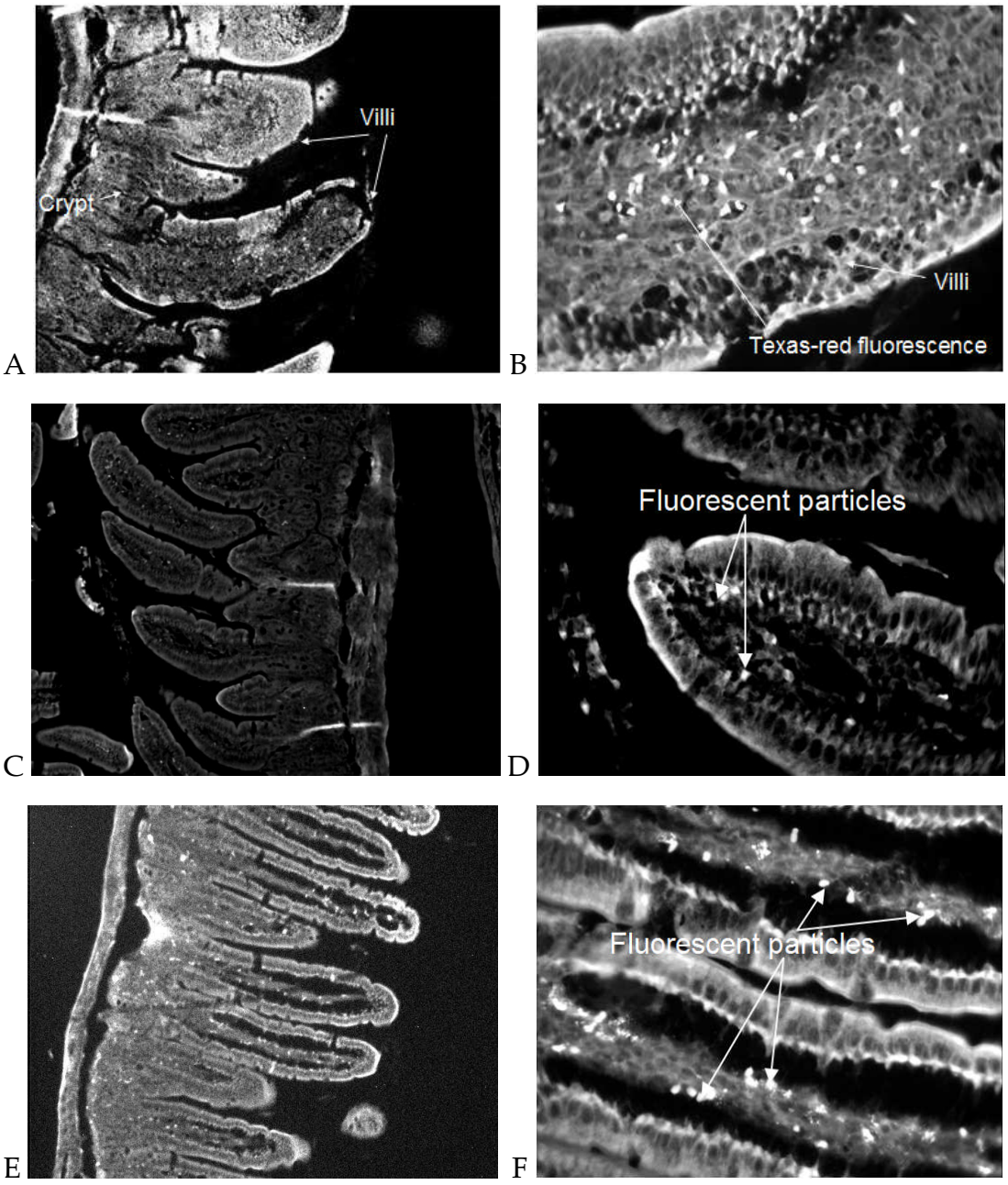


Figure 28 – Fluorescence (bright spots) visible in sections of the small intestine removed and embedded in wax at time points after administration of 1000nm bilosomes loaded with Texas-Red labelled ovalbumin. A) 1h, 10x magnification, B) 1h, 40x magnification, C) 3h, 10x magnification, D) 3h, 40x magnification, E) 5h, 10x magnification, F) 5h, 40x magnification. Slides were viewed using a Nikon Eclipse E600 with a FITC filter and a Photometrics Coolsnap FX camera.

