Development of a Mucosal Vaccine Delivery System

By

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Dedication

I would like to dedicate this thesis to Libyan martyrs and heroes who sacrificed their lives to give Libyan people freedom and dignity.

To the memory of my nephew WESAM, a brave young man who gave his body and soul to defend his family and his country. Thank you for your love and care, may you rest in peace.

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Abstract

Vaccine delivery systems that target the mucosal immune system carry important advantages in terms of accessibility and acceptability. Lipid based nanoparticles with and without the incorporation of bile salts were formulated by novel preparation methods in order to provide suitable immunogen carriers for delivery to mucosal surfaces. The nanoparticles were physically characterised by determination of size, zeta potential, and morphological appearance using a variety of analytical tools. A new method for the analysis of the chemical lipid components by HPLC separation was implemented, which can be adopted for quality control in future. The adjuvant and delivery properties of the nanoparticles were evaluated with different immunogens incuding non-pathogenic peptides and immunogenic proteins originating from pathogenic organisms. In particular, the reproductive self-hormone gonadotrophin releasing hormone (GnRH) conjugated with different carrier proteins was used as an immunogen in *in vivo* studies. Various mucosal administration routes were compared against parenteral vaccination, including oral, nasal and vaginal. Systemic and local specific antibodies (IgG, IgG subclasses and IgA) were evaluated in body fluids obtained from immunised mice. The effect of immunisation was evaluated by assessment of reproductive hormone levels after immunisation. In addition, pathogen-derived immunogens were used to evaluate whether the lipid nanoparticles were suitable for vaccination via mucosal surfaces. The results showed that lipid-based nanoparticles enhanced antigen delivery via subcutaneous and nasal routes with nasal delivery proving to be successful in terms of producing systemic and mucosal antibodies. Whereas, vaginal and oral routes showed a very low immune responses when using GnRH conjugates. However, oral administration of

pathogen-derived antigens showed promising results. The possible reasons for the limited capability of orally delivered nanoparticles were investigated using LC/MS analysis and fluorescent multiphoton microscopy. The study suggests that lipid based nanoparticles possess adjuvant properties and can be potential candidate for enabling vaccine delivery via the nasal route.

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Book Chapters

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Table of Contents

List of Abbreviations

AFM	Atomic force microscopy
APC	Antigen-presenting cell
BALT	Bronchus-associated lymphoid tissue
BCA	Bicinchoninic acid
BCG	Bacillus Calmette Guerin
BSA	Bovine serum albumin
BSA-AuNCs	BSA protected gold nanoclusters
CHOL	Cholesterol
cmc	Critical micelle concentration
cpp	Critical packing parameter
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DCP	Dicetyl phosphate
DLS	Dynamic light scattering
DOC	Sodium deoxycholate
EB	Empty bilosome
EC	Epithelial cells
EE%	Entrapment efficiency percentage
ELISA	Enzyme-linked immunosorbent assay
EN	Empty NISV
FAE	Follicle-associated epithelium
FGT	Female genital tract
GALT	Gut-associated lymphoid tissue
GIT	Gastrointestinal tract
GnRH	Gonadotrophin releasing hormone
HA	Hemagglutinin
Hib	Haemophilus influenza b
HPLC	High-performance liquid chromatography
IFNγ	Interferon gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL	Interleukin
IM	Intramuscular
ISCOM	Immune stimulating complexes
KLH	Keyhole limpet haemocyanin
LALT	Larynx-associated lymphoid tissue
LB	Loaded bilosme
LC/MS	Liquid chromatography/mass spectrometry
LN	Loaded NISV

LNP	Lipid nanoparticles
MALT	Mucosa-associated lymphoid tissue
MGT	Male genital tract
MHC	Histocompatibility complex
MIS	Mucosal immune system
MLN	Mesenteric lymph nodes
MM	Mann’s modified method
MPG	1-monopalmitoyl glycerol
MW	Microwave method
NALT	Nasal-associated lymphoid tissue
NISV	Non-ionic surfactant vesicles
O.D	Optical denisty
OPV	Oral polio vaccine
OVA	Ovalbumin
PDI	Polydispersity index
PPs	Peyer’s patches
PPSV	Pneumococcal polysaccharide vaccine
RSV	Respiratory syncytial virus
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
SEM	Scanning electron microscopy
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
Th	T helper
TNF-β	Tumor necrosis factor beta
TT	Tetanus toxoid
VLPs	Virus-like particles
Xn	Xanthan gum
ζ-potential	Zeta potential

List of Figures

List of Tables

Chapter one: Literature review

1.1 Introduction

One of the most significant public health interventions of the last century has been the application of vaccines to control many serious diseases, prevent disability and decrease death in millions of people (Ehreth, 2003; Andre *et al.*, 2008). Vaccines have also been successful in eradicating infectious diseases such as small pox, and is expected to eradicate poliovirus in coming years. It has also controlled the distribution of many fatal diseases such as, measles, pertusis, tetanus, diphtheria, mumps, paralytic poliomyelitis, rubella and Hepatitis B and A (Soni and Sawant, 2008). Moreover, recent advances in vaccine technology have allowed its use as a potential therapeutic approach in cancer therapy, addictions, allergies and autoimmune diseases, and to prevent pregnancy (Ferro *et al.*, 2004b).

1.2 Types of vaccines

Traditional vaccines are mostly composed of inactivated bacteria or virus (Plotkin, 2005; Plotkin, 2009). This vaccine approach has been verified as effective in controlling and even eradicating many infectious diseases. However, vaccines formulated by conventional approaches where live-attenuated bacteria or virus have been incorporated as antigens, have led to some undesirable side effects such as mutations which can cause a reversion to virulence (Shimizu *et al.*, 2004) and it can cause complications in immunocompromised patients (Kroger *et al.*, 2011). Recent advances in the understanding of the pathogenesis of infectious diseases have opened up

doors for newer techniques. For example, the genomic approach for antigen identification followed by its chemical synthesis has started the trend of genetic immunisation, which has evolved as an attractive alternative to live attenuated pathogens in terms of safety, ease of mass production and cost (Soni and Sawant, 2008). Another example is the recent accomplishment in immunology known as reverse vaccinology (Rappuoli, 2001). This approach applies a combination of *in silico, in vitro* and *in vivo* methods and is an efficient way to screen for and to assess universal vaccine candidates. By using such methods, studying of the genomic information of organisms (*in silico*) using bioinformatics tools allows the determination of antigenic peptides. These peptides can be expressed (*in vitro*) and used in animal model immunisation (*in vivo*) to assess whether or not the selected peptides produce an appropriate immune response in the host (Rappuoli, 2001). The following section looks at the different types of vaccines in more detail.

1.2.1 Live, attenuated vaccines

Live, attenuated vaccines such as the Sabin polio and measles vaccines, contain a living microorganism that has been weakened, so that it becomes immunogenic without causing the disease (Plotkin, 2005). They induce significantly high cellular and antibody responses and often confer permanent immunity with only one or two doses.

Attenuation of viruses is often achieved by cultivating the virus through a number of cell lines, which can create mutations that render the virus less virulent, and has been used for the measles and polio vaccines

(Enders *et al.,* 1962; Sabin and Boulger, 1973). Other attenuation methods involve generation of a strain which is capable of growing at lower temperatures (Ellis, 2001). Attenuation of bacterial pathogens can also be achieved by applying the sub-culturing methods. In 1921, Calmette and Guerin developed a vaccine against tuberculosis infection. The Bacillus Calmette Guerin, or BCG vaccine was constructed, by attenuating *Mycobacterium bovis* through 231 subcultures over 13 years (Ellis, 2001; Daniel, 2006).

In spite of the advantages of live, attenuated vaccines, there are several drawbacks. Mutation of the attenuated microbe in the vaccine could revert to a virulent form and cause disease. In 2009, approximately 153 Nigerian children became paralysed by polio. The outbreak occurred from the use of type 2 oral polio vaccine (OPV) that contained a live-attenuated form of the poliovirus (Wassilak *et al.,* 2011). In addition, people who have damaged or weakened immune systems cannot safely receive live, attenuated vaccines. Another limitation is that live, attenuated vaccines as a rule require to be refrigerated to stay effective which may prevent broad use in developing countries.

1.2.2 Inactivated vaccines

Inactivated vaccines such as typhoid fever and pertussis vaccines, are produced by killing the virulent microorganisms, with chemicals, heat, or radiation (Ulmer *et al.,* 2006; Plotkin, 2008). Unlike the live vaccines, microorganisms in the inactivated vaccines cannot mutate back to their

virulent state which makes the inactivated vaccines more stable and safer than live vaccines. Besides, they do not need refrigeration, allowing their storage and transportation in freeze-dried form, which makes them easily available to wide areas in developing countries (Plotkin, 2009).

However, inactivated vaccines induce weak immune responses and require several booster administrations to maintain continuous immunity. A recent study revealed that formaldehyde-inactivated respiratory syncytial virus (RSV) and measles vaccines caused a disastrous worsening of the disease during a later natural illness (Moghaddam *et al.*, 2006). The study claims that the induction of carbonyl groups on vaccine antigens by formalin treatment may have changed the immunogenicity, shifting the balance between protective and deleterious immune responses (Moghaddam *et al.*, 2006).

1.2.3 Subunit vaccines

These types of vaccines contain only major antigens (epitopes) of the pathogen which stimulate the immune system to produce antibodies against the microorganism (Plotkin, 2009). Some subunit vaccines use only one antigen such as haemophilus influenza b (Hib) and hepatitis B, whereas influenza is an example of a subunit vaccine with two antigens (haemagglutinin and neuraminidase). The immune response to a subunit vaccine differs according to whether the vaccine antigen is a protein-based or a polysaccharide subunit vaccine, for example hepatitis B and influenza, are T-dependent protein vaccines, whereas polysaccharide-containing

vaccines such as the pneumococcal polysaccharide vaccine (PPSV) generate a T-independent response (Baxter, 2007).

1.2.4 Toxoid vaccines

Toxins secreted by bacteria can be inactivated or detoxified by treating them with formalin or modified genetically (Pizza *et al.,* 1996). The resultant detoxified toxins, called toxoids, are safe for use in vaccines against bacterial diseases. These vaccines are used when illness is mainly due to the bacterial toxin. The immune system produces antibodies that bind to and inactivate the toxin. Examples include vaccines against diphtheria and tetanus (Bizzini *et al.,* 1970; Del Giudice *et al.,* 1998). There are three principal advantages of toxoid vaccines. Firstly, they are safe because they are unable to cause the disease and there is no risk of mutation into a virulence state. Secondly, they cannot spread to unimmunised individuals. Thirdly, they are generally stable and long-lasting as they are less liable to alteration in temperature, humidity and light. However, toxoid vaccines have two disadvantages, they usually need an adjuvant and require several doses, and furthermore, local reactions at the vaccine site are more common (Baxter, 2007).

1.2.5 Conjugate vaccines

Bacteria have an outer coating of polysaccharides that cover the bacterial antigens so that the immature immune systems of infants and younger children cannot recognise or respond to them (Wood, 2006).

Conjugate vaccines, are a special type of subunit vaccine that get around this problem by the linkage of antigens or toxoids from a microbe to an adjuvant so that an infant's immune system can recognise microbial polysaccharides. The linkage helps the immature immune system react to polysaccharide coatings in order to mount a defence against the pathogen (Wood, 2006). Examples of conjugate vaccines include Hib vaccine, pneumococcal and meningococcal vaccines in which a non-toxic tetanus toxoid or non-toxic diphtheria protein is used as the link to stimulate the immune system (Pletz *et al.*, 2008).

1.2.6 DNA vaccines

The advance leap in DNA immunisation came in 1990 when Wolff *et al*. (Wolff *et al.*, 1990) established gene transfer *in vivo* after performing intramuscular (IM) injections with naked DNA plasmids. DNA strategies have been successfully applied commercially in veterinary medicine (Dunham, 2002), aquaculture (Heppell and Davis, 2000) and are in progress in clinical trials in human medicine against tuberculosis, malaria, HIV, hepatitis B, influenza, and rabies (Webster and Robinson, 1997; Lalosevic *et al.*, 2001; Cavenaugh *et al.*, 2011; Buddle *et al.*, 2013). DNA vaccination is referred to as direct immunisation with the gene of interest located on an appropriate eukaryotic expression plasmid. The DNA travels to the nucleus of the host cells and is expressed in the host system. The DNA immunogen takes over the host's endogenous transcription, translation and post-

translational machinery, resulting in an immunogenic protein structure with high quality presentation inducing Th1 cellular immunity (Zhu *et al.,* 2003).

1.3 Mucosal vaccination:

Most current vaccines are administered by parenteral injection and only a few are commercially available for mucosal route administration (Hao *et al.,* 2008); emphasizing the hurdles encountered in targeting the appropriate sites to induce protective immunity at both mucosal and systemic levels. A vast expanse of literature exists describing how the mucosal immune system (MIS), the first line of defence against pathogenic organisms entering the body via mucosal surfaces, induces mucosal immune responses to provide constant vigilance and critical protection (Mossad, 2003; Bernstein, 2007; Plotkin, 2008). Understanding how these responses are induced can help rational design of mucosal vaccine formulations, not only for protection against pathogens, but also in non-infectious applications such as malignancies (Peek *et al.,* 2008), reproductive conditions (Torchilin, 2005) and allergies (Katre, 2004).

The MIS includes the barrier of the mucous membranes that line the digestive, respiratory and reproductive systems, as well as the conjunctiva and tear-ducts. These mucosal surfaces vary according to tissue function and through their specialisation each provides their own challenges to development of site-specific mucosal vaccines. The MIS can be broadly divided into inductive and effector sites; the former being organised into mucosa-associated lymphoid tissue (MALT) and lymph nodes, while the effector sites are formed in part by the lamina propria and epithelial surface (Bernstein, 2007). Together with the physical barrier, there is a

complex and intimate interaction between innate and adaptive components that together mount an immune reaction. Recognition receptor molecules play an important role in innate immunity and they regulate the adaptive response as well as help to generate a memory response (Dwivedy and Aich, 2011). The key for producing an adaptive immune response is antigen processing and presentation by different cells that are involved in immune reactions. Recent extensive reviews of immune cell interactions have been published that describe defence against pathogens, antigen sampling from mucosal surfaces either with the help of antigen-presenting cells (APCs), or by the M cell that then stimulate naive T and B lymphocytes (Pearse and Drane, 2005; Stewart and Devlin, 2006). There are several vaccines against major mucosal infections that have been internationally licensed, these are listed in Table 1.1 (Czerkinsky and Holmgren, 2012).

Infection and vaccine(s)	Route	Trade name (producer)
Polio		
Live attenuated vaccine (OPV)	Oral	Numerous
Cholera		
Cholera toxin B subunit + inactivated V. cholerae O1 whole cells	Oral	Dukoral® (Crucell, Canada)
V Cholera Classical biotype, ELTor and O 139.	Oral	ORC-Vax® (VaBiotech, Vietnam)
Typhoid		
Ty21a live attenuated vaccine	Oral	Vivotif™ (Berna Biotech, Switzerland)
Rotavirus		
Live attenuated human rotavirus, monovalent pentavalent	Oral	RotaRix™ (GlaxoSmithKline, Belgium)
		RotaTeq® (Merck, USA)
Influenza		
Live attenuated cold-adapted influenza virus	Nasal	NASOVAC (Serum Institute of India Ltd., India)
		FluMist™ and Fluenz™ (MedImmune, USA)
Adenovirus		
Live, adenovirus Type 4 and Type 7	Oral	Barr Labs, USA

Table 1.1: Internationally licensed vaccines against mucosal infections (Dietrich *et al.*, 2003; Yuki and Kiyono, 2009; Czerkinsky and Holmgren, 2012).

1.4 Cellular structure of the mucosal immune system

Generally the mucosal surfaces can be divided into type I and type II mucosal tissue based on epithelial surface structure and presence of MALT, summarised in Table 1.2. The presence of organised or diffuse MALT is significant as organised MALT serves as the main immune inductive site where immune responses against processed antigens are initiated, while antibody production (mainly IgA) occurs in diffuse MALT (Soloff and Barratt-Boyes, 2010).

	GALT	**NALT**	**FGT**
Epithelial surface	Single	Single pseudostratified	Single/stratified squamous
Inductive sites	+ (PP)	+ (tonsils)	- to +
Effector sites	++	++	++
APC	++ (DC)	++ (DC)	++ (DC)
T cells	++	++	++
B cells	IgA>>IgG	IgA>>IgG	IgG>>IgA

+ (present), - (absent), >> (principal)

Table 1.2 Comparison of the cellular structure in GALT, NALT and FGT. Abbreviations: GALT; gut-associated lymphoid tissue, NALT; nasal-associated lymphoid tissue, FGT; female genital tract

1.4.1 Oral and intestinal tract

The oral cavity is divided into the front region (sublingual and buccal sub-regions) and the back region (includes the pharynx and the Waldeyer's ring

consisting of three types of tonsil) (Moore *et al.*, 2010). The mucosal surface of the back region consists of a single lymphoepithelial cell layer composed from ciliary and non-ciliary epithelial cells, goblet cells and M cells (Yu and Vajdy, 2010). Below the mucosa, numerous dendritic cells (DCs) are located with close contact with B and T cells (Pope, 1999). Antigen uptake through the tonsils suggests its role as an inductive immune site (Challacombe and Naglik, 2006; Brandtzaeg, 2011). The mucosal surface of the front region is lined by stratified squamous epithelium, which varies in thickness from about 40-50 cells in the buccal sub-region, and becomes thinner in the sublingual sub-region (Garg *et al.*, 2011). Keratinisation affects the permeability of the buccal mucosa to some extent, resulting in tissue of the sublingual sub-region being more permeable than the buccal sub-region (Challacombe and Naglik, 2006). This difference in permeability means that small immunogenic peptides can be used more readily in sublingual (s.l.) immunisation strategies (Squier, 1991). DCs take up small antigens in the submucosa and migrate to regional lymph nodes for antigen presentation to T-lymphocytes and induction of adaptive immune responses (Song *et al.*, 2009). S.l. immunisation has also been reported to induce significant antibody production in distal sites (nasal, bronchial and oral mucosa), making it a potentially useful site for protection against respiratory and oral pathogens (Song *et al.*, 2008; Zhang *et al.*, 2009). In recent years, s.l. immunisation has received more attention, and this may in part be due to ease of administration and reduction in manufacturing costs since it is known that lower antigen doses (10-50 fold) can be used compared with for example oral administration (Czerkinsky *et al.*, 2011).

Historically, the oral route appeared the best approach to mucosal vaccine development, particularly in light of the success of the oral polio virus vaccine (licensed over 50 years ago) in helping to eradicate poliomyelitis (Sabin, 1985). It offered the advantages of ease of administration, and potential for mass and pain-free vaccination. This led to the GALT being the most studied mucosal site for vaccine delivery. The lining of the GALT is covered by a single cell layer (Figure 1.1); the epithelia have intercellular spaces that are sealed by tight junctions, and M cells (Mishra *et al.,* 2010). IgA has an important neutralising antibody role as it is mainly found on epithelial surfaces, which lack complement and phagocytes. Antibodies can also prevent adhesion to cell surface molecules and receptors (Russell, 2007). GALT have several types of lymphoid nodules, including Peyer's patches (PPs), isolated lymphoid follicles, cryptopatches and lymphoglandular complexes found on the follicle-associated epithelium (FAE) and are covered with M cells. The main role for M cells is to sample luminal antigens by receptor-mediated uptake allowing the presentation of the antigen to underlying APCs in the lamina propria, which contains dense populations of lymphocytes, macrophages and DCs (Mason *et al.,* 2008). Several studies have demonstrated the ability of M cells to transport viruses efficiently to PPs by transcytosis (Wolf *et al.,* 1983; Sicinski *et al.,* 1990; Amerongen *et al.,* 1991). M cells are therefore highly transcytotic and are able to transfer a wide range of materials, including nanoparticles (Misumi *et al.,* 2009). In addition, DCs can extend projections between the epithelial tight junctions into the lumen to capture antigens for presentation to intraepithelial lymphocytes (Rescigno *et al.,* 2001). DCs express major histocompatibility complex (MHC) class-I molecules, low levels of MHC-class II molecules and can also present antigen to CD4+ and CD8+

T-cells (Ryan *et al.*, 2001). Once the MIS has been primed, subsequent mucosal boost immunisations provoke stronger responses than parenteral boost immunisations (Schmucker, 1999). After stimulation, antigen-specific B and T cells migrate to mesenteric lymph nodes (MLN) and the spleen to proliferate (Garside *et al.*, 2004). Proliferating B and T cells enter the circulation and migrate to mucosal effector sites as well as to peripheral lymphoid organs where they can differentiate into IgA- and IgG-secreting plasma cells (Mowat, 2003; Kiyono and Fukuyama, 2004).