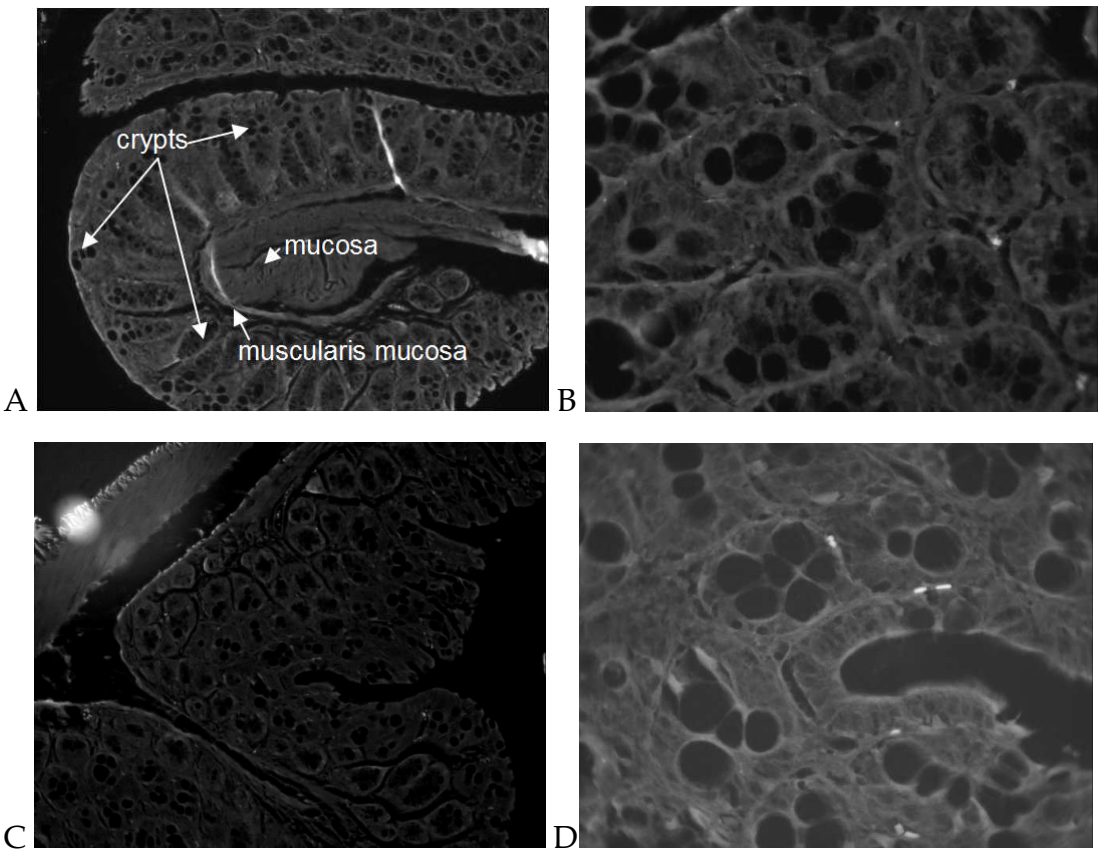


Figure 29 – Fluorescence visible in sections of the large intestine removed and embedded in wax at time points after administration of bilosomes loaded with Texas-Red labelled ovalbumin. A) 200nm bilosomes, 3h, 10x magnification, B) 200nm bilosomes, 3h, 40x magnification, C) 1000nm bilosomes, 3h, 10x magnification, D) 1000nm bilosomes, 3h, 40x magnification. Slides were viewed using a Nikon Eclipse E600 with a FITC filter and a Photometrics Coolsnap FX camera.

4.3.3.2 Uptake of Texas-red labelled ovalbumin in excised PP.

Due to difficulties in visually identifying and excising PP tissue in mice, it was decided to use Sprague-Dawley rats to facilitate this process. After incubation of small intestinal tissue from rats with Texas-red ovalbumin loaded bilosomes, fluorescence was mainly observed in the PP areas, with little or background levels in non-PP areas (Figures 30, 31). Movement down the z-axis showed the spread of fluorescence in a pattern indicative of localisation on the surface of a dome-type structure. Minimal fluorescence was observed in control areas incubated with empty bilosomes (Figure 32).

→ descending through z-axis left-to-right →

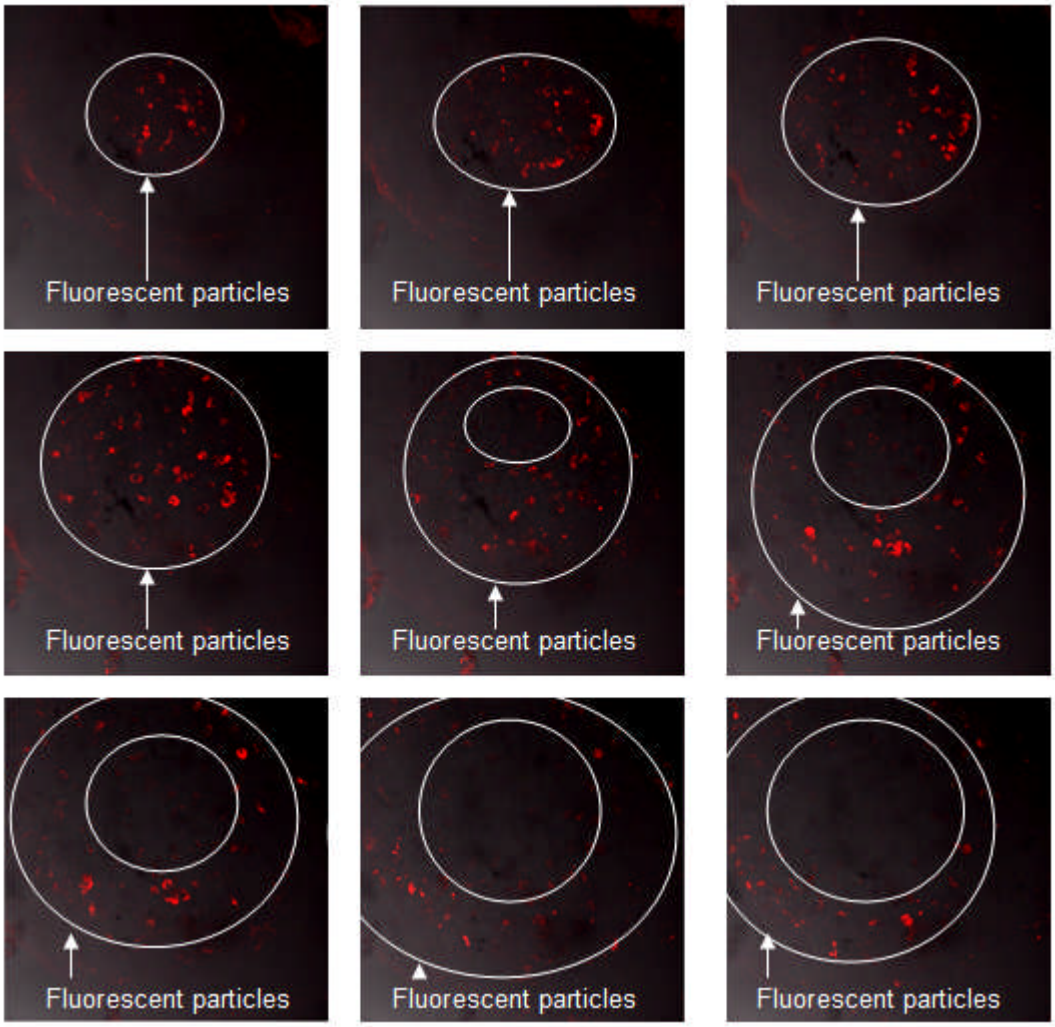


Figure 30 – Combined bright field / fluorescent images taken in the vertical axis of the dome of a PP area of rat intestine, incubated with Texas-red ovalbumin loaded bilosomes, moving down z axis in 5µm steps (left to right). Images were taken using a Leica SP5 confocal microscope at excitation 514nm, emission 520-577nm using a Leica HCX/Apo/L/UVH dipping lens at 20x magnification.

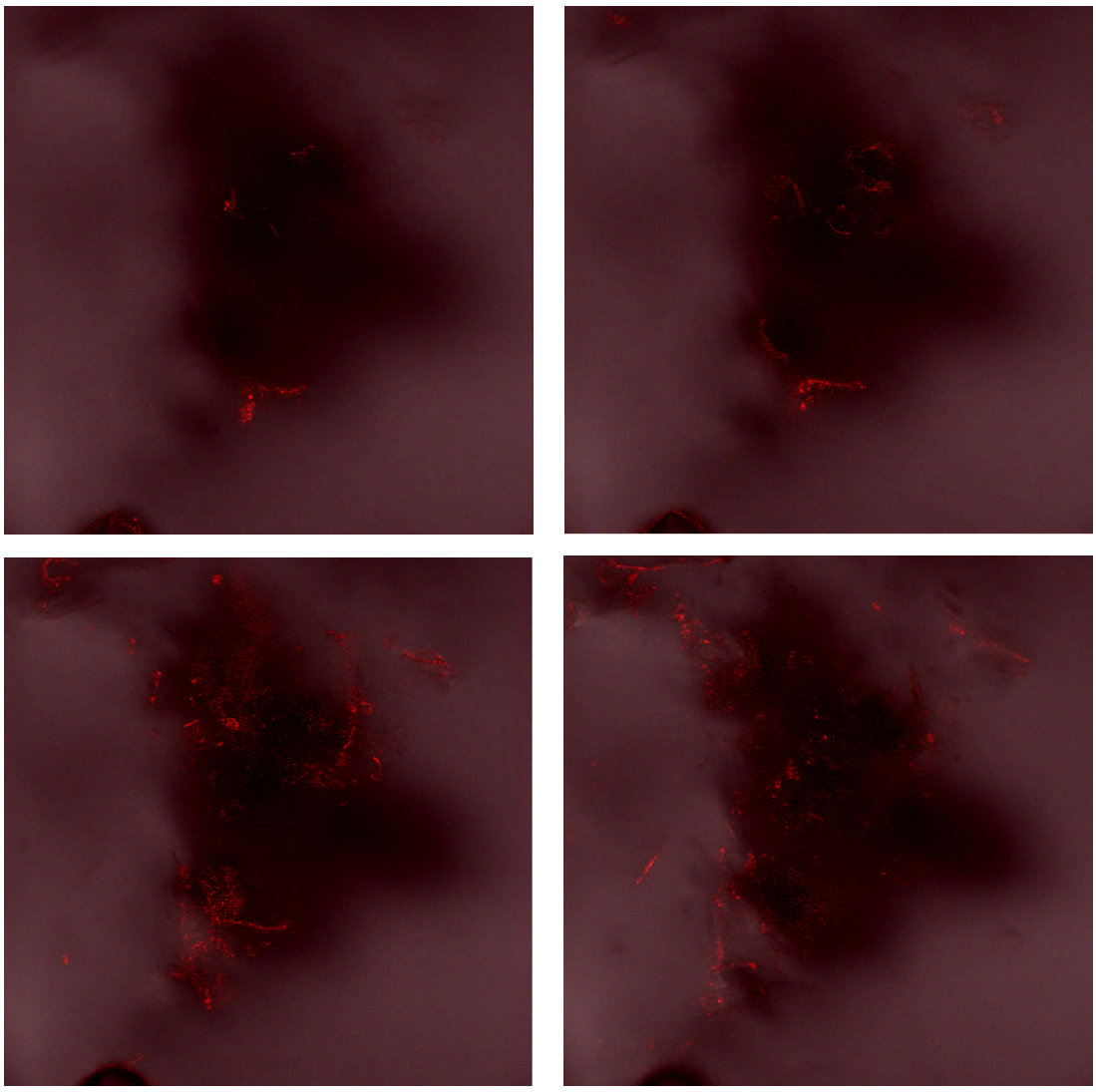


Fig 31 – Combined bright field / fluorescent images of non-PP area of rat intestine incubated with Texas-red ovalbumin loaded bilosomes, moving down z axis in 5µm steps. Little to no fluorescence was observed. Images were taken using a Leica SP5 confocal microscope at excitation 514nm, emission 520-577nm using a Leica HCX/Apo/L/UVH dipping lens at 20x magnification.