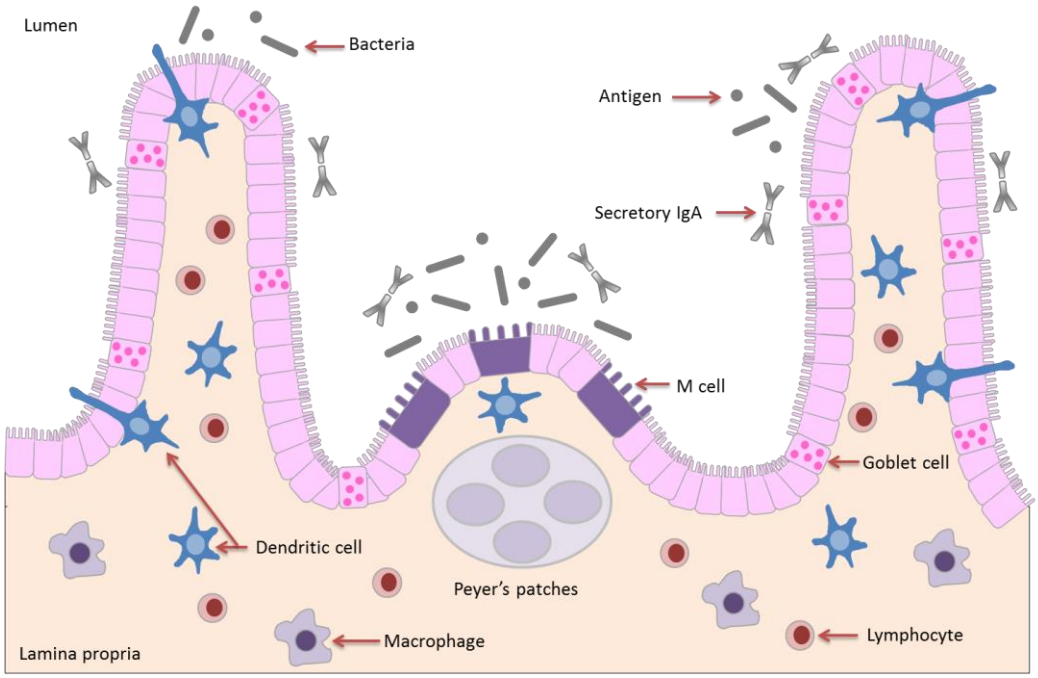


Figure 1.1: Intestinal tract showing a single cell surface layer with intercellular spaces that are sealed by tight junctions. M cells are present with the layer that enable delivery of antigen by active mechanisms from the lumen to organized lymphoid tissues and underlying antigen presenting cells (including dendritic cells, macrophages and lymphocytes). Covering the mucosal surface is a mucus layer, secreted by Goblet cells, that poses an additional barrier that needs to be penetrated by vaccine formulation delivered via the oral route (adapted from Gebril *et al.*, 2012).

The limited availability of oral vaccines reaching the market demonstrate the challenges associated with this type of vaccine design, including: difficulties in the collection and processing of external secretions, differences in experimental animal models compared with humans, a lack of standardised assays, the potential risk of induction of tolerance, damage to antigens caused by the harsh environment of the GI tract, poor transport of antigens across the intestinal epithelium, antigen–microbiota interactions and particle-mucous interactions (Cone, 2009; Czerkinsky and Holmgren, 2009). Live vectors have been used successfully to overcome these issues with some safety payback (Masuet *et al.*, 2011), therefore, the ideal scenario is to be able to induce effective responses with non-living vaccines. Thus, technological developments have focussed on delivery systems and novel adjuvants (Ferro, 2011).

1.4.2 Nasal and respiratory tract

Intranasal vaccination is an attractive mode of immunisation, for practical reasons such as ease of administration (particularly for mass immunisation programmes) and because of the high vascularisation in this area, the absence of acidity, lack of proteolytic enzymes (excluding the antimicrobial enzymes such as lysosome and lactoferrin) and a relatively small mucosal surface area that allows low doses of antigen to be administered.

The epithelial surface of the upper and lower respiratory tract and the underlying connective tissue contain immunocompetant cells that form the lymphoid structures of NALT, bronchus-associated lymphoid tissue (BALT), larynx-associated lymphoid tissue (LALT) and lymph nodes draining the respiratory system (Davis, 2001). NALT, located in the upper respiratory tract, extends the function of the

Waldeyer's ring, allowing NALT to sample antigens entering both the gastrointestinal tract (GIT) and the respiratory tract. The NALT is preferentially drained by the posterior cervical lymph nodes. The main cells involved in NALT inductive immune sites are similar to those found in GALT (Kiyono and Fukuyama, 2004). The epithelium of the NALT contains ciliated epithelial cells, mucous goblet cells and specialised non-ciliated cells similar to the M cells present in the PP in the MALT (Figure 1.2). In addition to M cells and DCs, the epithelial layer of the respiratory tract contains two other cell types, the macrophages and intraepithelial lymphocytes (Hameleers *et al.,* 1989; Csencsits *et al.,* 1999). The interaction of antigens delivered intranasally with the MIS, is determined by the nature of the antigens. Small soluble antigens can penetrate the nasal epithelium and reach DCs, macrophages and lymphocytes (intraepithelial and subnasal) (Kuper *et al.,* 1992), while particulate antigens are mainly transferred by M cells in the NALT and presented to underlying APC to prompt local or distant mucosal responses (Zhou and Neutra, 2002). So far BALT has only been found in rabbits, rats, guinea pigs, children and adolescents, but is thought to be absent in healthy adult humans where it is mainly substituted by the presence of the Waldeyer's ring (Challacombe and Naglik, 2006; Pabst and Tschernig, 2010).

As with s.l. immunisation, lower antigen doses can be used and studies have established that nasal administration of antigen induces specific mucosal IgA responses in the salivary glands, respiratory tract, genital tract and small intestine (Velasquez *et al.,* 2011). Nevertheless, to date only two nasal vaccines have been licensed, both of which are live-attenuated viral vaccines (Mann *et al.,* 2009a).

In the lower respiratory tract, features of the lungs such as high vascularisation, a large surface area and a thin epithelial layer have attracted interest in pulmonary immunisation (Al-Hallak *et al.,* 2010; Muttil *et al.,* 2010), which enables avoidance of first-pass metabolism and offers both systemic and local effects. Epithelial cells mediate and activate the transport of IgA into the airway lumen as a first line of defence related to adaptive immunity, express MHC receptors, recruit lymphocytes and regulate their activity through cytokine release (Nicod, 1999). In the lower airways, similarly to the upper airways, a tight network of DCs underlies the superficial mucosal layer responsible for sensing and capturing any invading pathogen to be carried to the lymph nodes around the airways or in the hilum (Nicod, 1999), whereas in the alveolar region, particles are engulfed by alveolar macrophages (AMs). The trigger of an immune response by AMs is initiated by the secretion of oxygen reactive species, the release of inflammatory cytokines and chemokines. This causes an inflammatory cascade with the activation of neighbouring cells and recruitment of neutrophils and lymphocytes (Siekmeier and Scheuch, 2008).

However, as with any drug delivered by the pulmonary route, deposition in the lungs in quantities sufficient to produce a therapeutic effect is essential (Taylor and Kellaway, 2001). This requires the delivery system to be nebulised or aerosolised with a mass median aerodynamic between 1 and 5μm to deposit in the alveolar region. Particles larger than 5μm impact on the airway walls whereas particles smaller than 1μm reach the alveoli, but are diffuse and constantly in Brownian motion leading to their exhalation (Carvalho *et al.,* 2011). An advantage of aerolisation is that the vaccines can be formulated into stable dry-powders. This means that particle size can be controlled to enable better targeting of the vaccine to

appropriate lung areas (Amorij *et al.*, 2010). Furthermore, this removes the need for cold storage. Inhalation devices, such as nebulisers and metered-dose inhalers, the limitations of these technologies include shear forces and the need for propellants that can damage protein antigens. Pulmonary delivery of influenza antigens has been investigated since the 1960s and found to give similar responses to intradermal and better than intranasal vaccination (Amorij *et al.*, 2010). However, to date there are no commercial pulmonary influenza vaccines. Other pulmonary vaccines tested in patients have included pneumococcal polysaccharide (Meyer *et al.*, 2006) and measles antigen (Fernandez-de Castro *et al.*, 1997).

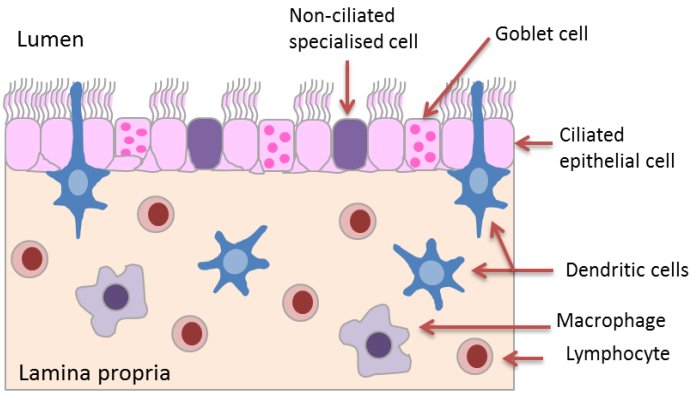


Figure 1.2: Nasal epithelium with ciliated epithelial cells, mucus-secreting goblet cells and specialised non-ciliated cells analogous to the M cells present in the intestine. Small soluble antigens penetrate the nasal epithelium and reach DCs, macrophages and lymphocytes (APCs), located below the single epithelial cell layer, while particulate antigens are mainly transferred by M cells and then presented to the underlying APC to prompt local or distant mucosal responses (Gebril *et al.*, 2012).

1.4.3 Genital tract

Although the female genital tract (FGT) is considered to be part of the MIS, it shows some features that are not common to other mucosal tissues or the systemic compartment. Among these differences include distinct regional epithelial layers, mucus that is inhabited by microflora that only occupy the reproductive tract, hormonal variations that may change mucosal immunity, and the ability to accept histoincompatible sperm and an allogenic foetus without decreasing the capacity to react to sexually transmitted pathogens (Mestecky *et al.,* 2005; Wira *et al.,* 2005; Hickey *et al.,* 2011).

The FGT employs a mixture of defence mechanisms against the threat of infection, which appear to be both complementary and synergistic. These include non-immune and pre-immune strategies, such as environment pH, mucus, epithelial barrier, and also inflammatory reactions and secretion of humoral soluble factors. The cellular defence and pre-immune humoral strategies may also be involved in rapid protection prior to antigenic stimulation occurring. If these early defences fail, additional acquired and antigen-specific strategies begin together with associated humoral responses with SIgA/IgM and locally produced IgG as well as cellular immune responses (Mestecky *et al.,* 2005). Thus, the innate immune cells provide protection against pathogens and epithelial cells are active participants in mucosal defence. They function as sensors that detect dangerous microbial components through pattern recognition receptors (PRRs), which are essential for adaptive immunity responses.

The FGT mucosal surface is covered mainly by squamous epithelium, varied from multilayer form in the vaginal mucosa to pseudosquamous with simple columnar cells in the mucosa of uterus, cervix, and fallopian tubes (Vajdy, 2008) (Figure 1.3). There are no M-like cells in the reproductive tract, however, the underling lamina propria contains a large range of B cells, CD4+ and CD8+ T-cells and APCs that form the inductive and effector immune sites for the reproductive tract (Vajdy, 2008). Priming occurs solely in the draining lymph nodes found in the abdomen and pelvis, including the common iliac, interiliac, external iliac and inguinal femoral lymph nodes (Iwasaki, 2010).

Intraepithelial γδ T cells, macrophages, Langerhans cells (LCs) and submucosal DCs are found in type II epithelia in the vaginal canal. While, natural killer (NK) cells and regulatory T cells in the uterus provide antiviral protection. Post infection, neutrophils, monocytes, plasmacytoid DCs (pDCs) and NK cells move to the vagina, leading to antigen-specific T and B cells entering the tissue to provide defences that are pathogen-specific. LCs and DCs are able to recognize a wide variety of microorganisms and following pathogen recognition these cells mature and migrate to the draining lymph nodes to prime naive T and B cells (Romani *et al.*, 2010).

Studies have demonstrated that the reproductive tract is an inductive site for immune responses resulting in production of specific local IgA and IgG antibodies in uterine and cervicovaginal secretions after immunisation by inactivated polio vaccine in the uterus and vagina of women (Ogra and Ogra, 1973) or animals (Prabhala and Wira, 1995; Wira and Rossoll, 1995; Fahey *et al.*, 1999). Isolated uterine epithelial cells (ECs) as well as APCs from the basolateral subepithelial stroma express MHC

class II antigen and can process and present antigens to T cells (Wallace *et al.*, 2001). In addition to MHC class II molecules, ECs also express CD1d and CD40 glycoproteins (antigen-presenting molecules) that confirm that these cells interact with CD8+ T cells. However, ECs and stromal APCs are regulated by the influence of hormonal changes in the reproductive tract (Prabhala and Wira, 1995; Wira and Rossoll, 1995; Kaushic *et al.*, 1996; Wira *et al.*, 2005). For example, the stromal antigen presentation process is suppressed by TGF-β cytokine produced by uterine ECs in response to oestrogen (Wira and Rossoll, 2003).

Nevertheless, it should be noted that despite various advantages of vaginal delivery, this mode of vaccination is not as readily put into practice. Consideration needs to be given to accessibility to different areas of the tract, and alterations of tissue structure in response to hormonal fluctuations during the menstrual cycle (Kozlowski *et al.*, 2002), as well as societal and cultural concerns. In addition, the FGT lacks inductive mucosal sites equivalent to PPs and these issues therefore require different approaches. A recent study compared the protective activity resulting from immunisation of mice via intranasal (i.n), intravaginal (i.vag.) or subcutaneous (s.c) routes with a HPV type 16 E7 polypeptide vaccine. Only the s.c. route of immunisation induced complete regression of established genital tumours (Decrausaz *et al.*, 2011), suggesting that for certain vaccines the mucosal route may not be the most appropriate. Other hurdles include availability of suitable pre-clinical experimental models since it is known that rodent genital epithelia differ significantly to that of humans in terms of keratinisation. The non-keratinised human vaginal mucosa tends to make this tissue more permeable and less resistant to damage compared to rodent tissue (Squier *et al.*, 2008).

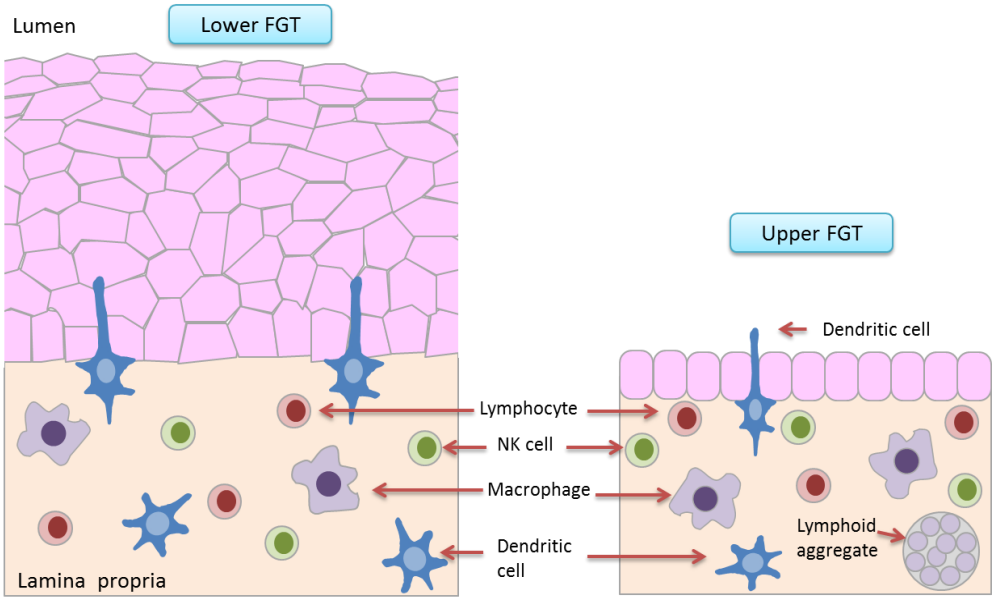


Figure 1.3: The lower female genital tract (vagina) is covered with multilayered squamous epithelia, while the uterus, cervix, and fallopian tubes are covered with pseudosquamous and simple columnar epithelial cells. There is no equivalent to M cells however; the lamina propria contains a large range of B and T cells, as well as APCs that form the inductive and effector immune sites. Priming occurs in various draining lymph nodes (adapted from Gebril *et al.*, 2012).

In contrast to the FGT, the male genital tract (MGT) character and immune responses have not been widely researched, reviewed by Moldoveanu *et al.* (2005) and challenges arise since studies provide contradictory reports on the types and levels of antibodies produced in the MGT and the lack of organised inductive sites. However, probably far more compelling is the inconvenience of mucosal administration to this area and since systemic immunisation effectively stimulates humoral responses in the MGT there is little advantage in pursuing the development of mucosal vaccines for this site.

1.4.4 Cross-talk between the mucosal tissues

A recent review has described the chain of events following antigen sensitization of the B and T cells (Czerkinsky and Holmgren, 2010) and offers an explanation for cross-talk between different mucosal sites. For example it is known that the gut and mammary glands are linked, similarly the nasal mucosae are linked to respiratory and genital areas. B and T cells encounter antigen, leave through the lymph into the circulation and then re-locate in the mucosa of origin where they differentiate into memory or effector cells. The ability to do this is believed to be due to "homing receptors" or integrins on their surface, tissue-specific receptors or addressins on vascular endothelial cells and chemokines that promote cell migration by chemotaxis. Differences in expression of the integrins, addressins and chemokines may control cross-talk. This is an important consideration when targeting a particular mucosal area (Brandtzaeg, 2010; Pasetti *et al.*, 2011; Czerkinsky and Holmgren, 2012).

1.5 Mucosal vaccine delivery systems

One approach to widening the choice of vaccination without the use of live-attenuated micro-organisms is the development of adjuvants and delivery systems. These have recently been reviewed under the categories of mineral salts, oil-in-water emulsions, microbial derivatives, natural products, endogenous immunostimulatory factors, inert vehicles and lipid particulates (Ferro, 2011). Some of these are more appropriate for mucosal delivery, for directly targeting the MIS. For example aluminium salts (referred to as 'alum'), which is used in many parenteral vaccines, does not induce mucosal immunity. Delivery systems can also be used in

combination with adjuvants and are designed to increase delivery, targeting, protection against degradation and controlled release of antigen at a particular site.

Microparticles are spheres with diameters ranging from nanometres up to several micrometres. They may be constructed from different polymers, are easy to produce and are stable. Depending on their size, they are taken up by M cells or even epithelial cells (Tabata *et al.,* 1996; Ryan *et al.,* 2001). When formulated with biodegradable and biocompatible polylactide-co-glycolide (PLG) polymers, they are capable of releasing incorporated antigens. Incorporation of additional immunomodulators or carrier molecules can enhance their potency. There are noticeable differences between soluble and particulate antigens. In general, soluble antigens are less immunogenic and tend to induce tolerance rather than immune system activation. Particle size is vital to immunogenicity, since small particles are non-specifically taken up by epithelial cells (Tabata *et al.,* 1996). Recent studies revealed that macrophages respond in different ways according to the adjuvant particle size after endocytosis (Brewer *et al.,* 2004; Mann *et al.,* 2009a; Mann *et al.,* 2009b); more specifically the macrophage production of cytokines can be manipulated towards a Th1 or a Th2 phenotype. Some mucosal routes are better suited for particulate delivery. For example in pulmonary immunisation, the large surface area of the lungs allows greater interaction between the antigens and the immunoreactive cells (Thomas *et al.,* 2010). The slow clearance and subsequent migration of the antigen from the alveolar region to lymphoid tissues can enhance vaccine efficiency in comparison to other routes associated with rapid vaccine clearance (Muttil *et al.,* 2010). However, alternative adjuvants other than alum are required to be administered with antigens as alum is not approved for pulmonary

delivery since it causes immunotoxicological responses in the lungs and requires large doses of antigen to be used (Muttil et al., 2010). As a result, the use of polymeric carriers such as chitosan, PLGA and PLA have shown success as adjuvants and have been used as delivery carriers for vaccines in the form of microparticles and nanoparticles (Thomas *et al.,* 2011). Reported use of PLGA include spray-dried nanoparticles as carriers for diphtheria CRM-197 antigen (Lu *et al.,* 2010) and recombinant hepatitis B surface antigen (Muttil *et al.,* 2010), spray-dried microparticles containing recombinant antigen 85B against tuberculosis (Lu *et al.,* 2010) and wet microspheres containing hepatitis B surface antigen (Thomas *et al.,* 2010).