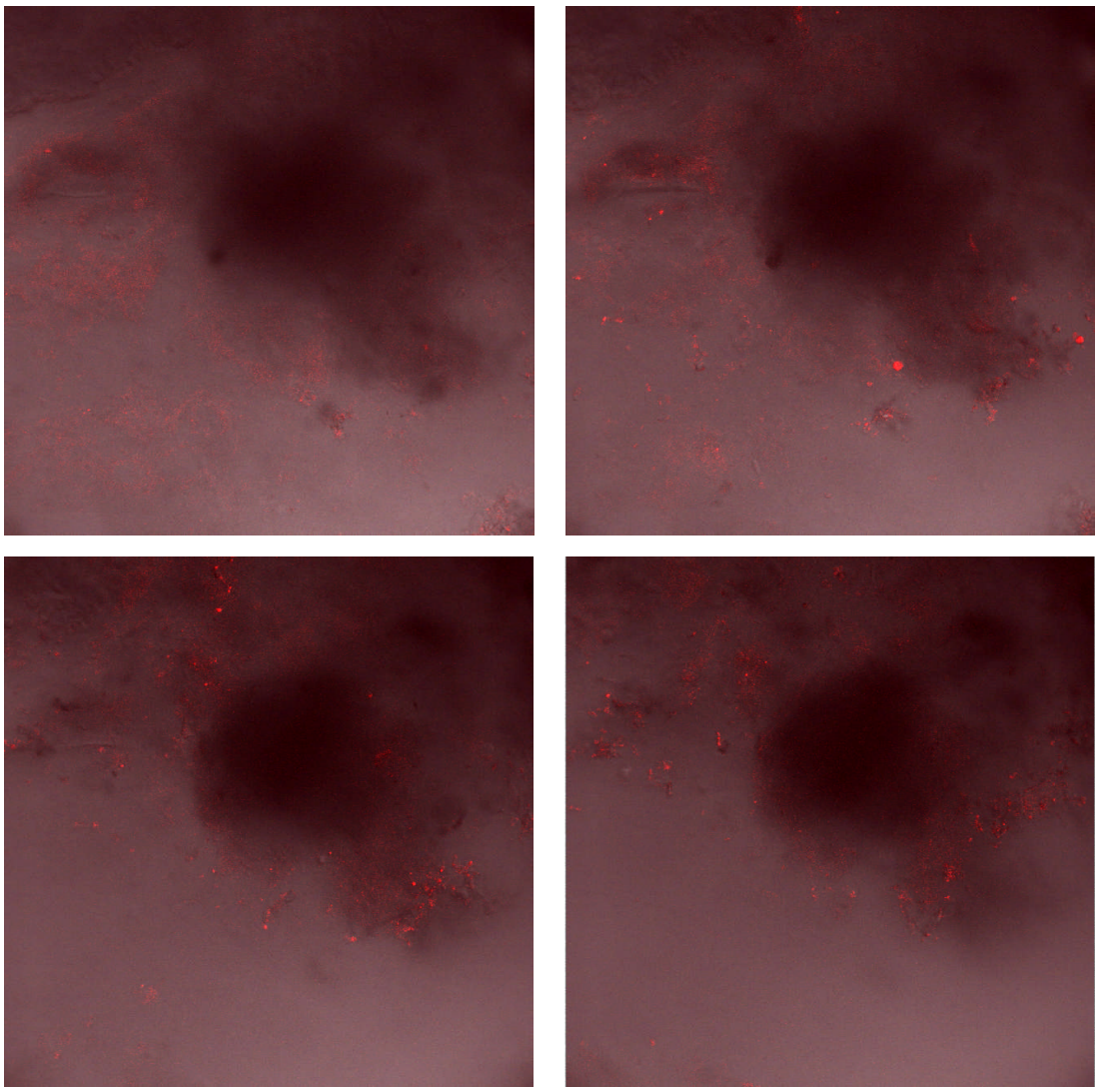


Fig 32 – Combined bright field / fluorescent images of PP area of rat intestine incubated with empty bilosomes, moving down z axis in 5µm steps. Little to no fluorescence was observed. Images were taken using a Leica SP5 confocal microscope at excitation 514nm, emission 520-577nm using a Leica HCX/Apo/L/UVH dipping lens at 20x magnification.

4.3.4 Analysis of lung cytokines by RT-PCR after oral immunisation

After examining uptake in the intestine, the next site evaluated was the lungs, as it was thought that immunisation could provoke some change in the levels of cytokines here.

Extraction of RNA from lung tissue was found to be of good quality, with a mean concentration of 137.7 ± 31.1ng/μl and a mean RNA quality indicator (RQI) of 7.1 ± 0.3 (Fig. 33). Analysis of the relative levels of expression of TNF-α in mouse lungs could not be performed due to the presence of genomic DNA, indicated by a second band in the agarose gel results. It was not possible to remove this despite multiple treatments with DNAse. Analysis of the relative expression levels of Il-10 in the lungs indicated no significant differences between empty (p = 0.164 vs. IM, 0.100 vs. bilosome), IM injection (p = 0.437 vs. bilosome), or bilosome groups.

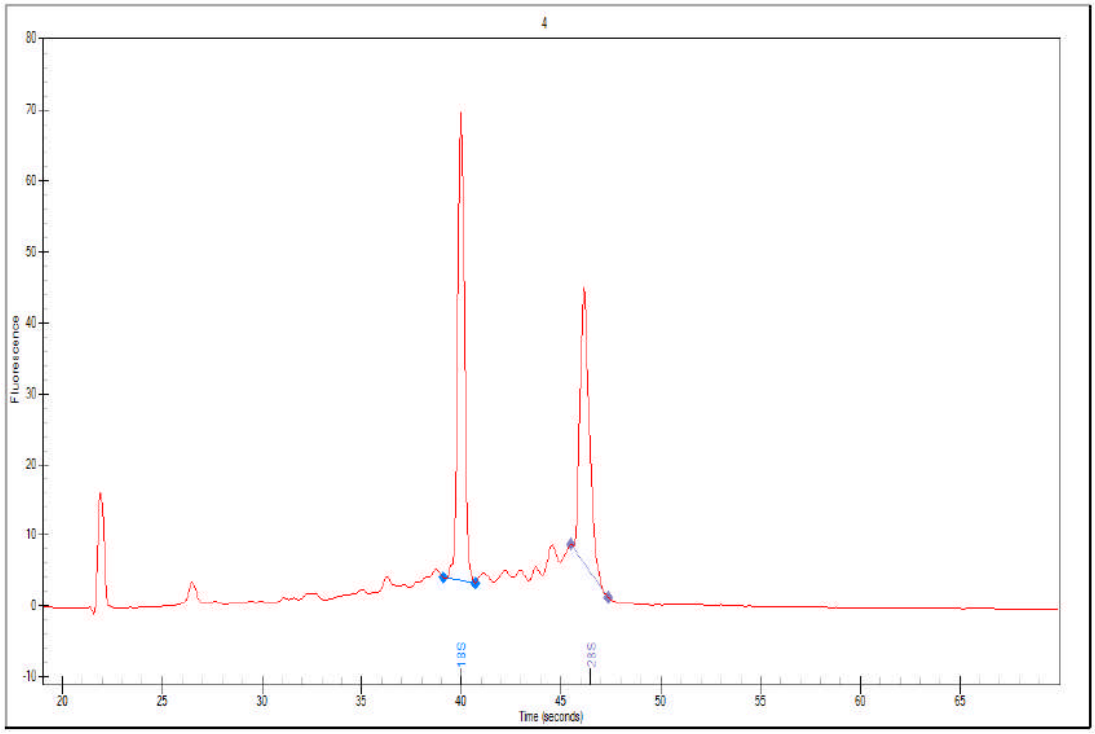


Fig. 33 – Total RNA recovered from mouse lungs after extraction, processed through an Experion chip. 1st major peak = alignment marker, 2nd major peak = 18S RNA, 3rd major peak = 28S RNA. RQI = 7.8, indicating good quality for PCR amplification.

4.4 Discussion

4.4.1 Effect of buffer pH

It has previously been reported that gastric pH is significantly lower in mice (3.0 fed, 4.0 fasted) than in man [197]. It was therefore postulated that the mildly alkaline buffer pH used in the bilosome formulation, and the use of a volume, which is close to the capacity of a mouse stomach, could potentially lead to neutralisation of stomach acid. To examine whether buffer pH would alter the efficacy of the bilosome two formulations (HM) were tested, the standard pH 9.7 and a lower, neutral pH 7.