1.5.1 Liposomes

Lipid particulates are readily altered in size, charge, lamellarity and membrane fluidity. Liposomes are vesicular structures composed of one or more lipidic bilayer(s) surrounding an aqueous core and into which antigens can be incorporated. They are stable in acidic, bile and pancreatin solutions. Liposomes have been studied extensively and are currently found in a number of FDA approved vaccines for different conditions (Nordly *et al.,* 2009; Yang *et al.,* 2011) at least 8 vaccines approved for clinical use or undergoing clinical trial (Watson *et al.,* 2012) and have been developed for mucosal delivery (Romero and Morilla, 2011). As microparticles, they are taken up due to their small size and their efficiency can be enhanced by addition of immunomodulatory or carrier molecules (Ryan *et al.,* 2001). Addition of immunostimulatory components is of particular relevance to cationic liposomes, which in general are poorly immunostimulatory. However, in recent years a number

of adjuvants based on cationic liposomes have been successfully developed reviewed by (Christensen *et al.,* 2011).

1.5.2 Non-ionic surfactant vesicles (NISV)

Another class of lipid-based vesicles are prepared from self-assembly of hydrated mixtures of cholesterol and non-ionic surfactants molecules such as monoalkyl or dialkyl polyoxyethylene ether. These are niosomes and they exhibit more chemical stability than liposomes as non-ionic surfactants are more stable than phospholipids (Baillie *et al.,* 1985). Vesicles vary from unilamellar to multilamellar structure and have the ability of entrapping hydrophilic and hydrophobic solutes (Florence, 2005). They have been used in a number of mucosal routes including nasal (Atherly *et al.,* 2005), pulmonary (Marianecci *et al.,* 2010; Moazeni *et al.,* 2010) and vaginal (Ning *et al.,* 2005; El-Ridy *et al.,* 2011). A simple adaptation has enabled oral administration, with the incorporation of bile salts as an integral part of the vesicle (Mann *et al.,* 2009b), resulting in formation of bilosomes (Figure 1.4). This is believed to stabilise the vesicles upon subsequent exposure to endogenous bile salts in the gastrointestinal tract (Singh *et al.,* 2004). Bilosomes have been investigated with a number of different antigens including synthetic peptides, BSA, haemagglutinin antigen, tetanus toxoid, hepatitis B and Human enterovirus 71 (HEV71) (Mann *et al.,* 2004; Singh *et al.,* 2004; Mann *et al.,* 2006; Shukla *et al.,* 2010; Shukla *et al.,* 2011; Premanand *et al.,* 2013).

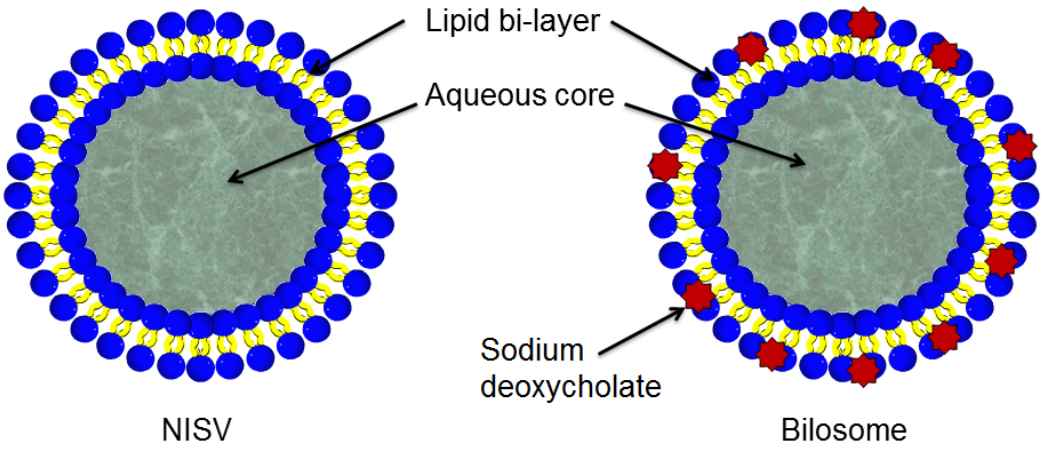


Figure 1.4: Schematic diagram showing the proposed structure of non-ionic surfactant vesicle (NISV) and vesicles with incorporated bile salts (Bilosome).

1.5.3 Immune stimulating complexes (ISCOMs)

Immunostimulatory fractions of *Quillaja saponica* (Quil A) have been included into lipid particles such as liposomes to form ISCOMs. The antigen-free formulation, contains negatively charged oval nano-structures of about 40 nm in size and are termed ISCOM matrix, while when an antigen is incorporated into the nano-particles, the term ISCOM is used. ISCOMs were first saw by researchers using transmission electron microscopy but were regarded as microscopic artefacts (Horzinek and Mussgay, 1971; Kersten and Crommelin, 1995). In 1984, Morein *et al.* studied the nano-structures and investigated their potential as vaccine delivery systems (Morein *et al.*, 1984). Since then, ISCOMs have been investigated in great depth. Hydrophobic or membrane-associated proteins can be incorporated into ISCOMs, while incorporation of non-hydrophobic proteins is more difficult, but not impossible. ISCOMs are resistant to both temperature and low pH and their particulate nature assist uptake by M cells or epithelial cells. Their efficacy as systemic and mucosal vaccine delivery system has been demonstrated in several studies in both animal models and humans for diseases such as influenza , herpes HSV-1, rotavirus and tuberculosis (Sun *et al.*, 2009). ISCOMs can provoke cytotoxic T lymphocytes (CTLs) and stimulate cells of the innate immune system (Ryan *et al.*, 2001).

1.5.4 Virus-like particles and virosomes

Virus-like particles (VLPs) are constructed by self-assemble of viral capsid proteins into particulate structures closely resembling immature virus particles (Buonaguro *et al.*, 2009). The primary form of virosome was the generation of lipid vesicles

incorporating viral spike proteins (Almeida *et al.,* 1975). Currently, virosomes are formed by detergent solubilisation and reconstruction of enveloped viruses (Abdoli *et al.,* 2013). VLPs proved to produce strong stimulation of the innate immune system and activating mature DCs (Sailaja *et al.,* 2007). The presence of the antigens at the surface of VLPs stimulate the immune system to produce antigen specific reaction (Huckriede *et al.,* 2005). In addition, several efforts have already been made using VLPs for protection against different non infectious diseases like obesity, smoking and high blood pressure with antigens against ghrelin, nicotine and angiotensin, respectively (Dyer *et al.,* 2006). Recently, they have been studied in HPV vaccination and likely to play a part in the future vaccines against HIV (Young *et al.,* 2006).

1.6 Mucoadhesives

As part of the technological development of particulates, consideration has been given to targeting the delivery systems to appropriate sites in the MIS. This has involved use of M cell targeting ligands to promote uptake of the formulation and enhance immune responses (Lo *et al.,* 2012). Other studies have also reported the development of mucoadhesives as part of the mucosal vaccine delivery system, of which the majority are considered biodegradable or from natural sources such as chitosan and xanthum gum (Table 1.3). The mucosal membranes lining the gut, respiratory and reproductive tracts are characterised by moist surfaces due to the mucus layer secreted by epithelial cells. This mucus layer provides protection of epithelial cells from chemicals and mechanical damage as well as affording lubrication to the mucosal surface (Khutoryanskiy, 2011). Mucoadhesive

formulations were first reported by Scrivener and Schantz in 1947 (Scrivener and Schantz, 1947), when they administered penicillin to oral mucosal surfaces using formulations containing a mixture of gum tragacanth and dental adhesive. Since then, a range of mucoadhesives has been used with polymer delivery systems to enhance the bioavailability of antigens to various mucosal administration routes. In particular, the need for prolonged retention of antigens at surfaces such as the nasal cavity or vagina necessitate the use of formulations that enhance mucoadhesiveness (Han *et al.*, 2006). In addition mucoadhesives protect antigens from degradation particularly via the oral route, they also allow an increase in antigen concentration at application sites, thus reducing the amount of antigen needed for induction of an immune response (Mishra *et al.*, 2010). Mucus forms a protective layer varying from 1 micron to several hundred microns depending on the site. It mainly contains mucin fibres formed from cross-linked mucin monomers, secreted by goblet cells and submucosal glands (Lai *et al.*, 2009). Conventional large particles are most likely to be trapped by mucus through adhesive forces and promptly removed through mucociliary clearance. For instance, orally delivered large nanoparticles may associate with chyme or adhere to mucin fibres in the GI tract , leading to elimination with faeces, while small nanoparticles penetrate the mucus mesh toward the underlying epithelia for possible entry through mucosal membrane surface (Galindo-Rodriguez *et al.*, 2005). Mucoadhesion may occur by either or both physical and chemical interactions or a combination of both. These can be further categorised as hydrogen bonds, Van der Waals forces and hydrophobic bonds (physical interactions e.g. formation of gel structures when aqueous solutions of polyvinyl alcohol and

glycine are mixed), whereas ionic and covalent bond formation are considered chemical interactions (alginate and chitosan solutions in water) (Woodley, 2001).

There is an argument that particulate delivery systems need to be mucus penetrating rather than mucoadhesive, reviewed by (Atherly *et al.,* 2005). Since human mucus is known to be 10^3-10^5 times more viscous than water, this means that particulate matter cannot move through it readily. This enables muciliary clearance to remove the particulate material and so it is important for delivery systems to overcome this hurdle. Size and charge are thought to play an important role, and manipulation of the interactions with mucus components, in particular mucin, will feature greatly in the future.

Mucoadhesives classification	
Based on origin	
Synthetic	Cellulose derivatives, poly (acrylic acid) polymers, poly (hydroxyethyl methylacrylate), poly (ethylene oxide), poly (vinylpyrrolidone), poly (vinyl alcohol)
Natural	Tragacanth, sodium alginate, karaya gum, guar gum, xanthan gum, soluble starch, gelatin, pectin, chitosan
Based on nature	
Hydrophilic polymers	Poloxamer, hydroxypropyl methyl cellulose, methylcellulose, poly (vinyl alcohol) and poly (vinyl pyrrolidone)
Polysaccharides and their derivatives	Chitosan, methyl cellulose, hyaluronic acid, hydroxy propyl methylcellulose, hydroxy propyl cellulose, xanthan gum, gellan gum, guar gum, and carrageenan
Novel polymers	
Lectins	Merolectins, hololectins, chimerolectins
Thiolated polymers	Chitosan–iminothiolane, poly (acrylic acid)–cysteine, poly (acrylic acid)–homocysteine, chitosan–thioglycolic acid, chitosan–thioethylamidine, alginate–cysteine, poly (methacrylic acid)–cysteine and sodium carboxymethylcellulose–cysteine
Other novel mucoadhesives	Bioadhesive nanopolymers, alginate-polyethylene glycol acrylate, poloxomer, pluronics.

Table 1.3: Classification of mucoadhesives (reviewed by (Mythri *et al.,* 2011).

1.7 Aims of the study

The main scope of this thesis is the development of a mucosal vaccine delivery system based on lipids. The mucosal administration routes present numerous rewards beside the immunological ones: the uncomplicated procedure to administer them, the low cost in terms of components and manufacturing, and the potency to increase patient comfort and compliance. On the other hand, these routes are also coupled with some challenges such as, for instance, the poor intestinal absorption of vaccines and the aggressive environment in the gut which destroys a significant quantity of orally administered vaccines. Furthermore, MIS mucus reduces the competence of delivered antigens. Thus, a mucosal vaccine requires not only efficient delivery across epithelial barriers, but also the use of strategies that elicit a protective immune response without undesired inflammatory reactions.

The major aim of this study is to develop a vaccine delivery system that can provide effective protection at sites distal to the gut e.g. genital (immunocontraception, infectious disease, cancer). The challenge will be to release the vaccine at an appropriate mucosal site, without destruction of antigen, in order for maximum interaction to take place with specialised immune cells.

The principal objectives of this study were: to synthesis and characterise lipid based nanoparticles (Chapter 2), evaluate these nanoparticles for oral and parenteral delivery for non-pathogenic immunogen model against gonadotrophin releasing hormone (GnRH) (Chapter 3), improve and develop this delivery system for application in alternative mucosal routes (Chapter 4). In addition, studying the application of this delivery system in mucosal immunisation against pathogen

derived-toxin and in toxin challenge trials (Chapter 5), and the investigation of mechanisms of the oral delivery system (Chapter 6).

Chapter two: Lipid nanoparticles: method of preparation and characterisation

2.1 Introduction

The innovation of liposomes as vesicular structures by Horne and Bangham in the early 60s of the 20th century (Bangham, 1978), unwrapped a path for numerous lipid-based formulations to be used as drug delivery systems. Lipid-based vesicles are prepared from self-assembly of hydrated mixtures of cholesterol and surfactant molecules such as monoalkyl or dialkyl polyoxyethylene ether. The key forces are hydrophobic and hydrophilic interactions at the interface between the monomer and the aqueous phase. The conflict of these forces to each other create a system where the hydrophobic nonpolar tails aggregate together to exclude water (Tanford, 1987), with the hydrophilic polar heads on the outside. Cholesterol is used for cessation of the gel to liquid phase transition of lipid bilayer system created, effectively hardening and provides the structure integrity (Uchegbu and Vyas, 1998a). In addition, cholesterol improves homogeneity to form more mono-dispersed formulations (Parasassi *et al.*, 1994) along with additional components that can be added to enhance the system, such as charged molecules like dicetyl phosphate (DCP), which act as system stabilisers and prevent aggregation (Uchegbu and Vyas, 1998b). The stabilising properties of DCP arise due to its negative charged regions repelling other cholesterol-based vesicles (Uchegbu, 1999).

There are two factors essential for the formation of vesicles. The first is the critical micelle concentration (cmc), which is the minimum concentration of premicellar monomeric amphiphiles to spontaneously form amphilic micellar aggregates in monomeric solution. The second factor is the critical packing parameter (cpp) of lipids which depends on their optimal area (a_0), the hydrocarbon chain volume (v) and the length of maximum critical chain (lc) (Kumar, 1991).

Consequently, the equation [cpp = v/a_0 x lc] is used to define the shape and size of structures into which lipids may pack (Israelachvili, 2011). Thus, cpp values determined < 1/3, forms micelles, while cpp values > 1/3 and < 1/2 results in non-sperical micelles or worm-like micelles (Chu *et al.*, 2013), vesicles produced when cpp value > 1/2 and < 1, while bilayers formed when cpp value about 1, finally cpp value >1 generate inverted micelles or bilayer sheets (Israelachvili, 2011). Figure 2.1 demonstrates the relationship between cpp and the morphology of self-assembled surfactant aggregates.

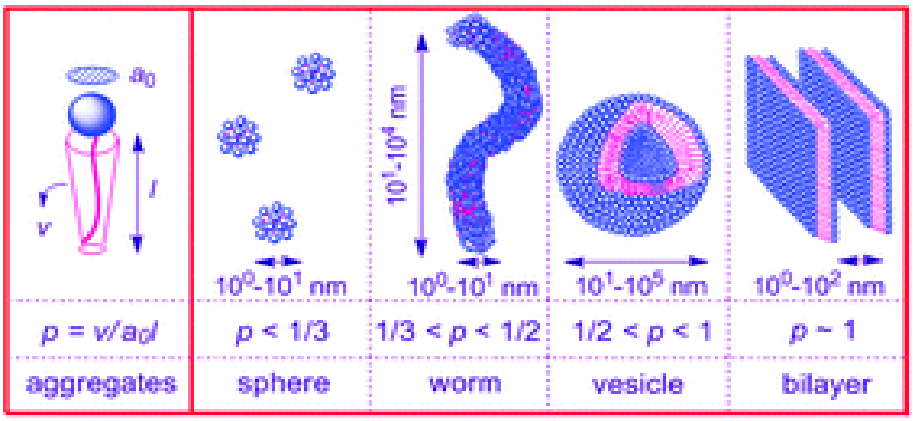


Figure 2.1: Relationship between the packing parameter p and the morphology of self-assembled surfactant aggregates (adapted from (Chu *et al.*, 2013).

2.1.1 Methods of preparation of LNP vesicles

Lipid-based particulates such as NISV and bilosomes are of great interest as they can mimic the particulate nature of micro-organisms that become a target of the APCs (Perrie *et al.*, 2008). NISVs have been proved to have adjuvant activity with some antigens, such as HSV-1 (Hassan *et al.*, 1996) and A/Texas (Walker *et al.*, 1996). Brewer et al. (1998) reported the possibility of immune response modulation by altering the size of the vesicle, with NISVs over 225nm producing a Th1 biased

response, and those under 155nm a Th2 response (Brewer *et al.*, 1998). However, NISV may not allow oral administration of vaccines, as they are unstable in the presence of bile salt (Conacher *et al.*, 2001). The incorporation of bile salts at a low concentration into liposomes improve the stability of the resultant vesicles (Schubert *et al.*, 1983). Similar effects have also been observed with NISV, with addition of sodium deoxycholate (DOC) shown to provide protection against degradation in the GI tract (Conacher *et al.*, 2001); the resulting vesicle was called a bilosome. Conacher *et al.*, found that bilosomes retain 85% of entrapped antigen after treatment with 20mM bile salt solution *in vitro*, whilst NISV retained only around 40% (Conacher *et al.*, 2001).

The conventional process of preparing colloidal suspensions of LNP vesicles involves evaporation to produce a lipid film followed by hydration with medium containing antigen. Vesicles can be then separated from un-entrapped antigens and the final products characterised and analysed by various methods to determine size, shape and entrapment efficiency (Torchilin and Levchenko, 2003). Several methods have been developed since then to produce lipid-based vesicles listed in Table 2.1.

In this thesis, vesicle production was based on methods involving melting lipids by heat. 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 (Mozafari, 2005). Similarly, formation of unilamellar vesicles was achieved by melting lipids at 120°C, followed by hydration with aqueous solution, homogenisation and incubation at 30°C for approximately 3h. Aqueous solution containing the active immunogen was then added and the system homogenised again,

forming small unilamellar vesicles (Mann *et al.,* 2004). This formulation method has been successfully used in a murine model for oral vaccine delivery against both influenza (A/Panama, N/Cal (Mann *et al.,* 2004; Bennett *et al.,* 2009)) and tetanus toxoid (Mann *et al.,* 2006).

Several techniques were used for removal of un-entrapped antigens when required. However, this adds an expensive step to the final method and could cause loss of or changes to the formulated vesicles. Sephadex column chromatography may lead to loss of lipids and vesicles due to retention on the column, vesicle leakage and permeability changes that may occur. Purification using centrifugation, is considered suitable and effective in research purposes, but expensive to be used in industrial scale-up (Torchilin and Levchenko, 2003).

Sterility of final products is also important when developing vaccines for commercial purposes. However, it is often considered only to be possible with liposomes via filtration, which does not remove most viruses, imposing costly and time consuming microbiological control (Mozafari, 2005). Heat sterilisation (121°C, 20min) can be used for liposomes (Kikuchi *et al.,* 1991). However, this is not relevant where a heat sensitive antigen is entrapped, making it inappropriate in the case of most protein vaccines.

Technique	Structure	Reference
Hand shaken multilamellar vesicles	multilamellar vesicles	Bangham *et al.*, 1965
Injection methods	large unilamellar vesicles	Batzri and Korn, (1973)
Emulsion method	unilamellar liposomes	Meure, (2008)
Reverse phase evaporation	large unilamellar vesicles	Szoka and Papahadjopoulos, (1978)
Microfluidic channel method	liposomes	Jahn *et al.*, (2007)

Table 2.1: Organic solvent based techniques for production of lipid particles with exposure to organic solvents

2.1.2 Characterisation of vesicles

Vesicle structure and shape can be characterised by several types of microscopy including optical (Wadhe *et al.*, 2009), freeze fracture electron (Mokhtar *et al.*, 2008), surface electron, scanning electron and negative staining transmission electron (Bibi *et al.*, 2011). Size and charge can be assessed using a multifunctional zeta potential analyser (Bhaskaran and Lakshmi, 2009). Entrapment efficiency is determined by measuring the difference between the un-entrapped and total amounts of drug. Un-entrapped antigen is determined by various techniques such as exhaustive dialysis (Baillie *et al.*, 1985) and centrifugation (Uchegbu and Vyas, 1998a). Centrifugation allows the determination of antigen concentration in supernatant or pellets and consequently, the antigen entrapment efficiency can then be calculated.

Aims in this chapter

In this chapter; two different methods (heat and microwave) were used to prepare lipid nanoparticles (LNP). Different physical characteristics of the LNP, such as size and zeta potential, antigen loading, associated lipid concentration, the morphological appearance and shape characteristics were compared.

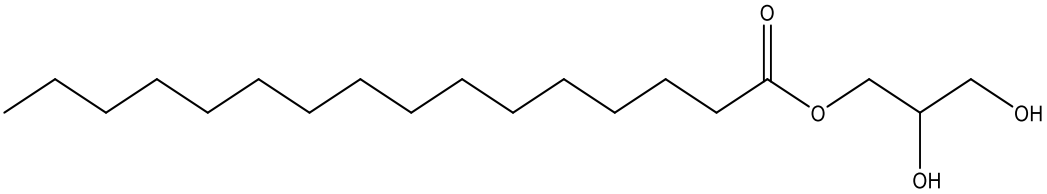
2.2 Methods

2.2.1 Lipid nanoparticle (LNP) preparation

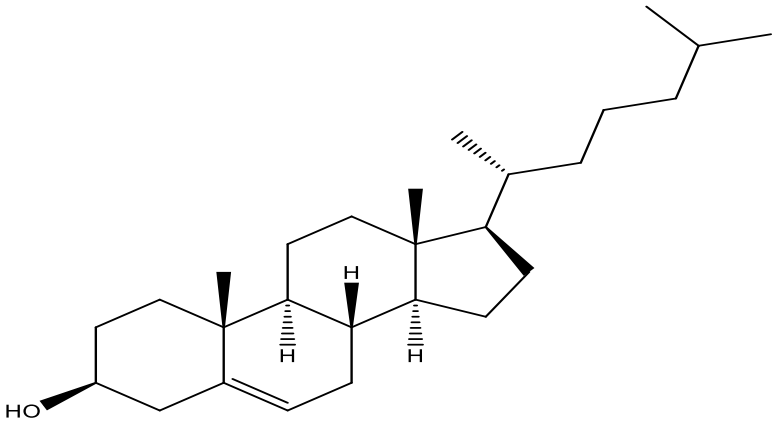
Two methods were used in vesicle preparation; the first method was initially described by Mann *et al.,* 2004 and modified by Bennett *et al.,* 2009. The second preparation method used microwave irradiation in order to melt the lipids. Chemical structures of LNP lipid components are illustrated in Figure 2.2.

2.2.1.1 Mann modified method (MM)

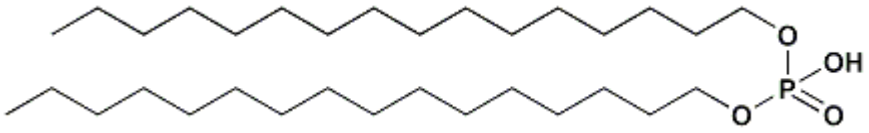
Briefly, 248mg 1-monopalmitoyl glycerol (MPG, 150µM, Larodan AG, Sweden), 234mg cholesterol (CHOL, Sigma-Aldrich, UK) and 82mg dicetyl phosphate (DCP, Sigma-Aldrich, UK) (5:4:1 molar ratio for a final volume of 10ml) were melted in a 25ml round bottomed flask using an oil bath at 120°C then hydrated by addition of 3.78ml of 0.025M carbonate buffer, pH 9.7 (preheated to 60°C), then homogenisation for 2min at 8000 rpm (Silverson Machines Ltd., UK). Bile salt solution (1ml of 100mM of sodium deoxycholate (DOC, Sigma-Aldrich, UK) in 0.025M carbonate buffer pH 9.7) preheated to 60°C was added and the mixture homogenised for 8min at 8000 rpm before being cooled to 30°C in a water bath over 2hr. Finally, 2mg of protein (in these preparation bovine serum albumin, BSA, was used to determine entrapment efficiency) in 5.22 ml carbonate buffer pH 9.7 preheated to 30°C was added to the bile-lipid mixture and homogenised for 1min at 8000 rpm.



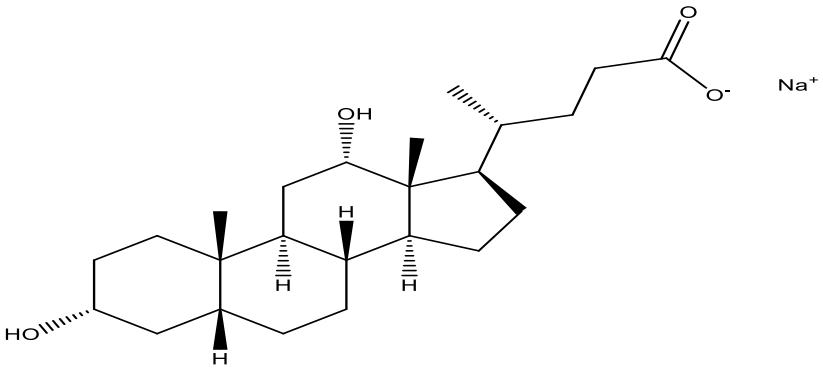
1-monopalmitoyl glycerol



Cholesterol



Dicetyl phosphate



Sodium deoxycholate*

Figure 2.2 Major components used in LNP preparation: 1-monopalmitoyl glycerol, cholesterol and dicetyl phosphate. * involved in bilosome preparation. Structures were drawn using ChemBioDraw V13.0.

2.2.1.2 Microwave method (MW)

The lipid molar ratio of 5:4:1 was used in this method for preparation of NISVs or bilosomes. All solid ingredients were added to 8ml of carbonate buffer (0.025M, pH 9.7) in a 20ml microwave glass tube and sealed. For bilosome preparation, 1ml of carbonate buffer was replaced by 1ml of 100mM bile salt solution before adding lipid ingredients. The mixture was irradiated in a Biotage Initiator microwave (Biotage, Upsala, Sweden) for 2min at 140°C under pressure with continuous stirring. After cooling to 30°C, 2ml of antigen solution in carbonate buffer was added slowly with continuous stirring for 1h. The mixture was vigorously vortexed for 5min at high speed. The formed emulsion was kept at 4°C until used in immunisation or lyophilised for long-term storage.

2.1.3 Lyophilisation of vesicles

Lyophilisation was achieved using an Edwards Modulyo freeze drier at -45°C under pressure for 24h. The dry powder was stored at room temperature.

2.2.4 Vesicle size and zeta potential (ζ-potential)

Particle sizing and zeta potential (ζ-potential) measurements were determined by dynamic light scattering (DLS) and phase analysis light scattering (PALS), respectively, using a Nano ZS® (Malvern, UK) at 25°C. Each measurement was carried out for three runs and the average taken. Bilosome and NISV samples were diluted 1:50 in 0.025M carbonate buffer, pH 9.7 immediately before measurements were taken.

2.2.5 Scanning Electron Microscopy (SEM)

SEM was carried out in Electron Microscopy Facility, School of Life Sciences, University of Glasgow, UK. Briefly, diluted LNP samples were deposited onto a carbon-tape substrate attached to aluminium pin stubs and coated with gold/palladium with a Polaron SC515 sputter coater and imaged on a JEOL 6400 scanning electron microscope with an ADDA3 digital interface at 3-10kV.

2.2.6 Quantification of the protein entrapped within LNP vesicles:

Entrapment of protein was quantified using two different methods. During initial experiments a modified ninhydrin assay was used. This method reported to be unaffected by lipid interference and was previously described by Brewer *et al.*, (1995). For determination of un-entrapped antigens, a bicinchoninic acid (BCA) based method was used for protein quantity determination in the supernatant. For both methods, samples were subjected to ultracentrifugation in order to separate entrapped antigen from free antigen. Briefly, a 0.11ml sample of vesicles, diluted in 4ml of 0.025M carbonate buffer, pH 9.7, was spun in a Beckman tube in a Beckman XL-90 ultracentrifuge (Beckman RIIC, UK) at 35,000 rpm for 2h. The pellet was resuspended in 0.11ml 0.025M carbonate buffer, pH 9.7, and then transferred to 1.5ml microfuge tubes for the ninhydrin protein quantification assay. The supernatant was collected and antigen concentration determined using the BCA assay.

2.2.6.1 Modified ninhydrin assay

The samples, along with standards were prepared with 0.1, 0.25, 0.5, 0.75, 1.0, 1.5 and 2mg of antigen, all in 1.5 ml microfuge tubes, and placed in an oven at 90°C overnight. One hundred and fifty microlitres of 13.5M NaOH was then added to each tube, with a pinhole made in the lid before autoclaving at 121°C/131 kPa 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 5 seconds. To each tube, 500µl of ninhydrin reagent (Sigma–Aldrich, UK) was then added to each sample, vortexed and placed in a water bath at 90°C for 20min. Two hundred and fifty microliters 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. Absorbance was read at 540nm in a microplate reader (SpectraMax M5, Molecular Devices, USA), with test sample levels determined by linear regression from the standard calibration curve.

2.2.6.2 BCA assay

Supernatants obtained from ultracentrifugation were used in this assay to determine the quantity of un-entrapped antigen. Briefly, BSA was dissolved in 0.1M NaOH/1% (w/v) sodium dodecyl sulphate (SDS) to a final concentration of 2mg/ml to prepare a stock standard solution. Twenty five microliters of each sample was added to 75µl of 0.1M NaOH/1% (w/v) SDS. Samples and BSA were heated in a block set at 90°C for 15 min and then allowed to cool before being centrifuged at 13000 rpm for 15 min. Ten microlitres of each sample and different BSA standard dilutions (125-2000µg/ml) was transferred into a microtitre plate wells.. To each

well, 190µl of BCA combined reagent (Thermo Fisher Scientific Inc., UK) was added and the plate was incubated at 37°C for 30min. The samples were read at absorbance 560nm in a SpectraMax M5 plate reader. The quantity of antigen in the supernatant was then subtracted from the total antigen amount added to the LNP in order to calculate the entrapment efficiency percentage (EE %).

2.2.7 Entrapped bile acid quantification

In order to determine the amount of bile acid entrapped in the vesicle, an enzymatic colorimetric assay for quantification of total bile acids (Randox laboratories, UK) was used following the manufacturer's protocol. Two reactions are combined in this kinetic enzyme cycling method. In the first reaction bile acids are oxidised by 3-α hydroxysteroid dehydrogenase with the subsequent reduction of thio-NAD to thio-NADH. In the second reaction the oxidised bile acids are reduced by the same enzyme with the subsequent oxidation of NADH to NAD. The rate of formation of thio-NADH is determined by measuring the specific absorbance change at 405nm. Briefly, a 0.11ml sample of vesicles, diluted in 4ml of 0.025 M carbonate buffer, pH 9.7, was spun in a Beckman tube in a Beckman XL-90 ultracentrifuge (Beckman RIIC, UK) at 35,000 rpm for 2h. The pellet was resuspended in 0.11ml 0.025M carbonate buffer, pH 9.7. In a flat-bottomed 96-well plate, 3µl from each sample and from a calibration solution (cal, 100µM bile salt solution) was added into wells containing 200µl of reagent 1 and mixed well. The samples were then left for 5min to equilibrate. Sixty microlitres of reagent 2 was added to each well and mixed. After 1min an initial absorbance (A_1) was read at wavelength 405nm at 37°C

measured against a blank (distilled water), then after a further 2min a second reading (A_2) was taken against the blank well.

The concentration of bile acids was determined using the following equation:
Concentration = A_2-A_1 (sample)/ A_2-A_1 (cal) X conc. of cal.

2.2.8 High-performance liquid chromatography (HPLC) analysis of lipids

The lipid content of LNP was analysed using a modified gradient normal phase HPLC method (Alsaadi, 2011). One millitre of LNP suspension in 1.5ml micro-tubes were spun at 13000 rpm for 10min, the supernatant was discarded and pellet dried by lyophilisation. Prior to the lipid analysis, 10mg of the lyophilised LNP was dissolved in 10ml of chloroform stabilised with ethanol to prepare a 1mg/ml solution. To obtain standard curve for each ingredient, stock solutions were prepared from 10mg of the individual constituents dissolved in 10ml of chloroform stabilised with ethanol to prepare a 1mg/ml stock solution. Then six dilutions (12, 25, 50, 100, 250 and 500µg) were separated using HPLC to obtain a high R^2 value and accurate equation.

2.2.8.1 HPLC instrumentation and chromatographic conditions

The HPLC system consisted of a Gynkotek® HPLC pump series P580 and autosampler model GINA50 (Macclesfield, Cheshire, UK) operated by Chromeleon™ software version 6.30 SP3 Build 594, Dionex (Surrey, UK). Separation was carried out on a YMC-PVA Silica column (100 × 3.0mm i.d. and 5µm particle size) from Hichrom Limited (Berkshire, UK) attached to a guard column packed the same as the column with PVA-Sil (10 × 3.0mm i.d. and 5µm

particle size) from Hichrom Limited (Berkshire, UK). Detection was facilitated by an evaporative light scattering detector (ELSD) model 500 (Alltech, UK) supplied with 5l of nebulisation gas by a compressor and optimised at 80°C and gas flow rate of 2.90 standard litres per minute (SLPM).

A gradient ternary elution was used for separation of the lipids, where solvent A was isohexane, solvent B was ethyl acetate and solvent C was 60% (v/v) propan-2-ol, 30% (v/v) acetonitrile, 10% (v/v) methanol, 142µl/100ml glacial acetic acid and 378µl/100ml triethylamine (Table 2.2). The gradient elution was run for 15min at a flow rate of 1ml/min where ingredients eluted within 10min and the final 5min was for column regeneration.

Time (min)	A	B	C
0	80	20	-
2	72	25	3
3	64	30	6
4	56	35	9
5	48	40	12
6	35	45	20
7	35	45	20
8	35	45	20
9	72	25	3
10	80	20	-
15	80	20	-

Table 2.2 Gradient elution sequence used in lipid analysis using 100% isohexane (A), 100% ethyl acetate (B) and a mixture of 60% (v/v) propan-2-ol, 30% (v/v) acetonitrile and 10% (v/v) methanol, 142μl/100ml glacial acetic acid and 378μl/100ml triethylamine (C).

2.3 Results

2.3.1 Vesicle sizing/ zeta potential

Measurement of particle size and ζ-potential values of formulated LNP by both adopted methods have shown that there was an overall slight increase of size in the MW compared with the MM which was not significant ($p>0.05$, Table 2.3). However, loaded LNP showed a significantly larger mean size than empty ones in both methods ($p<0.05$ and $p<0.01$ for bilosomes and NISV, respectively, Figure 2.3). Lyophilisation caused a significant ($p<0.001$) increase in the size of empty bilosomes (EB), loaded bilosmes (LB) and empty NISV (EN). While loaded NISV (LN) showed a significant ($p<0.05$) increase of vesicles size after lyophilisation (Figure 2.4). The ζ-potential values recorded for all formulations indicate that they were negatively charged and was in the range between (-80 to -130mV) with no significant variation between the type of LNP or protocol used (Table 2.3 and Figure 2.5).

Preparation method	LNP	Size (nm)±SD	PDI	ζ (mV)	EE%
Mann modified	**EN**	265±33	0.34	-90±8	NA
	LN	375±105	0.65	-108±21	25±3.2
	EB	198±10	0.27	-100±13	NA
	LB	385±30	0.31	-97±10	35±2.6
Microwave	**EN**	320±80	0.46	-116±17	NA
	LN	506±186	0.71	-104±9	24±2.2
	EB	236±68	0.64	-109±12	NA
	LB	316±14	0.44	-96±7	31±6.8

Table 2.3 Size, Zeta (ζ-potential) values and entrapment efficiency percentage (EE%) for various lipid nanoparticles (LNP).
Abbreviations; PDI: Polydispersity index, EB: empty bilosome, LB: loaded bilosome, EN: empty NISV and LN: loaded NISV.

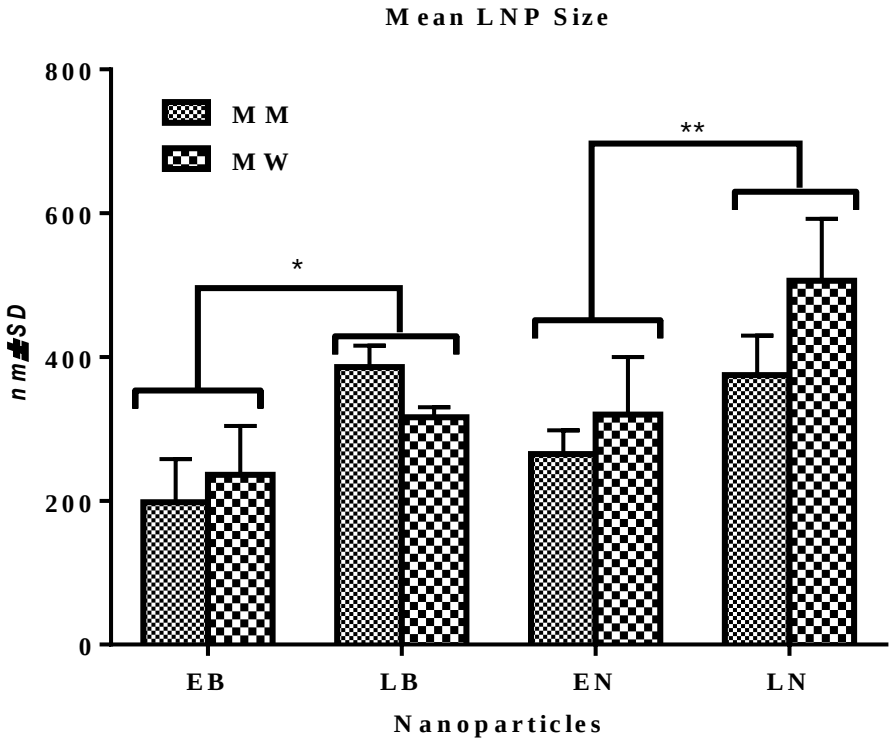


Figure 2.3 Mean sizes for different lipid nanoparticle formulations. Bars represents the mean of triplicate samples reading ±SD. * ($p<0.05$) and ** ($p<0.01$) indicates significance level between compared particles.

Abbreviations; LNP: Lipid nanoparticles, MM: Mann modified method, MW: microwave method, EB: empty bilosome, LB: loaded bilosome, EN: empty NISV and LN: loaded NISV.

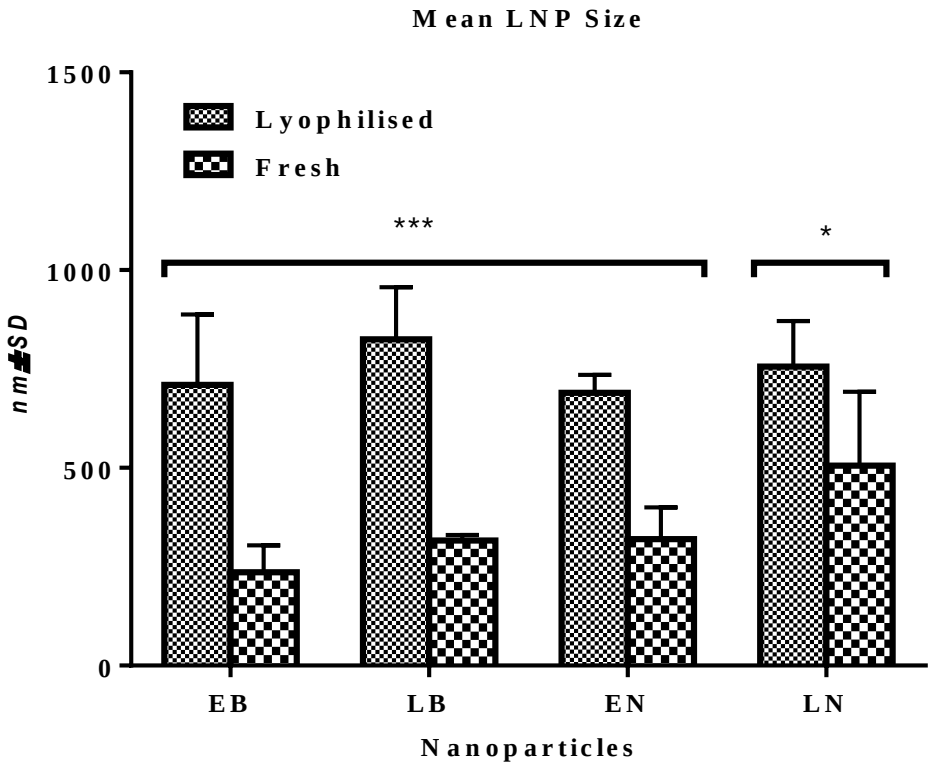


Figure 2.4 Mean sizes for fresh and lyophilised lipid nanoparticle formulations. Bars represents the mean of triplicate samples reading ±SD. * ($p<0.05$) and *** ($p<0.001$) indicates significance level between compared particles.