There was no difference in either bilosome size or entrapment efficiency with either pH 9.7 or pH 7.6 buffer, and both were consistent with previous work [66]. Entrapment efficiency and vesicle size are known to be highly variable depending on the formulation methods, antigens and lipids used [40, 43, 44, 141, 142, 146], thus, with the same constituents in both formulations, and varying only the buffer pH, it would be expected that size and entrapment would not vary significantly.

There was no significant difference in the immune responses between the different pH formulations. This indicated that there was no significant effect from the pH of the buffer, and it was therefore decided that the effect of gastric acid upon the bilosome itself, and the immune response *in vivo*, would be the next factors examined.

4.4.2 Effect of gastric acid on bilosome integrity in vitro

The harsh conditions of the GI tract represent a significant barrier to an oral vaccine, as protein antigens are likely to be quickly degraded before uptake

can occur in the intestine. This is the reason that such carrier systems as liposomes, and the bilosome, were developed, and it is therefore important to assess the effect that such conditions have on the bilosome, and the entrapped antigen. This was examined *in vitro,* using SDS-PAGE to assess antigen integrity after acid and SGF incubations. This indicated that, although stable in pH 1.2 HCl, the addition of pepsin and NaCl to this to create SGF resulted in a complete loss of any ovalbumin bands. As previous work with the bilosome has indicated that an immune response is generated [66, 137], this result is therefore somewhat surprising. Previous work by Hermida *et al*. (2009) has shown that liposomes containing cholesterol are relatively stable at pH 2, and maintain their internal pH above 4.5 for over an hour. This correlates with the data here, where ovalbumin was detected in bilosomes incubated for 1h at pH 1.2, but in the Hermida study pepsin was not used and no comparison can be made under the SGF conditions. SDS-PAGE has previously been used by Tiwari *et al.* (2009) to evaluate the integrity of antigen after encapsulation [198], and over a period of time in storage, however, no examination after simulated digestion was made. Such a study was performed by Mohanraj *et al.* (2010), using SGF at pH 2, which indicated that less than 5% of the entrapped protein (insulin) was released after 1h [199]. This study was performed using a modified Bradford assay, and such a method of protein analysis for future digestion studies is therefore to be considered. However, it is the integrity of the protein entrapped within the bilosome, which was the main concern of this work, and protein release studies may not be ideal for this. Given the indication that entrapped protein is completely degraded after 1h in SGF, and the obvious disparity with the immune response previously observed in chapters 2 and 3, the design of the experiment may therefore be questionable.

In any future work, consideration of the physical characteristics of the bilosome should also be made, as this was not performed here, and may yield useful information. An attempt to optimise the process for neutralization and disruption of the bilosome samples would also be useful, as this is the most likely source of any error.

4.4.3 The effect of gastric acid suppression on the immune response to the bilosome.

It has been shown previously [61, 63, 66, 137] that bilosomes with antigen can effectively produce both systemic and mucosal immune responses, however the systemic responses are generally weaker than those observed with IM injection. One possible reason for this could be degradation of the antigen in the stomach, therefore, if stomach acid is suppressed, an enhanced immune response may be possible. This was examined *in vivo* by administering ranitidine hydrochloride, a gastric acid suppressant, prior to oral dosing with bilosomes.

Animals were allowed access to water containing 5mg/ml ranitidine hydrochloride for 24h prior to immunisation, and upon completion of the study significant antibody titres were induced for both antigen loaded bilosome groups for both IgG1 and IgA versus the empty controls. For IgG2a only the non-pre-treated group induced any significant titres.

This data indicates that there was no significant effect on the immune response with stomach acid suppression, which contrasts with the data from the digestion experiments, which seemed to suggest that the entrapped protein, whilst stable at pH 1.2, would be degraded by gastric fluid containing pepsin. Gastric emptying times for liquids in mice and humans

are roughly similar [200, 201], with around 75% processed to the intestine after 15-30min, and the pepsin concentration in a murine stomach is likely to be higher than that used for the SGF in this study [202]. These factors are therefore unlikely to impact on the design of future experiments; however, other differences in the mouse model compared with humans may be highly significant. The apparently contradictory findings in the *in vitro* and *in vivo* studies highlight some of these issues, as it is possible that they can be explained by the difference in murine gastric fluid pH, around pH 3.5 [197], compared with the human SGF used, around pH 1.2. In addition, the relatively low volume of gastric fluid present, compared with the oral dose of 0.4ml, could also play a part in this effect. The suitability of an animal model for medical research is an issue, which will always be of great significance, and it appears that, for this particular study, a murine model may be unsuitable. Any research following on from this should therefore consider another model, such as ferrets, which are known to have more similar gastric conditions to humans [203].

4.4.4 Fluorescent particle uptake

While oral vaccination has been shown to be possible by induction of significant antibody titres (as in Chapters 2 and 3), one of the most contentious issues surrounding it is exactly how it is occurring. There is still some debate as to whether microparticle uptake is mainly villous [134, 204, 205] or if the M cells of Peyer's patches play a significant role [149-151], though it is clear that particle size and characteristics are key regulators of this [134, 179, 206]. As the bilosome has been shown to consistently induce a significant immune response, with variations in the Th1/Th2 bias depending on the size range used [137], it was decided that some investigation of the

mechanism of uptake was required. Subsequently, the uptake of both small (~200nm) and large (~1000nm) bilosomes *in vivo* was examined using Texas-red labelled ovalbumin as a fluorescent marker. As was expected, the majority of absorption occurred in the small intestine for both sizes, mainly in the villi, with minimal fluorescence observed in the large intestine. Areas of intense fluorescence were visible for both large and small bilosomes in the small intestinal sections, although no difference was noted in their location. This may be due to the effects of churning in the stomach, which could potentially act to reduce the size of the larger bilosomes. Future work may be needed to address this issue, using DLS to establish the size of the vesicles after incubation with SGF and simulated churning. Another explanation is that after uptake, the bilosomes are being degraded to release the entrapped ovalbumin, giving the more diffuse fluorescence observed in these images. Previous work involving solid fluorescent microparticles [134, 179] has produced well-defined bright spots which correspond to the size of particle used. The bilosomes used in this work, however, are fluid and lipid based, and as such will be subject to the body's natural mechanisms for dealing with such particles, which could explain the diffuse appearance of the fluorescence. While this work does indicate the presence of Texas-red in the villi of the intestine, it does not provide information on whether this is still associated with the ovalbumin or bilosome. This could be provided by using fluorescent labelled lipids in the formulation process, or using radiolabelling with both the lipids and protein, as studied by Henriksen-Lacey *et al.* (2010) [207].

Uptake of nano- and micro-particle scale vesicles in the intestine will be determined by how they interact with its various cells and lymphoid tissues.

The predominant cell type is enterocytes, which are part of a cell mono-layer which, along with the mucus secreted by goblet cells, provides a barrier against entry into the body. Lymphoid tissues are less prevalent in the intestine, and are often localised into structures such as the Peyer's patch, which sits below the intestinal epithelium, and contains M cells, which are specialised in sampling particulate matter [208]. The uptake of particles in the intestine has been the subject of a substantial amount of research, using methods such as tissue histology, *in vitro* studies on ligated intestinal loops, and cell culture models using the human Caco-2 cell line [179]. Despite this, there is still much debate about whether this is primarily via enterocyte sampling through the microvilli [134, 204, 205], or via the M cells of the Peyer's patch [149-151]. This is in part due to the range of particles used to study the effects, which affect the results depending on size, hydrophobicity, and constituents [208]. In order to understand the mechanism of action of a vaccine delivery system, such as the bilosome, it is therefore important that studies on their uptake are individually tailored in order to obtain pertinent results. The results obtained from examining uptake of the bilosome have indicated that uptake appears to occur through the microvilli, with sampling by enterocytes the most likely process for this. No apparent difference in uptake was observed between small and large bilosomes, although in future work it would be desirable to quantify this in a larger study. This would be facilitated by using a fluorescent marker in the blue range of the spectrum, which would be simpler to pick out from autofluorescence, and the use of tissue staining. Studying uptake in a model human system using Caco-2 cells should also be a priority [208], as one can never be certain that results in an animal model will translate to humans. Such a model would also facilitate the study of M cells, which was the next *in vitro* experiment.

As uptake in the small intestine has been shown to occur in the microvilli, as predicted by Doyle McCullough *et al*. (2007) [134], Hodges *et al*. (1995) [204], and Smyth *et al*. (2008) [135], further investigation into whether uptake also occurred in PP regions was necessary. Targeting of these regions has been shown to enhance the T_H2 response [154], and, with the strong IgG1 response consistently observed with the bilosome, study of uptake in PP was deemed appropriate. To simplify this it was decided to use Sprague Dawley rats, as identification and excision of PP areas proved problematic in BALB/c mice. The images obtained showed that in the PP itself a large amount of fluorescence could be observed when compared to the non-PP areas, with the fluorescence outlining a dome-shaped structure. The fluorescence appeared to be localised on the surface of the dome, with an increasing blank area observed in the centre as the camera descended through the z axis. It should be noted the results obtained here were limited in their scope by time considerations, and as such represent only very basic preliminary findings. The system used here appears to indicate a significant amount of uptake of the bilosome in the PP, which has previously been identified with microparticles by Buda *et al.* (2005) [149], Soni *et al.* (2006) [150], and Wang *et al.* (2010) [151]. However, in future work it would be useful to use a live cell stain to attempt to delineate the cell boundaries of the underlying tissue, as a major problem with this model was the relative absence of visual markers for identifying structures. It would also be interesting to compare this system with a cell culture model which expresses M cells, such as that described by des Rieux *et al.* (2007) [208].

As previously stated, the physical characteristics of microparticles play a significant part in how they are absorbed, with hydrophobic, negatively charged particles (as per the bilosome) shown to be better transported by PP

[209]. Given the data generated here, as well as that in the literature, it therefore seems to be the case that uptake of the bilosome occurs both in the epithelial villi and the PP, which should be considered in future work should any surface modification or targeting be necessary.

4.4.5 Analysis of lung cytokines by RT-PCR after oral immunisation

In this study many problems were encountered, including finding an effective method of RNA extraction from the mouse lungs, and finding primers, which would work with this RNA. The initial design of the study called for an examination of (1) IFN-α, IL-1, and TNF-α, pro-inflammatory cytokines which can indicate the degree of viral replication [210], (2) IL-4, IL-5, IL-6, and IL-10, cytokines which can indicate the degree of T_H2 response [211], and (3) IFN-γ, which can indicate a T_H1 response [212]. However, given the problems encountered in RNA extraction and primer design, time was limited, and it was decided to use off the shelf Taqman primers from Applied Biosystems and Eurofins. Due to expense considerations, this was initially limited to one housekeeping gene (B2M) and two cytokines (TNF-α and IL-10), with the intention of broadening the scope of the investigation should the system prove successful. It was found, however, that the TNF-α primer gave a genomic DNA band on the agarose gels, which could lead to a misquantification of the RNA. Despite repeated attempts to remove this, by treatment with DNAse, it was not possible to eliminate the DNA contamination, and it was decided that it must be excluded from the study.

After earlier attempts at RNA extraction using a Trizol protocol and various miniprep kits, it was found that RNA quality and yields were highest with the Midi kit from VWR, and the RNA used in this study had an average RQI of 7.1. IL-10 was chosen for the study due to evidence of high levels of this

cytokine being involved in enhanced susceptibility to influenza and post-influenza infection [213, 214]. In this study, however, there was found to be no difference in the relative expression levels of IL-10 for empty, IM injection or bilosome groups, indicating that it is unlikely to play a part in the immune response to an oral vaccine.

Despite the wide-spread use of RT-PCR for genetic analysis, little work has been done on cytokine levels after influenza infection in mouse lungs. There are therefore few relevant comparisons, and the majority of studies have used PCR for analysis of the viral load following immunisation, rather than cytokine levels [215-217]. The inability to examine more than one cytokine in this study, due to time and expense constraints, as well as the TNF-α primer genomic DNA contamination, mean that there is a lack of data from which to draw significant conclusions. However, the groundwork has been laid for follow-up studies to use the methods described here, which have been shown to work for the mouse lung model, to examine a range of other cytokines. This can provide information on the differences in the immune response to the antigen between IM injection and oral delivery, and should be regarded as an important step in the development of the bilosome system.