Abbreviations; LNP: Lipid nanoparticles, MM: Mann modified method, MW: microwave method, EB: empty bilosome, LB: loaded bilosome, EN: empty NISV and LN: loaded NISV.

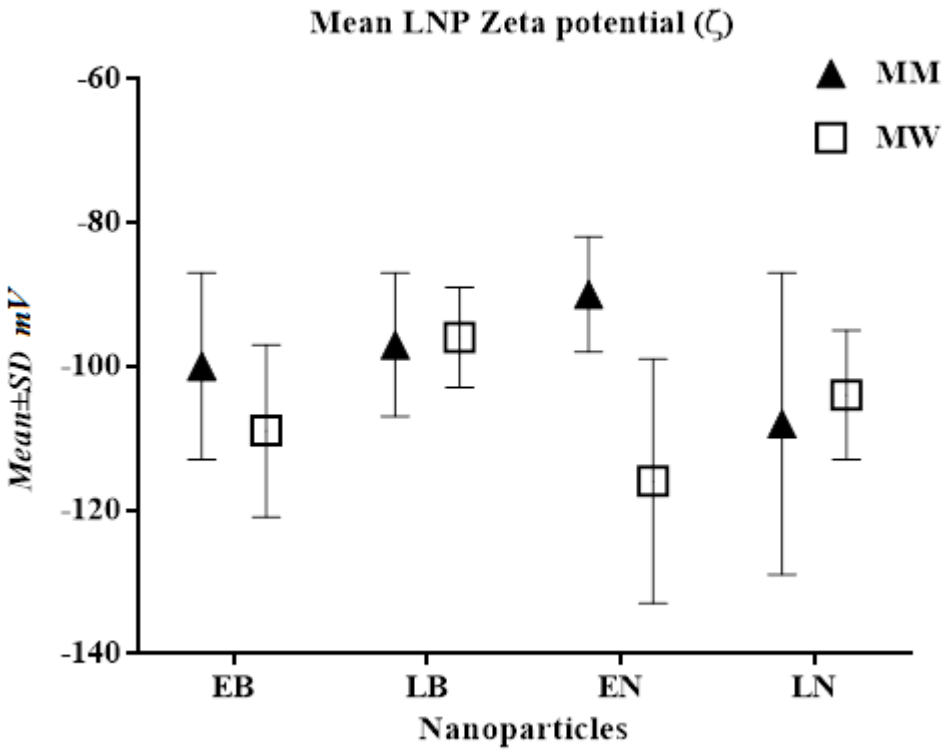


Figure 2.5 Mean ZP (ζ-potential) for different lipid nanoparticle formulations. Bars represent the mean of triplicate samples reading ±SD.

Abbreviations; LNP: Lipid nanoparticles, MM: Mann modified method, MW: microwave method, EB: empty bilosome, LB: loaded bilosome, EN: empty NISV and LN: loaded NISV.

2.3.2 LNP microphotograpghy by SEM

There was no difference detected between images of NISV or bilosomes. Both formulations contain nanoparticles that are spherical structures without lipid sheets or crystalline bodies, with a diameter range from 150nm to 2microns (Figure 2.6).

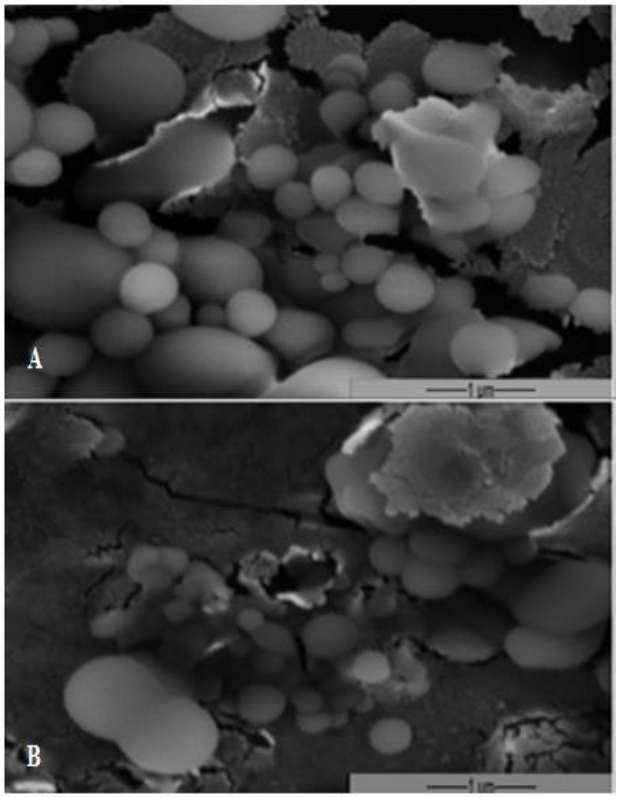


Figure 2.6 Scanning electron micrograph showing typical range of (A) bilosomes and (B) NISV. Samples were coated with gold/palladium with a Polaron SC515 sputter coater and imaged on a JEOL 6400 scanning electron microscope with an ADDA3 digital interface at 3-10kV to enhance surface imaging.

2.3.3 Estimation of antigen entrapment percentage

Standard curves, with high R^2 factors, for both assays used in this research allowed the determination of protein concentration from LNP samples (Figure 2.7). The ninhydrin assay showed that the range of antigen loading into LNP was between 22-38% of the original amount of proteins added to the mixture. Entrapment efficiency was significantly higher in the bilosome than in the NISV ($p<0.05$, Table 2.3). Determination of protein concentration by a BCA assay in the supernatant after ultra-centrifugation also confirmed the EE percentage observed by ninhydrin assay (Figure 2.8).

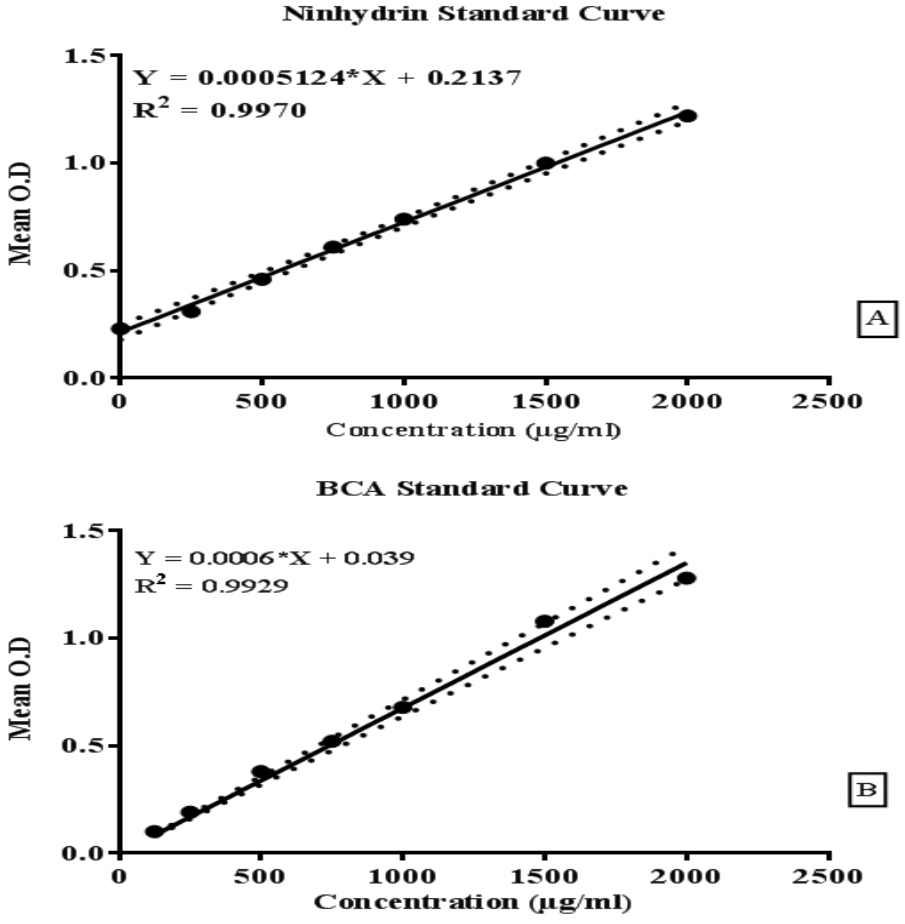


Figure 2.7 Typical standard curves generated for the ninhydrin (A) and BCA (B) assays using serial dilutions of a known amount of BSA.

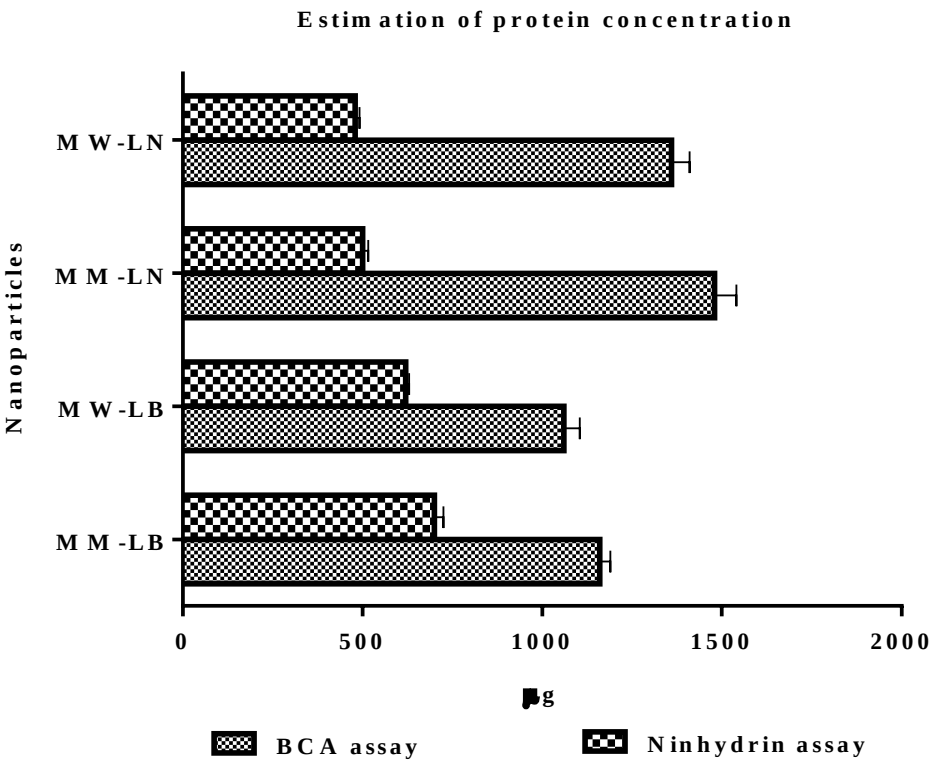


Figure 2.8 Protein concentration in various LNP prepared by the MM and MW methods. The ninhydrin assay was used for estimation of protein concentration entrapped in the LNP. While the BCA assay used for estimation of protein concentration in the supernatant of formulation mixtures.

Abbreviations; LNP: Lipid nanoparticles, MM: Mann modified method, MW: microwave method, LN: loaded NISV, LB: loaded bilosome.

2.3.4 Estimation of entrapped bile acid in bilosome formulation

The amount of entrapped DOC was determined using an enzymatic colorimetric assay for total bile acid quantification. Diluted samples were ultra-centrifuged, the amount of bile acid in pellet, supernatant and whole mixture showed that >75% of DOC is present in the pellet and <10% is free (un-entrapped) in the supernatant (Figure 2.9).

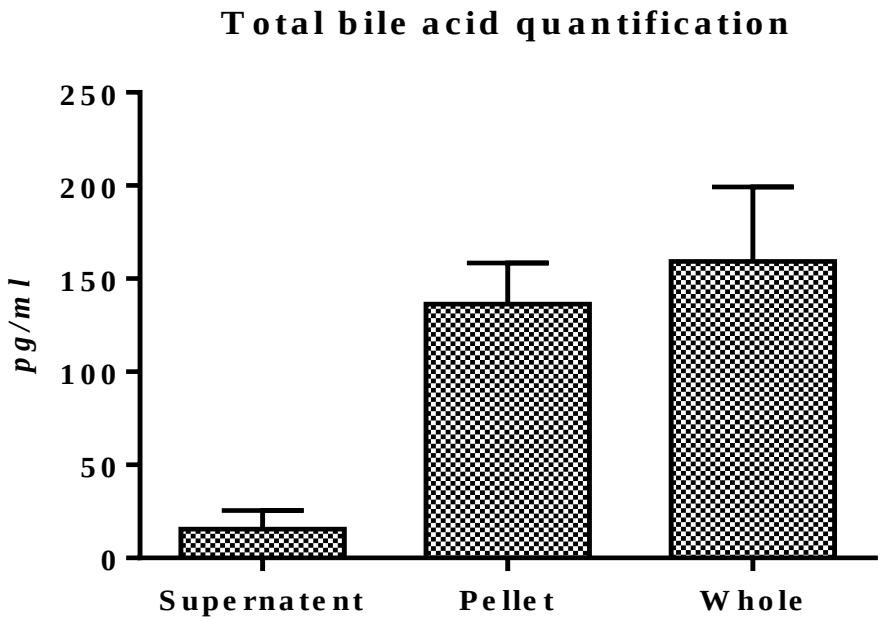


Figure 2.9 Amount of DOC in the supernatant, pellet and whole bilosome formulation quantified by an enzymatic colorimetric assay for total bile acid quantification.

Abbreviation: DOC: Sodium deoxycholate.

2.3.5 Analysis of lipids by HPLC

A standard curve was created for each ingredient using 6 dilutions (Figure 2.10) which allowed the calculation of the concentration of each compound in the lipid layer of the LNP (Table 2.4). The retention time for CHOL, MPG, DOC and DCP were 1.87, 4.64, 9.04 and 9.54min, respectively (Figure 2.11). There was no significant difference in the amount of lipids between freshly prepared and freeze dried bilosome or NISV formulations. HPLC analysis of the chloroform layer of EN and EB revealed the incorporation of DOC in the lipid layer of the bilosomes (Figure 2.12 and Figure 2.13).

	Amount mg/ml			
Sample	**CHOL**	**MPG**	**DOC**	**DCP**
Fresh Bilosome	44.13	38.98	4.18	6.68
FD Bilosome	42.37	39.21	4.03	6.29
Fresh NISV	43.82	41.33	-	7.24
FD NISV	41.03	39.54	-	6.86

Table 2.4 Estimated amounts in mg/ml of CHOL, MPG, DOC and DCP in fresh prepared and freeze dried LNP.
Abbreviations; LNP: Lipid nanoparticles, CHOL: cholesterol, MPG: 1-monopalmitoyl glycerol, DOC: Sodium deoxycholate, DCP: Dicetyl phosphate.

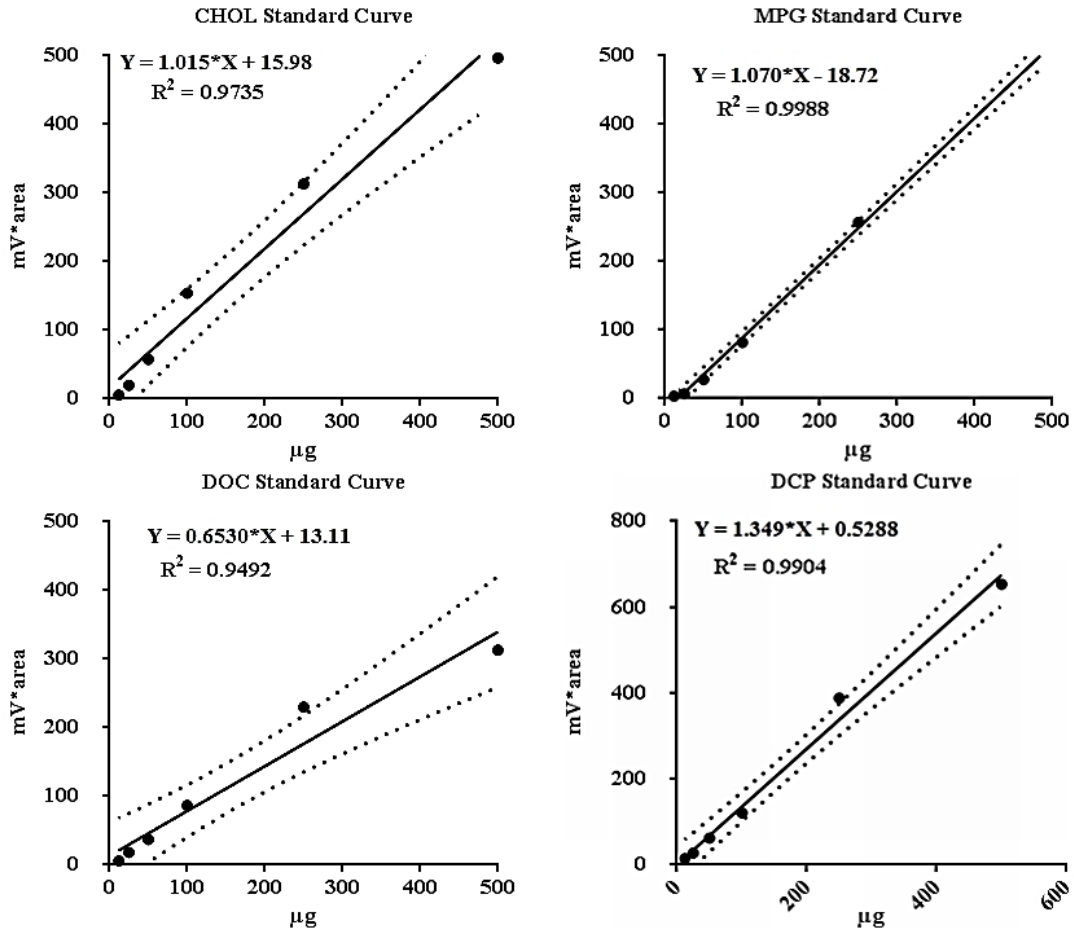


Figure 2.10 Standard curves created for each ingredient using 12, 25, 50, 250 and 500µg/ml for the calculation of the concentration of each compound in LNP formulations.

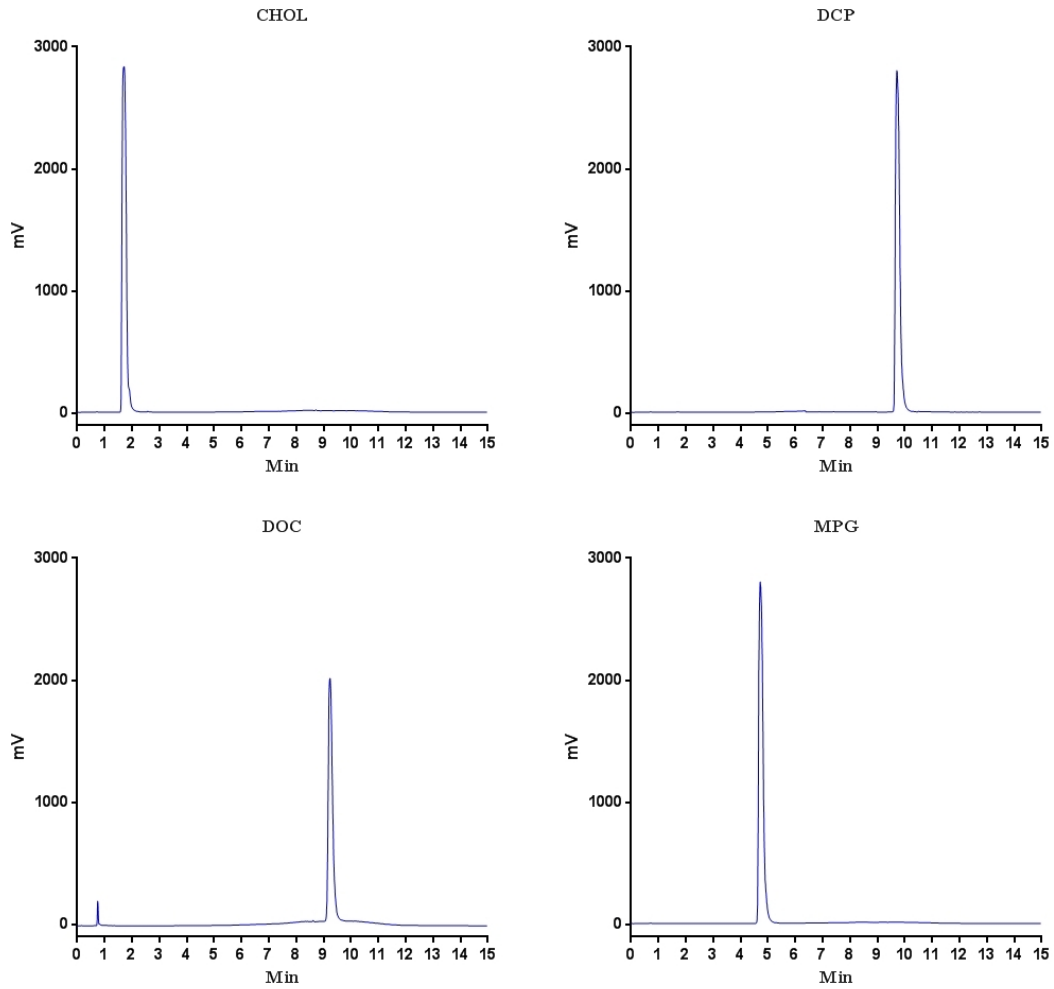


Figure 2.11 chromatograms illustrating elution (retention time) of individual standards of CHOL, MPG, DOC and DCP at 1.87, 4.64, 9.04 and 9.54min, respectively. Injection volume was 10µl of 1mg/ml in chloroform stock solution.

Abbreviations; CHOL: cholesterol, MPG: 1-monopalmitoyl glycerol, DOC: Sodium deoxycholate, DCP: Dicetyl phosphate.

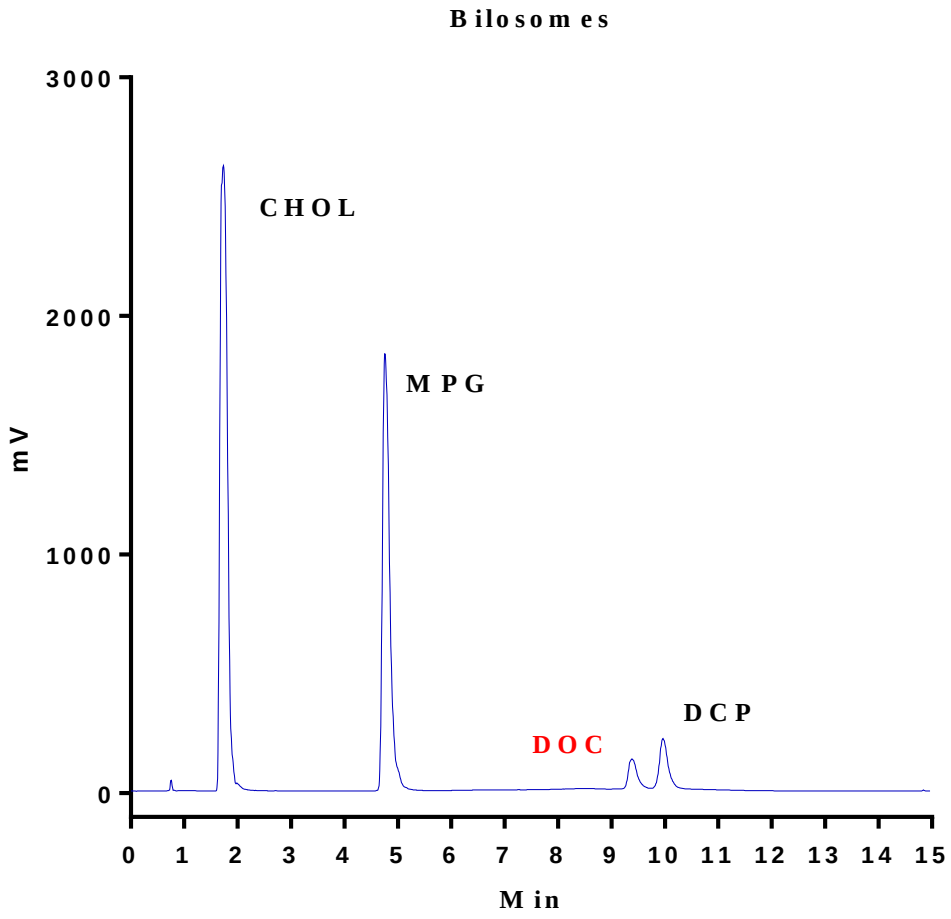


Figure 2.12 A chromatogram illustrating the separation and elution of CHOL, MPG, DOC and DCP at 1.87, 4.64, 9.04 and 9.54min, respectively. Injection volume was 10µl of bilosomes dissolved in chloroform.

Abbreviations; CHOL: cholesterol, MPG: 1-monopalmitoyl glycerol, DOC: Sodium deoxycholate, DCP: Dicetyl phosphate.

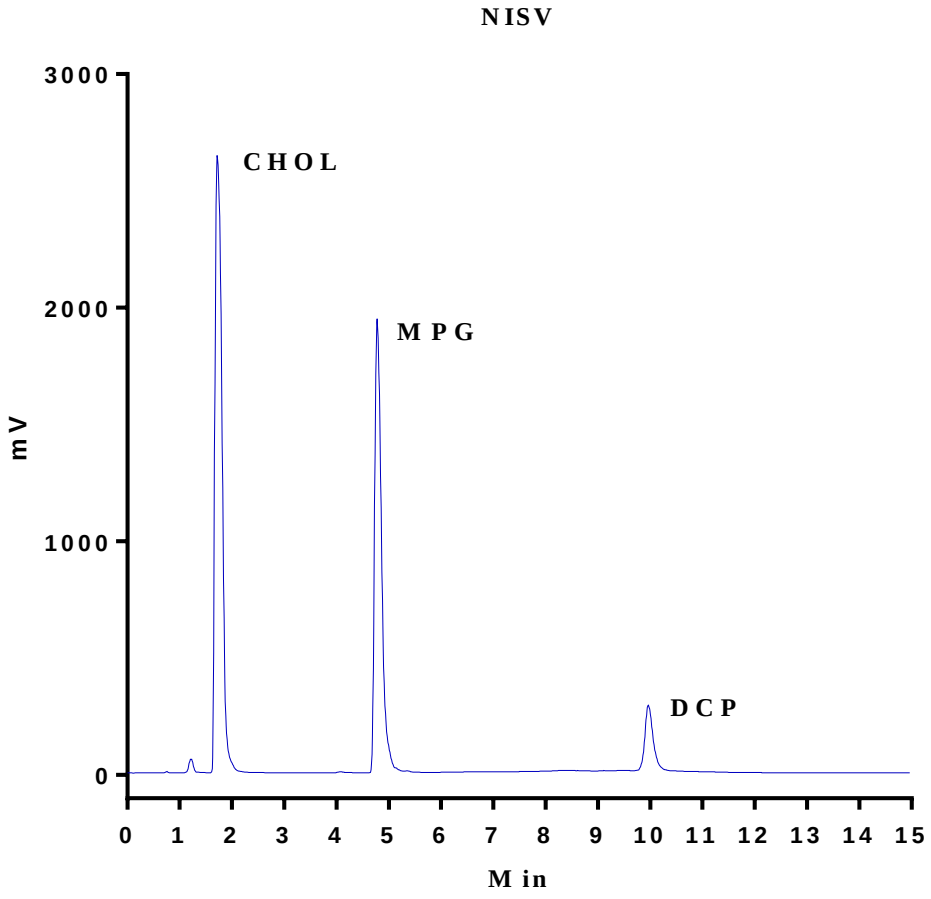


Figure 2.13 A chromatogram illustrating the separation and elution of CHOL, MPG, and DCP at 1.87, 4.64 and 9.54min, respectively. Injection volume was 10µl of NISV dissolved in chloroform.

Abbreviations; CHOL: cholesterol, MPG: 1-monopalmitoyl glycerol, DOC: Sodium deoxycholate, DCP: Dicetyl phosphate.

2.4 Discussion

Bilosome and NISV vesicles were successfully constructed using established protocols; the Mann modified method, MM (Bennett *et al.,* 2009) and the microwave method, MW (Bennett, 2010). The final product, a white emulsion of vesicles in carbonate buffer was stable for 3-4 weeks at 4˚C, after this period the vesicles tended to sediment to the bottom leaving a semi-turbid supernatant. However, the vesicles were easily suspended by hand shaking. The sedimentation rate was very low as it was not noticed in batches used for vaccination due to frequent handling for immunisation purposes. This could be due to ζ values observed in the mixtures which ranged between -80 and -130mV causing the particles to be in continuous movement. Particles in colloidal dispersions with high positive or negative ζ values are resistant to aggregation and become electrically stabilised (Hanaor *et al.,* 2012). The average size of empty bilosomes varied according to the method used. Bilosomes produced by the MM method gave small vesicles sized between 90 to 300nm (average ~ 200nm). However, the bilosome average size produced by the MW method was slightly larger, about 130 to 420nm (average ~ 240nm). These variations in vesicle size indicate that small vesicles are obtained by the mechanical homogenisation procedure, which can be applied to the MW method to decrease the vesicle size if desired. Similarly, empty NISV produced by the MW method were slightly larger than those produced by the MM method. A recent review described the average vesicle size produced by a lipid film hydration method to be between 1-3 μm (Myschik, 2009). Recently, Bennett *et al.,* pointed out that vesicle size varied depending on the homogenisation time (Bennett *et al.,* 2009). 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 (Brewer *et al.*, 1995; Kersten and Crommelin, 2003; Singh *et al.*, 2004). However, the size range obtained in this study is consistent with bilosomes formed by earlier studies (Mann *et al.*, 2004). A marked increase in size was observed after lyophilisation in all formulations. Earlier studies showed that lyophilisation of liposomes increased their size by about 2-7 fold on rehydration (Bridges and Taylor, 2001; Mohammed *et al.*, 2006), therefore the increase observed in this study is within the reported range.

Estimation of EE% revealed that bilosomes produced by both protocols showed high antigen entrapment efficiency compared with NISV. Although, this increase was not significant, this could be to the presence of DOC which is a known agent used routinely for isolation of membrane proteins and lipids (Zhou *et al.*, 2006). Earlier methods for bilosome preparation used freeze-thaw cycles to improve entrapment, and gave EE% between 40-50% (Mann *et al.*, 2004; Mann *et al.*, 2006). However, this increased the manufacturing time and was difficult to be scale up for commercial purposes. In addition, EE% is also known to vary with different antigens (Torchilin, 2005; Aguilar and Rodriguez, 2007; Peek *et al.*, 2008), and the values obtained in this study are within the expected range given the absence of the freeze-thaw steps.

Bilosomes are derivatives of NISV formulation with a lipid bilayer. However, the actual position of DOC in the lipid layer was not fully confirmed. HPLC was used for analysis of the lipid in the pellet after ultracentrifugation. Due to the poor UV absorbance of the lipids, several HPLC methods based on ELSD were used to monitor the separation (Felgner, 1997; Descalzo *et al.*, 2003; Simonzadeh, 2009). The analysis confirmed that most of the DOC is associated with the lipid portion of

the particle. A probable answer is that DOC maybe forms strong bond such as covalent bonds to the phosphate moiety of DCP. Otherwise, the dilution steps in the preparation of samples prior to ultracentrifugation could remove DOC from the lipid phase into the aqueous phase. This was not the case as demonstrated by the low amounts found in the aqueous supernatant phase using a total bile acid assay carried out in this study. This has also been confirmed from the HPLC analysis that showed the expected amounts of DOC in the chloroform lipid layer phase (Table 2.4). ELSD has been reported to be the best appropriate detector in combination with HPLC for the quantification of lipids (Zhong et al., 2010). To my knowledge, the present study is the first attempt to study the bilosomes lipid analysis by HPLC. In the present study it was possible to obtain a linear concentration response for MPG and DCP, while the CHOL concentration responses were less accurate (Figure 2.10). However, this method needs more optimisation in order to enable proper content analysis of other lipids.

After both NISV and bilosome LNP had been successfully synthesised and several analyses and characterisations carried out. The LNP vesicles should be examined *in vivo* for vaccine delivery via parenteral and oral routes. In the next chapter immunisation of mice model against non-pathogenic immunogen conjugates is described.

Chapter three: Comparison of non-pathogenic antigen vaccination using NISV and bilosomes

3.1 Introduction

Most previous work with the bilosomes has involved non-live, pathogen-derived immunogens (Bennett *et al.,* 2009; Mann *et al.,* 2009b). The aim of this study was to examine the suitability of bilosomes to be used with a model antigen such as GnRH. GnRH is a pituitary hormone that orchestrates the control of steroidogenesis and gametogenesis in both males and females. Vaccines against GnRH have been successfully commercialised for over a decade to control fertility and testosterone-associated meat taints in animals (Miller *et al.,* 2008; Andersson *et al.,* 2012). In contrast, developments in humans for treatment against testosterone-dependent prostate cancer have progressed more slowly (Naz and Shiley, 2012). Clinical trials in patients with locally advanced metastatic (T3/4) prostate cancer have shown that success of outcome can be correlated with achieving castrate levels of testosterone (the growth factor for androgen-sensitive prostate cancer) which is dependent on production of high titres of systemic anti-GnRH antibodies (Parkinson *et al.,* 2004b). It is believed that inhibition of the pituitary-gonadal axis depends on the ability of these antibodies to immunoneutralise circulating GnRH (Figure 3.1) to curtail steroid hormone production (Gual *et al.,* 1997; Talwar, 1997). The first gonadotropin-releasing hormone (GnRH) to be identified was isolated from pig (Matsuo *et al.,* 1971) and sheep (Burgus *et al.,* 1972). To date, there are 14 distinct forms of GnRH have been identified and sequenced from vertebrates and protochordates (Table 3.1). Twelve forms have been identified in vertebrates from jawless fish to humans (Sherwood, 1994; Jimenez-Linan *et al.,* 1997) and additional two from a protochordate (Powell *et al.,* 1996; Craig *et al.,* 1997). All known forms of GnRH are 10 amino acids in length with pyroglutamyl modified N-terminus, an

amidated C-terminus and conserved amino acids in positions 1,4,9 and 10 with greatest structural variation found between amino acids 5-8 (Table 3.1). Three GnRH forms (GnRH-I, GnRH-II and GnRH-III, Figure 3.2) were previously evaluated for immunogenic properties for vaccine production aimed at fertility control (Oonk *et al.,* 1998; Ferro *et al.,* 2004b; Khan *et al.,* 2007a). However, their distribution and complete physiological roles in humans have yet to be elucidated. Table 3.2 shows the known tissue localisation of GnRH-I, GnRH-II and GnRH-III isoforms and receptors. There is a particular interest in GnRH-III as it is found in very specific sites in the brain, whereas GnRH-I and GnRH-II appear to have a more ubiquitous distribution and presumably varied function throughout the body (Tan *et al.,* 2013). When immunising against the "self-peptide", there are integral immunogenicity difficulties and this requires different approaches to enhance the immune response. These have involved the use of carrier proteins (such as keyhole limpet haemocyanin (KLH) and diphtheria toxoid (DT) (Parkinson *et al.,* 2004a; Killian *et al.,* 2009)), molecular biology technologies (Khan *et al.,* 2008) and effective adjuvants (Saenz *et al.,* 2009; Aguilar *et al.,* 2012). The reason for using this antigen is to prove the concept and due to an established history of its development with NISV, administered via the parenteral route (Ferro *et al.,* 2004a), and thus far the adjuvant properties of the LNP and suitability for mucosal administration of GnRH has not previously been evaluated. In addition, two isoforms of GnRH (GnRH-I and GnRH-III), have been evaluated for their fertility control parenterally and shown the efficiency of these isoforms as an immunocontraception vaccine (Ferro *et al.,* 2004b; Turkstra *et al.,* 2005; Khan *et al.,* 2007b).

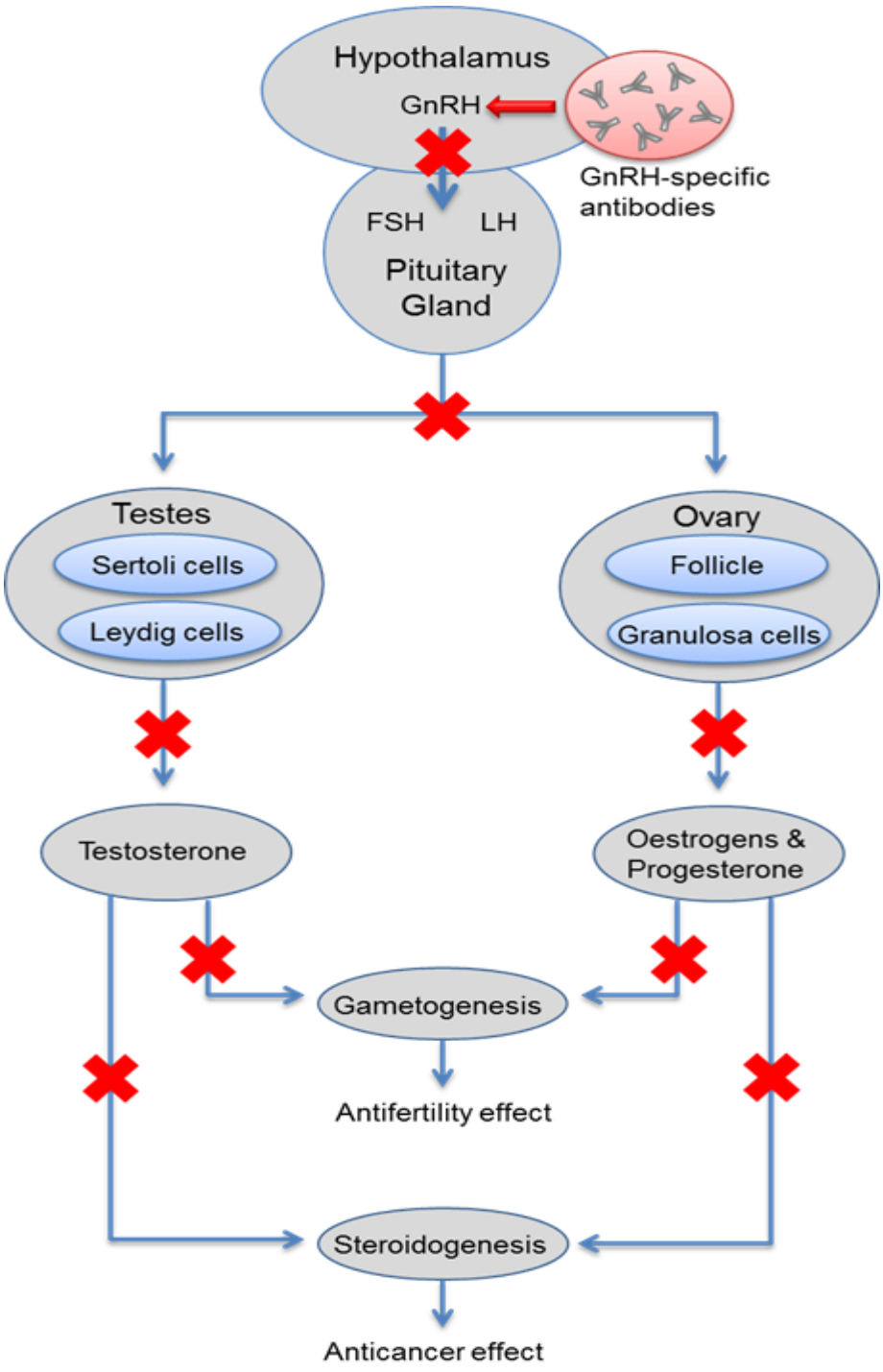


Figure 3.1 Schematic diagram showing immunoneutralisation of circulating GnRH, with potential anti-cancer and anti-fertility applications (Adapted from Gebril *et al.*, 2012)