5. Thesis summary

In recent years, one of the issues most concerning to the global medical community has been the potential for a highly virulent strain of influenza to cause a pandemic outbreak, such as was feared for "bird flu", H5N1, and caused much of the hysteria surrounding "swine flu", H1N1. Though H1N1 proved to be no more deadly than seasonal influenza epidemics, the memory of the 1918 "Spanish flu" pandemic, which caused between 20-100 million deaths [218], is a constant reminder that we must remain prepared for such a pandemic occurring. One of the pillars of any defence is anti-viral drugs such as Tamiflu®, which have been stockpiled around the globe in vast quantities. While effective in reducing symptoms and transmission of the disease, these drugs are expensive to produce, and their use may result in viral resistance spreading, rendering them useless in the future. Additionally, they do not provide a memory response to influenza, and therefore offer no future protection against the disease. The other approach is vaccination, which provides protection and a memory response, but offers no protection against heterogeneous strains and can take a significant amount of time to synthesise from the first outbreak. Vaccination is usually by IM injection, which can be time-consuming, causes a risk of needle-stick injuries and can reduce patient compliance, which may be critical in limiting the spread of a pandemic. For these reasons there is a drive to produce new vaccines and methods of delivery, capable of inducting cross-protection as well as oral administration, regarded as the most acceptable route to the majority of patients. This forms the rationale behind this thesis, where the intention was to improve and streamline the formulation of an oral vaccine delivery system, the bilosome, whilst examining its mechanism of action.

Oral delivery of the bilosome has been shown to be capable of inducing an immune response with influenza antigens [66, 137], as well as tetanus toxoid [63] and cholera antigens [141]. The focus of this thesis was therefore to refine the formulation method into one which would deliver the best immunological response possible, whilst offering the simplest, quickest and most flexible preparation in order to create a product which could easily be scaled-up by a pharmaceutical company.

Initial testing examined the effect of the bile salt concentration on the bilosomes characteristics and immunological effect, as all previous work has used a concentration of 10mM [61, 63, 66, 137]. Bile salt disrupts liposomes in the GI tract, however, when incorporated into the formulation process, it acts to harden them against future exposure, thereby reducing antigen leakage [61]. Determining the optimum concentration for this was therefore necessary, as it was possible that entrapment efficiency or immune response could be affected by it. It was found that the greatest antibody titres were generated by the 10mM formulation, and this was subsequently used in all other studies.

Simplifying the formulation process for the bilosome was a major aim of this thesis, and the first attempt to achieve this was via reducing the 3-step HM process to a 1-step manufacture. This proved unsuccessful, with no significant antibody titres observed with the 1-step process. The other modification examined in this study was lyophilisation, which proved more successful, giving mucosal and systemic antibody titres which were not significantly different from the unlyophilised 3-step HM bilosomes. While lyophilisation adds an extra step to the manufacturing process, and requires

drying overnight, it is relatively simple, and offers significant advantages. These include improved stability, increased entrapment, and the removal of the need for cold-chain storage, which would be highly relevant in less developed countries. The next study examined these properties, comparing the physical characteristics of lyophilised bilosomes, stored at room temperature, with a wet formulation, stored at 4°C, for a period of 9 months. While the entrapment efficiency of the two formulations was not significantly different at the end of the study, the size of the lyophilised bilosomes was found to decrease from around 1000nm to 300nm. It was thought that this was due to the hygroscopic nature of the powder, which could have absorbed water, either via ineffective sealing of the storage vessel, or the air retained within the vessel. Despite this change, no effect on the immunogenicity of the formulation was observed, and both of the stored formulations induced systemic antibody titres, which were not significantly different from their fresh counterparts. The lack of an IgA response with the stored formulations is of concern, however, as the induction of such a response is one of the key advantages of mucosal immunisation. In future this should therefore be examined, and it is possible this could be restored by using cryoprotectants [158].

As the results of Chapter 2 indicated that the attempt to simplify the HM process was unsuccessful, streamlining of the formulation process for bilosomes formed the basis of Chapter 3. The other key areas for this chapter were the incorporation of novel surfactants, which could be produced in-house, and at a lower cost than MPG, and enhancement of the immune response. The novel surfactants proved unsuitable for use with the HM method, due to their high melting points, and a new process was therefore

designed. This involved heating the lipids, bile salt, and buffer in a water bath, followed by homogenisation, which resulted in bilosomes which were similar in size (~200nm) to the HM method, but had a range of entrapments from 20-44%. Testing these formulations in an animal model indicated that one of the surfactants, hexadecylgluconamide, induced significant systemic and mucosal antibody titres. Despite this, the formulations produced by this method were inconsistent, and large granules of undispersed lipid were often observed. As this method also required a similar amount of time to the HM method, it was decided that a new formulation process would be pursued. This resulted in the MW method, which reduced formulation time to between 30-45min, and considerably simplified the process. The bilosomes formed in this way had a similar appearance to the HM method, with none of the large granules observed with the WB method. They had a greater range of sizes, however, which could be expected given the lack of homogenisation, although the size range was within that expected with lipid vesicles. Two surfactants were tested in an animal model with the MW method, hexyl- and hexadecyl-gluconamide, and wet and lyophilised variants of each were tested against the respective HM and MW formulations using MPG. While the fresh variants all induced significant mucosal and systemic antibody titres, the lyophilised formulations failed to induce an IgG2a response. This is of concern as a T_H1 response has been shown to give the best protection against influenza infection [155], and an attempt to enhance the immune response would form the basis of the next section. Despite this, the fresh MW formulations all induced antibody titres which were not significantly different to the HM method, or each other, and as the process is considerably simpler and quicker, it should form the next stage in the evolution of the formulation process.

Given that oral vaccination has consistently produced systemic antibody titres, which were significantly lower than those with IM injection, the use of an adjuvant to enhance these was proposed. The adjuvant selected was squalene, which is used with influenza vaccines in the commercially available MF59 (Novartis) [167-169]. This adjuvant has been shown to be effective in IM injections, and there is evidence that oral co-administration with an antigen produces an enhanced immune response [189]. Despite this, there was no apparent effect with squalene, and in order to enhance the immune response it will be necessary to test other adjuvants in future. This may, however, take the work on the bilosome outside of the remit of a medical devices EngD. A "medical device" can be defined as any instrument, apparatus, implement, machine, appliance, implant, *in vitro* reagent or calibrator, software, material or other similar or related article, intended by the manufacturer to be used, alone or in combination, for human beings for one or more of the specific purposes of…diagnosis, prevention, monitoring, treatment or alleviation of disease…" [219]. The bilosome, which acts as a carrier for the vaccine, can therefore be considered to be a medical device, as it has no apparent immunological or pharmaceutical effect on the body itself. A successful attempt to incorporate an adjuvant into the bilosome would therefore remove it from this definition, and make it a pharmaceutical treatment, as any such formulation would likely effect an immune response without any antigen.