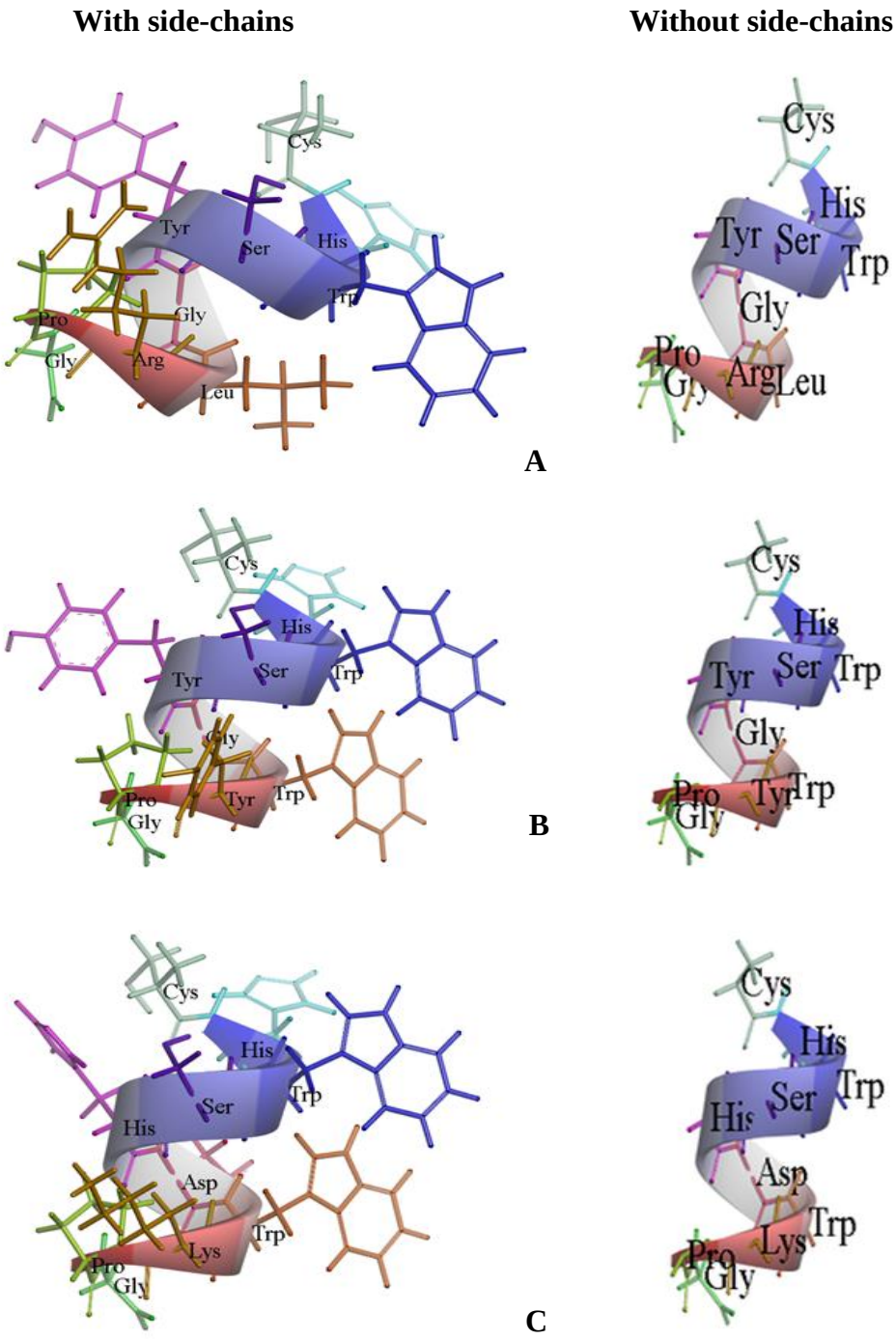


Figure 3.2 Ribbon and molecular simulating of GnRH-I (A), GnRH-II (B) and GnRH-III (C) showing the backbone amino acids with and without side chains. Constructed using Discovery Studio v3.1 (Accelrys, Inc., USA).

GnRH isoform	Amino acid sequences	Origin	Reference
Vertebrates			
GnRH, GnRH-I, mGnRH	pEHWS**YGLR**PG-NH_2	All mammals	(Matsuo *et al.*, 1971)
GnRH-II, cGnRH-II	pEHWSYG**WY**PG-NH_2	Chicken	(King and Millar, 1982)
GnRH-III, lGnRH-III	pEHWS**HDWK**PG-NH_2	Lamprey	(Sower *et al.*, 1993)
GnRH-IV, sGnRH-IV	pEHWSYG**WL**PG-NH_2	Salmon	(Sherwood, 1986)
cGnRH-I	pEHWSYG**LQ**PG-NH_2	Chicken	(Miyamoto *et al.*, 1984)
lGnRH-I	pEH**YS**L**G**W**K**PG-NH_2	Lamprey	(Sherwood, 1986)
dfGnRH-I	pEHWS**H**GL**N**PG-NH_2	Dog fish	(Ngamvongchon *et al.*, 1992)
dfGnRH-II	pEHWS**H**G**WY**PG-NH_2	Dog fish	(Lin *et al.*, 1998b)
cfGnRH-I	pEHWS**H**G**WL**PG-NH_2	Cat fish	(Lin *et al.*, 1998a)
hrrGnRH	pEHWS**H**GL**S**PG-NH_2	Herring	(Carolsfeld *et al.*, 2000)
sbGnRH	pEHWSYGL**S**PG-NH_2	Sea bream	(Powell *et al.*, 1994)
gpGnRH	pE**Y**WSYG**V**RPG-NH_2	Guinea pig	Leschied 1995
Protochordate			
tGnRH-I	pEHWS**DYFK**PG-NH_2	Tunicate	(Powell *et al.*, 1996)
tGnRH-II	pEHWS**LCHA**PG-NH2		

Table 3.1 Vertebrates and invertebrates isoforms of GnRH and their amino acids sequences expressed in single letter code (**bold** letters highlight variable amino acids sequences).

Molecule	**GnRH-I**	**GnRH-II**	**GnRH-III**
	Brain		
	Hypothalamus	Hypothalamus	Hypothalamus
			Midbrain
Receptors	***Extra-hypothalamic***		
	Reproductive system		
	Endometrium	Endometrium	
	Fallopian tube epithelium	Prostate	
	Ovarian GL* cells	Ovarian GL* cells	
	Ovarian surface epithelium	Ovarian surface epithelium	
	Ovarian carcinoma	Ovarian carcinoma	
	Placenta	Placenta	
	Preimplantation embryo		
	Other organs and tumours		
	Breast tissue	Breast tissue	
	Breast cancer	Breast cancer	
		Kidney	
		Bone marrow	

Table 3.2 Location of GnRH and GnRH receptors in higher mammals, *; Granulosa lutein, (Adapted from (Ramakrishnappa *et al.*, 2005; Tan *et al.*, 2013).

Aims of this chapter:

To demonstrate that GnRH isoforms conjugated to established immunogen carrier proteins could produce an immune response after immunisation via parenteral and oral routes. A set of experiments were carried out in male BALB/c mice with the following aims:

1- To compare GnRH-I, II and III conjugated with tetanus toxoid (TT) immunogens, administered subcutaneously, to evaluate antibody response (IgG in sera), cross reactivity of antibodies raised between different GnRH isoforms, testosterone levels, and cytokine release stimulation.

2- For proof of concept of suitability of GnRH conjugates to be administered orally. TT-GnRH conjugates loaded in bilosomes were administered by the oral route, and evaluation of IgG and IgA produced against TT-GnRH conjugates for systemic and local immune responses.

3- To compare the adjuvant effect of NISV versus alum-based adjuvant (Imject®) of GnRH-I and III immunogens conjugated to a different carrier protein, ovalbumin (OVA), and administered orally, and to observe the change of the carrier protein on immunogenicity.

3.2 Methods

3.2.1 Immunogen and coating antigen preparation

This section describes the methods used for preparation of immunogen (peptide conjugation with different carrier proteins) used in vaccination and used as coating antigen in ELISA. Peptides were conjugated to three carrier proteins: bovine serum albumin (BSA), TT and OVA.

3.2.1.1 Tetanus toxoid-GnRH peptide conjugation

In order to prepare TT-GnRH peptide conjugates for immunisation the following synthesised peptides were used: GnRH-I (CHWSYGLRPG-NH2), GnRH-II (CHWSYGWYPG-NH2) and GnRH-III (CHWSHDWKPG-NH2), purity greater than 95%, synthesised by Immune Systems Ltd., Torquay, UK. A conjugation procedure previously described by Ferro *et al.*, (1996) was adopted.

Briefly, 8μmol sulpho-maleimidobenzoyl-N-hydroxy-succinimide (S-MBS, Perbio Science Ltd., Cheshire, UK) was dissolved in 100μl of 0.05 M sodium phosphate buffer, pH 8.0, and added to TT solution (0.1μmol, a gift from Dr. Mark Lavery, Intervet Ltd., UK). The mixture was incubated in the dark, at room temperature, for 30 min, followed by dialysis overnight at 4°C against 0.9% (w/v) saline, pH 6.5. The peptide (4μmol) was dissolved in 100μl of 0.9% (w/v) saline (pH 6.5) and added drop-wise to the activated tetanus toxoid. The mixture was incubated in the dark at room temperature for 3h, followed by dialysis overnight at 4°C against 0.9% (w/v) saline, pH 7.2. The peptides conjugated were designated TT-GnRH-I, TT-GnRH-II and TT-GnRH-III and kept at -20°C for later use in animal

immunisation and preparation of antigen entrapped vesicles. The volume was recorded and peptide concentration calculated assuming that all the peptide bound in a 1:2 ratio.

3.2.1.2 Peptide conjugation to OVA and BSA

GnRH-I, GnRH-II and GnRH-III pure peptides were conjugated to OVA or BSA as described by Oonk *et al.*, (1998). Briefly, equal weights of both GnRH peptide and carrier protein (OVA or BSA) were dissolved separately in similar volumes of MilliQ water. Both solutions were thoroughly mixed by stirring for 1/2h at room temperature. Next, a tenfold excess based on weight equivalent of 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDC), was dissolved in MilliQ water and slowly added to the peptide/protein solution under continuous stirring for at least 6h at room temperature. The resultant solution was dialysed against MilliQ water over night at 4°C. An additional dialysis for 2h against MilliQ water was carried out at room temperature.

3.2.1.3 Loading of immunogen into LNP

Immunogens were loaded into freshly prepared LNP as described in sections 2.2.1.1 and 2.2.1.2.

3.2.2 Animal experiments

In vivo experiments were designed to compare the efficiency of immunisation using the GnRH conjugates loaded in LNP (bilosomes/ NISV) by the oral and

subcutaneous routes respectively. Comparison was made with subcutaneous immunisation of the conjugates mixed with Alum as an adjuvant.

3.2.2.1 Immune response against TT-GnRH conjugates by subcutaneous route.

In-house bred BALB/c male mice, 6–8 weeks old, were housed in a fully conditioned room, randomised, ear coded and placed into groups (n=5). Each mouse in the test groups received 0.2ml of the immunogen containing 50µg equivalent of the GnRH peptide, Group 1 (TT-GnRH-I), Group 2 (TT-GnRH-II), and Group 3 (TT-GnRH-III). Group 4 received no immunisation (control group). Previous work had shown that conjugate alone induced very low antibody titres therefore this control group was omitted (Ferro *et al.*, 1996). The required peptide dose was made upto 0.1ml with phosphate buffered saline (PBS; 0.02M sodium phosphate buffer, containing 0.14M NaCl, pH 7.4) and adsorbed with an equal volume of Imject® Alum (Fisher-scientific, UK) for 30min. The immunisation schedule was started on day 0 with boosters at 2 weekly intervals until the last immunisation on week 15 (Table 3.3). Starting from the 2nd immunisation, 5 days post-immunisation, tail bleeds were collected in 1.5ml microfuge tubes (Elkay, UK) and centrifuged at 1000g for 20min. The serum was transferred into fresh 0.5ml micro-centrifuge tubes, and stored at -20°C until antibody levels were determined by enzyme linked immunosorbent assay (ELISA). All mice were euthanised on week 16 by lethal CO_2 exposure according to Home Office regulations. Cardiac blood and testes were collected. One testis from each mouse was immediately transferred into 5ml falcon tubes containing RNAlater® solution (AMBION Inc., USA) and kept at -80°C for

later use in total RNA extraction and purification. The cardiac blood samples were used to determine testosterone serum levels for each group.

3.2.2.2 Immune response against TT-GnRH conjugates loaded in bilosmes.

BALB/c male mice, 6–8 weeks old, were housed in a fully conditioned room, randomised, ear coded and placed into groups (n=5). Each mouse in the test groups received 0.2 ml of the immunogen containing 50μg equivalent of the GnRH peptide, Group 1 (TT-GnRH-I), Group 2 (TT-GnRH-II), and Group 3 (TT-GnRH-III) loaded in freshly prepared bilosomes (prepared as described in section 2.2.1.1) administered by oral gavage. Mice were withheld from food 2h before dosing, but allowed water *ad libitum*. A total of 4 immunisations separated by 2 week intervals between dosing were carried out (Table 3.3). Tail blood was collected after 5 days from each dosing and processed as section 3.2.2.1 for ELISA. All mice were euthanised on week 7 by lethal CO_2 exposure.

3.2.2.3 Immune response against OVA-GnRH conjugates loaded in LNP (bilosomes and NISVs)

The third experiment was totally different in immunisation schedule, immunogens and delivery systems (Table 3.3). Ovalbumin was used as the carrier protein in preparation of the immunogen. Bilosomes, NISV (prepared as described in section 2.2.1.2) and alum were used as the immunopotentiators. The immunisation schedule for groups given NISV and Imject® Alum containing vaccines were administered subcutaneously in a similar manner to that used in previous experiments. The main difference in immunisation schedule was in the groups given

bilosomes orally, where the schedule was two immunisations over a 3 day period, repeated after 2 weeks. Only GnRH-I and GnRH-III conjugated to OVA were used as immunogens in preparation of the vaccines. Briefly, seven groups, each with 5x BALB/c 6–8 weeks old, male, were in this experiment. Group 1 and Group 2 were administered conjugate plus bilosomes orally, Group 3 and Group 4 administered conjugate plus NISV by the subcutaneous route and Group 5 and Group 6 were administered conjugate plus alum vaccine by the subcutaneous route. Group 7 received no immunisation (control group). Starting from the second dose, tail bleeds were collected after 5 days from each immunisation for Groups 3, 4, 5 and 6. Blood sampling for Groups 1 and 2 was carried out after 3 days from the second administration. On experiment termination, all mice were euthanised by lethal CO_2 exposure.

Group	Immunogen	Delivery system/Adjuvant	Route	Schedule
Experiment 1: To compare different GnRH isoforms immune responses				
1	TT-GnRH-I	Imject® Alum	Subcutaneous	Immunisation at day1, boosters at 2 weekly intervals until week 15
2	TT-GnRH-II			
3	TT-GnRH-III			
4	Control			
Experiment 2: To compare the mucosal immune response of different GnRH isoforms loaded into bilosomes				
1	TT-GnRH-I	Bilosomes	Oral	Immunisation at day1, boosters at 2 weekly intervals for a total of 4 immunisation
2	TT-GnRH-II			
3	TT-GnRH-III			
4	Control			
Experiment 3: To compare immune response of OVA-GnRH conjugates loaded LNP				
1	OVA-GnRH-I	Bilosomes	Oral	2 immunisations, over 3 day intervals, repeated after 2 weeks
2	OVA-GnRH-III			
3	OVA-GnRH-I	NISV	Subcutaneous	4 immunisations, over 2 week intervals
4	OVA-GnRH-III			
5	OVA-GnRH-I	Imject® Alum		
6	OVA-GnRH-III			
7	Control			

Table 3.3: Immunisation programme and schedule for *in vivo* studies using different GnRH isoform conjugates as immunogens, via different routes (subcutaneous and oral).

3.2.3 ELISA for detection of anti-GnRH antibodies in sera

In order to measure the antibody reactivity, an indirect ELISA was carried out. Half of a 96-well tissue culture plate (Iwaki, Japan) was coated with 2μg/well equivalent of modified GnRH-I, GnRH-II or GnRH-III peptides conjugated to BSA in 100μl PBS, pH 7.4. The plates were blocked with 3% (w/v) non-fat milk protein (Marvel, Premier Brands, UK) in wash buffer (PBS and 0.05%, v/v, Tween-20) for 1h at 37°C. The serum samples were diluted 1:1000 in PBS, pH 7.4, incubated for 1h at 37°C (all samples were carried out in triplicate in both coated and uncoated halves of the plate) and developed with goat anti-mouse peroxidise labelled antibody (1:3000 dilution, Perbio, UK) for 45min at 37°C. This was followed by incubation with 100μl of TMB substrate (250μl of stock tetramethyl benzidine 6μg/ml dissolved in dimethylsulphoxide (DMSO), added to 25ml of 0.1M sodium acetate citrate buffer, pH 5.5, with 4μl of 30% (v/v) H_2O_2). After 15min the reaction was stopped with 50μl/well of 10% (v/v) H_2SO_4, and the absorbance at 450 nm read on a SpectraMax M5 plate reader. The absorbance was calculated by subtracting the uncoated well reading from antigen coated well reading.

3.2.4 Determination of serum testosterone level

Testosterone levels were determined using a quantitative ELISA kit (Alpha Diagnostic Int., USA) validated by the manufacturer for use with mouse serum. Briefly, anti-rabbit IgG coated microwell strips (provided in the kit) were washed 3 times with 200μl washing buffer provided in the kit. One well was saved as a blank without any addition of test reagents. Ten microlitres of standards (0-20ng/ml) and serum samples obtained from the final bleed were added into appropriate wells, and

then 50µl of antibody solution (provided in the kit) added into each well followed by addition of 50µl of enzyme conjugate into each well except the blank and gently mixed. The plate was covered and incubated for 60 min at room temperature. The plate was washed 3 times and 100µl of TMB substrate was added to each well, mixed for 5-10 seconds and incubated for 30 min at room temperature before adding 50µl of stop solution to all wells. The absorbance at 450 nm was read on a SpectraMax M5 plate reader. A standard curve was constructed with log-logit fit with R^2=0.991 and used for calculating the amount of testosterone in the serum samples.

3.2.5 Detection of Th1 and Th2 cytokines using a reverse transcription polymerase chain reaction (RT-PCR)

In order to investigate the immunological response against the GnRH vaccine in the reproductive organs, the up-regulation in the release of Th1 and Th2 associated cytokines, was assessed using a RT-PCR method.

3.2.5.1 Total RNA isolation and purification

Briefly, testes obtained from immunised mice were immediately transferred into RNAlater® solution (AMBION, Inc., USA) to avoid any RNA degradation. A total RNA purification miniprep kit was used for RNA extraction and purification from testes tissue. A slice of the RNAlater® stabilised tissue weighing about 40mg was transferred into a DNase/RNase free microtube (Eppendorf, UK). After addition of 500µl of supplied lysis solution the tissue was homogenised for 1 min in a rotor-stator homogeniser (repeated twice to ensure total homogenisation of the tissue). The homogenised tissue was pipetted into a GenElute™ filtration column and centrifuged

at 13,000 x g for 2min. The filtration column was discarded and 500µl of 70% (v/v) ethanol added to the filtered lysate and mixed thoroughly before being transferred into a binding column tube. The lysate/ethanol mixture was centrifuged for 15s to allow RNA to bind to the column, and the flow-through was discarded. The column was washed twice for 15s with 500µl wash solution 1 and 2, respectively, and the flow-through discarded. An additional wash for 2min using wash solution 2 at a maximum speed was carried out to dry the binding column. Finally, the RNA was eluted in a new collection tube by the addition of 50µl of elution solution into the binding column and centrifuged at a maximum speed for 1min. Purified RNA was stored at -80°C for later use in the preparation of complementary DNA (cDNA).

3.2.5.2 Reverse Transcription (RT) procedure (First-Strand cDNA Synthesis)

Briefly, 2-3µl of RNA was added to a nuclease-free microcentrifuge tube containing: 1µl of oligo(dt) (MWG Biotech AG, Germany), 1µl of 10mM deoxyribonucleotide triphosphates (dNTP) (Invitrogen UK Ltd.) mix and made up to 13µl with nuclease-free water (Sigma-Aldrich UK). The mixture was heated to 65°C for 5min and incubated on ice for at least 1min. After brief centrifugation, the following were added: 4µl of 5 X First-Strand buffer, 1µl 0.1M dithiothreitol (DTT), 1µl RNaseOut™ (recombinant RNase inhibtor) and 1µl of SuperScript® III Reverse Transcriptase (RT) (Invitrogen UK Ltd.), and mixed gently by aspiration with a pipette. The reaction was started by incubation at 42°C for 60 min, and the reaction inactivated by heating at 72°C for 15min.