With a formulation process, which was now significantly streamlined, and could remove the need for cold-chain storage, an examination of the mechanism of action of the bilosome was performed. The initial stages of this involved examination of the effects, which buffer pH and gastric pH had on

the immune response. While no difference in antibody titres was observed using different buffer pH, it is possible that it may be the relatively large volume (0.4ml) of the dose, compared to the volume of gastric acid in a mouse, has a significant effect. Before definitively concluding that buffer pH plays no part in the immune response, it will therefore be necessary in future to test this theory using a range of different volumes, buffer pHs, and antigen concentrations. The results of testing oral vaccination following gastric acid suppression showed no difference between the various antigen groups, and some of the same issues as in the buffer pH study may apply here. Other issues that may explain this include the higher gastric pH of mice (pH 3.5) [197], and it will be necessary in future to determine the exact effect of gastric acid suppression on stomach pH.

With examination of the effect of gastric pH showing no apparent bearing on immunogenicity, the uptake of the bilosome in the intestine was examined, using a fluorescent labelled protein (Texas-red ovalbumin). The main aims of this work were to assess any differences in uptake between small and large bilosomes, as these have been shown to induce different T_H1 responses [137], and whether uptake was mainly villous, or via PPs. No apparent difference in uptake was observed between small and large bilosomes, although the limitations of this study should be noted. There were difficulties in consistently identifying bilosome-based fluorescence using analysis software, and thus no quantification of uptake was performed. This could be improved by using a more identifiable fluorescent probe, counterstaining the tissue, or using radiolabelling to identify whether the antigen and the vesicle are co-located within a given structure [207]. Analysis of the uptake of the bilosome within PPs via confocal microscopy also proved challenging, with initial

problems in identifying and excising PP from mouse intestines. In order to allow excision and mounting of viable tissue, rats were therefore used, and the data generated with these shows uptake of protein within a domed structure, which is presumed to be a PP. This work also has severe limitations, as the lack of counterstaining made identification of anatomical landmarks extremely difficult, and while protein uptake was observed it is unknown whether this is still within the bilosome. These studies, however, do show uptake of the protein within the villi of the small intestine, most likely via enterocytes, and also in the PPs of rat intestines. While the results are open to interpretation, and provide only empirical data, they can be used for future work on the bilosomes mechanism of action, and thus form a useful starting point.

The final study for this thesis was on the expression levels of cytokines within mouse lungs, after oral and IM immunisation. This study could have provided a great amount of information, and a lot of time and money were consequently spent trying to achieve the goal. Despite this, several problems plagued this work, such as successfully extracting RNA from lung tissue, supply problems with the kit which was found to be best for this, primer design, and the amplification of genomic DNA with an off-the-shelf primer. The results with this study were therefore not what had been hoped for, with the only cytokine examined (IL-10) showing no change in expression in comparison to the housekeeping gene (B2M). In spite of this, the majority of the work in developing the assay has now been achieved, and it is conceivable that follow-up work would require only the design of appropriate primers to expand this initial work into a large-scale study.

With around 90% of infections involving the mucosal surfaces of the body [35], it is apparent that induction of mucosal immunity (mediated mainly by IgA) would provide a great advantage in combating these infections. In elderly patients this is particularly relevant, as there is a reduction in immune response with age [220, 221], and it is estimated that only between 10-30% of over 65s are afforded protection from influenza vaccination [222]. Therefore, they are placed at a disadvantage by enabling the virus to gain a foothold on mucosal membranes before any protective response occurs. Induction of an IgA response has other advantages; it can neutralise the influenza virus itself, in the absence of serum IgG [118, 223, 224], and it has been shown that, whilst conventional parenteral administration of vaccine mediated by IgG provides adequate protection against homologous viral infections, it has little efficacy against emerging variant strains [225]. In this situation, where there may be an absence of a direct vaccine for these variants, or the patient may not be immunised against them, protection can still be achieved through induction of an IgA response [223, 225, 226], an issue which is particularly relevant for high risk demographics. Despite the clear benefits of a mucosal IgA response, it remains necessary for any vaccine to induce virus neutralizing antibodies in serum in order to attain regulatory approval [227], something which has not been achieved in clinical studies [228-231]. Throughout this work systemic antibody titres were induced, which in all cases were significantly lower than those generated with IM injection. While these titres are likely to be capable of inducing a protective response, it will be necessary to determine this in future work, possibly by virus-neutralizing assays or *in vivo* challenges in an appropriate animal model. If protective responses do not occur then it will be imperative to improve the immune response to the antigen, initial work on which was

attempted in the work with squalene (section 4.1.7), although this was unsuccessful in this case.

The bilosome provides a proven delivery system that offers the potential to preclude the need for IM injection of influenza vaccines, improving patient compliance with the benefits of mucosal as well as systemic protection. Since lyophilised versions offer similar levels of IgG1 and IgA, this removes the need for cold-chain issues, thus improving storage and distribution and allowing vaccine programmes to operate in areas of the world lacking in medical infrastructure. Whilst this may be less relevant in terms of influenza, it would provide a great benefit with other antigens which can be used with bilosomes, such as those derived from cholera toxin [141], hepatitis B [64] and tetanus toxoid [63]. The work contained in this thesis has shown how the formulation process for the bilosome can be simplified from a 3-step process taking ~3h which is unlikely to be viable at larger commercial scales, to a simple, ~30-45 min process which can be easily automated and scaled-up. This produces a product, which incorporates less expensive surfactants than MPG, and, when lyophilised, can be stored for up to 9 months at room temperature whilst retaining its immunogenicity. This represents a significant improvement on the initial HM bilosome formulation used, and is likely to be far more attractive to industry, particularly if an appropriate adjuvant can be incorporated. Examination of the biological pathways by which the bilosome delivers the antigen has provided information on the protection afforded to the antigen in the stomach, and has shown apparent uptake of the bilosome in the small intestine, in both villi and PPs. This work has therefore provided a superior formulation to the HM method, and has

provided an initial, although limited, insight into the bilosomes behaviour *in vivo,* which can form the basis for future investigation of this activity.

6. References

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