3.2.5.3 PCR and multiplex PCR procedure

Four primer sets were designed for detection of mRNA of cytokines; Interleukin 4 (IL4), Interleukin 6 (IL6), Interferon gamma (IFNγ) and Tumor Necrosis Factor Beta (TNF-β) using mouse genome sequences in GeneBank (International Nucleotide Sequence Database Collaboration), as shown in Table 3.4. Optimisation was carried out on one primer set followed by addition of another primer set, until all 4 primer sets were included in one multiplex PCR.

The PCR reaction mixture contained: 25µl of 2X HotStarTaq plus (Qiagen Ltd., UK), 5µl of each primer, 1µl of cDNA and the volume up to 50µl with nuclease-free H_2O. The PCR conditions were: initial activation heating at 95°C for 5min, followed by 35 cycles (95°C for 30s, 55°C for 30s and 72°C for 1min) and a final extension step at 72°C for 5min. The PCR products (20µl) were mixed with 2X gel loading buffer and loaded into wells of 1.5% agarose gel prepared in 1X Tris-Borate-EDTA buffer (TBE, 1M Tris, 0.9M boric acid, and 0.01M EDTA) and the separated DNA was visualised under UV.

Cytokine	Gene accession number	Primer set sequences	PCR product size
IL4	NM_021283.1	MIL4(For): TTGAACGAGGTCAC AGGAGAAGG	247bp(196-442)
		MIL4(Rev): ACTCATTCATGGTG CAGCTTATCG	
IL6	NM_031168.1	MIL6(For): CCGGAGAGGAGACT TCACAGAGG	345bp (121-465)
		MIL6(Rev): TCTCTCTGAAGGAC TCTGGCTTTGTC	
IFN-γ	NM_008337	MIFNγ(For): GCGGCCTAGCTCTG AGACAATG	445bp (90-534)
		MIFNγ(Rev): CAACAGCTGGTGGA CCACTCG	
TNF-β	NM_010735.1	MTNF-β(For): TACCCTGGTATCAG GCATCCCTC	550bp (57-606)
		MTNF-β(Rev): CGCACTGAGGAGAG GCACATG	

Table 3.4 Primer sets designed for detection of Th1 (IFN-γ and TNF-β) and Th2 (IL4 and IL6) cytokines by PCR. Abbreviations; For: forward PCR primer, Rev: reverse PCR primer.

3.2.6 Statistical analysis:

Significances and statistical differences compared with the control group were determined using an unpaired Student T-test with a value of $p \leq 0.05$ being considered significant, using Minitab®, v.15 software.

3.3 Results

3.3.1 Evaluation of GnRH-I, GnRH-II and GnRH-III antibody responses after subcutaneous immunsiation

IgG responses were measured by ELISA using serum samples collected following immunisation in study week 3, 5, 7, 9, 11, 13 and 15. The reactivity of the antibodies raised against each antigen was expressed as the mean of triplicate *A*450 ± SD readings after deduction of the background value. Figures 3.3-3.6 represents the antibody response and cross reactivity of the treatment groups determined on plates coated with BSA-GnRH-I, BSA-GnRH-II and BSA-GnRH-III. Group 1 (TT-GnRH-I) showed a fluctuating response to GnRH-I below 1.0 A_{450} in weeks 1-7 then rose until weeks 13 and 15 (1.8±0.2 and 2.0±0.4) (Figure 3.3). However, sera from this group showed very low cross-reactivity against BSA-GnRH-II and BSA-GnRH-III coated plates (Figure 3.4). Titres against GnRH-II from Group 2 (TT-GnRH-II) showed a gradual increase in weeks 3-9 and reached a peak at week 11 (3.1±0.4) before declining to (2.0±0.4) by week 15 (Figure 3.3). However, this Group's sera cross reacted with BSA-GnRH-II and BSA-GnRH-III coated plates with the highest OD values (0.461±0.1 at week 11 and 0.494±0.2 at week 15, respectively) (Figure 3.5). Similarly, sera from group 3 (TT-GnRH-III) showed antibody titres against GnRH-III with a gradual increase in weeks 3-9 and reached a peak at week 11 (2.7±0.7) before declining to (2.2±0.3) by week 15 (Figure 3.3). However, this Group's sera only showed slight cross reactivity to BSA-GnRH-I antigen with the highest level at week 13 (0.456±0.2), but no noticeable cross reactivity with BSA-GnRH-II coated plates (Figure 3.6).

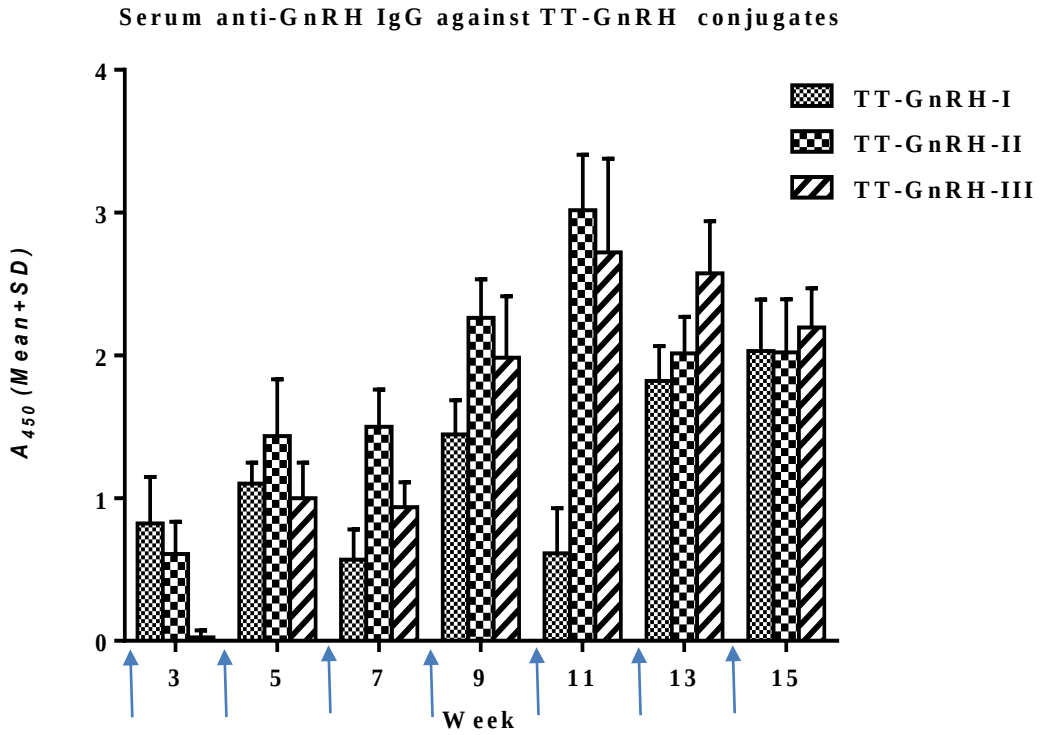


Figure 3.3 Specific anti-GnRH-I, GnRH-II and GnRH-III antibody levels from male mice (n = 5) immunised in study weeks 0, 2, 6, 8, 10, 12, and 14 (arrows). Each point represents the mean *A*450 ±SD readings, measured by indirect ELISA.

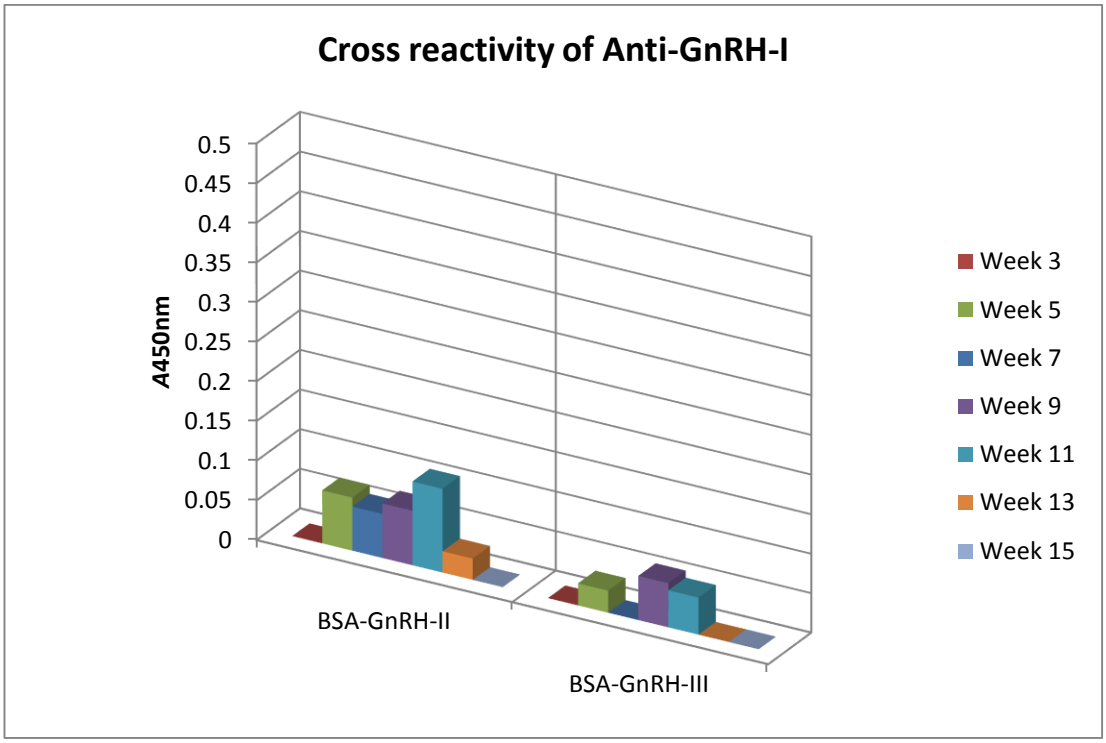


Figure 3.4 Cross-reactivity of TT-GnRH-I antiserum, determined on plates coated with (2µg/well) BSA-GnRH-II and BSA-GnRH-III. The antiserum was obtained from male mice (n = 5), immunised in study weeks 0, 2, 6, 8, 10, 12, and 14 with 50µg equivalent of (TT-GnRH-I). Each point represents the mean *A*450 readings, measured by indirect ELISA.

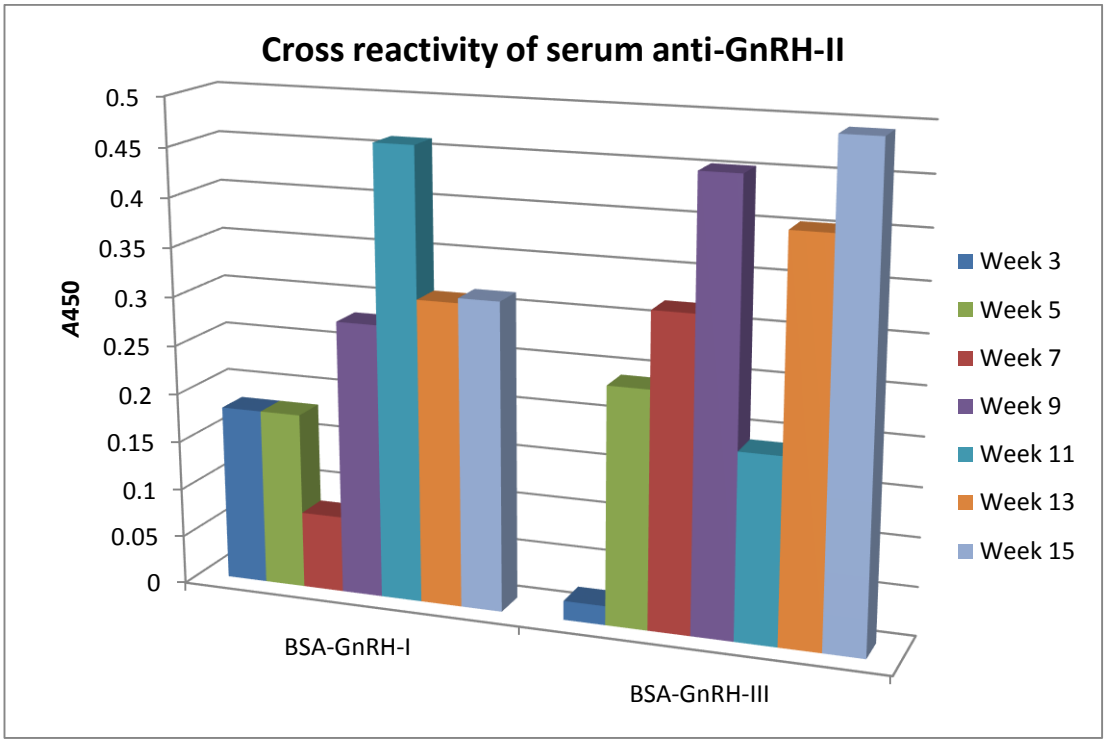


Figure 3.5 Cross-reactivity of TT-GnRH-II antiserum, determined on plates coated with (2µg/well) BSA-GnRH-I and BSA-GnRH-III. The antiserum was obtained from male mice (n = 5), immunised in study weeks 0, 2, 6, 8, 10, 12, and 14 with 50µg equivalent of (TT-GnRH-II). Each point represents the mean *A*450 readings, measured by indirect ELISA.

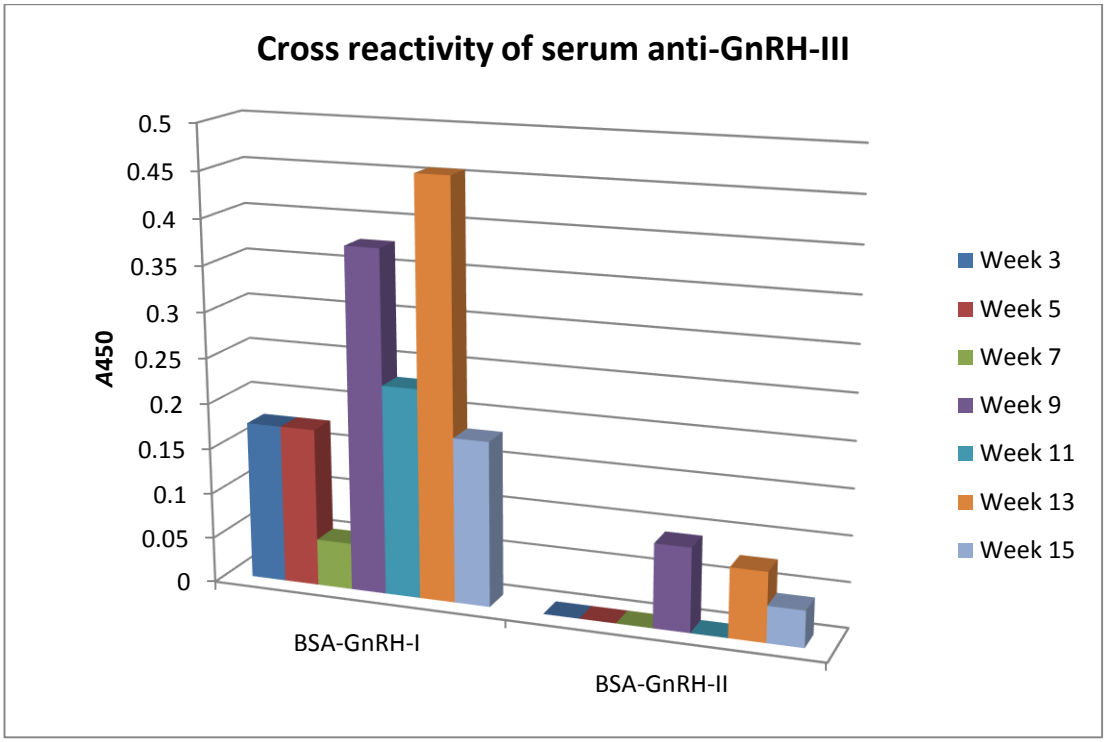


Figure 3.6 Cross-reactivity of TT-GnRH-III antiserum, determined on plates coated with (2µg/well) BSA-GnRH-I and BSA-GnRH-II. The antiserum was obtained from male mice (n = 5), immunised in study weeks 0, 2, 6, 8, 10, 12, and 14 with 50µg equivalent of (TT-GnRH-III). Each point represents the mean *A*450 readings, measured by indirect ELISA.

3.3.2 Detection of anti-GnRH antibody in mice sera immunised by oral route

There was no immune response detected by ELISA to oral immunisation with any of the three antigens (TT-GnRH-I, TT-GnRH-II and TT-GnRH-III) used in this experiment. Serum from all treatment groups did not react to any of plate coating antigens used in the ELISA assay.

3.3.3 Detection of anti-GnRH antibody in mice immunised with OVA-GnRH conjugates loaded in LNP

Specific IgG responses were measured by ELISA using serum samples collected following immunisation in study weeks 3, 5 and 7 (Groups 3-7) and in study weeks 3 and 7 (Groups 1, 2 and 7) . The reactivity of the antibodies raised against each antigen was expressed as the mean of triplicate *A*450±SD. Figures 3.7 and 3.8 represent the antibody response of the treatment groups determined on plates coated with BSA-GnRH-I or BSA-GnRH-III. Mice received GnRH-I or GnRH-III with alum subcutaneously showed the highest response (2.6±0.3 and 2.7±0.4 respectively) as noted in week 7 (Figures 3.7 and 3.8). A comparable IgG level observed from mice received GnRH-I or GnRH-III loaded in NISV subcutaneously (2.6±1.0 and 2.5±0.2 respectively) as noted in week 7 (Figures 3.7 and 3.8). Meanwhile, in groups immunised orally, there were very low non-specific antibody response to both GnRH-I and GnRH-III from samples obtained in the study week 3 and were decreased in the samples obtained in study week 7.

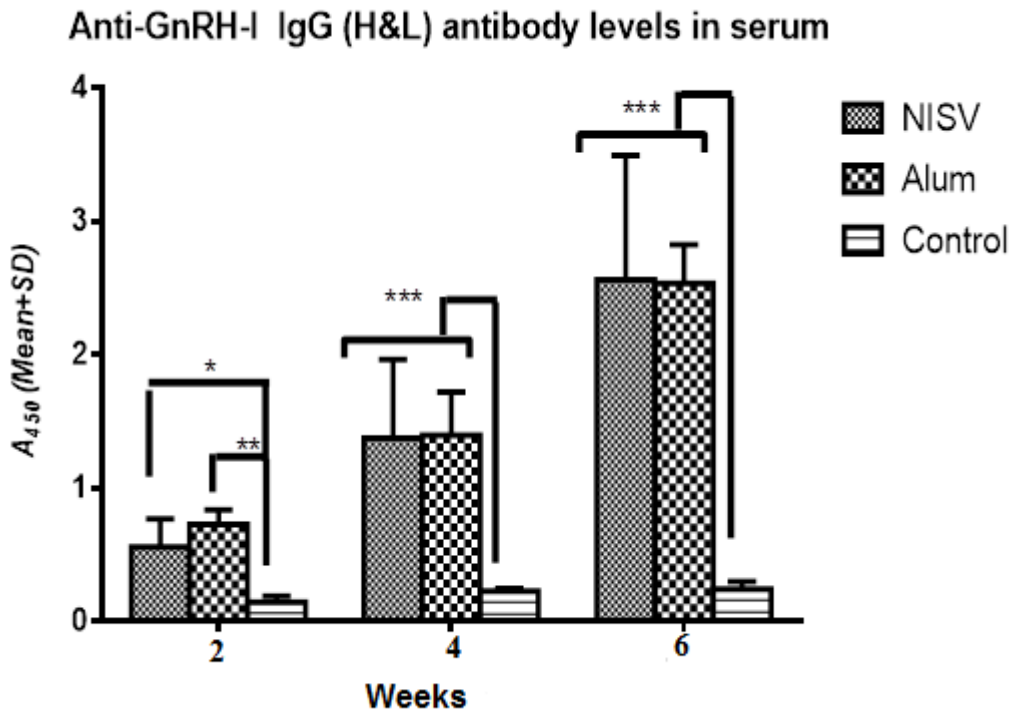


Figure 3.7 Anti-GnRH-I antibody response from male mice (n = 5) immunised subcutaneously OVA-GnRH-I. Each point represents the mean *A*450 ±SD readings, measured by indirect ELISA. *(p<0.05), **(p<0.01), ***(p<0.001) indicates significance level against the Control group.

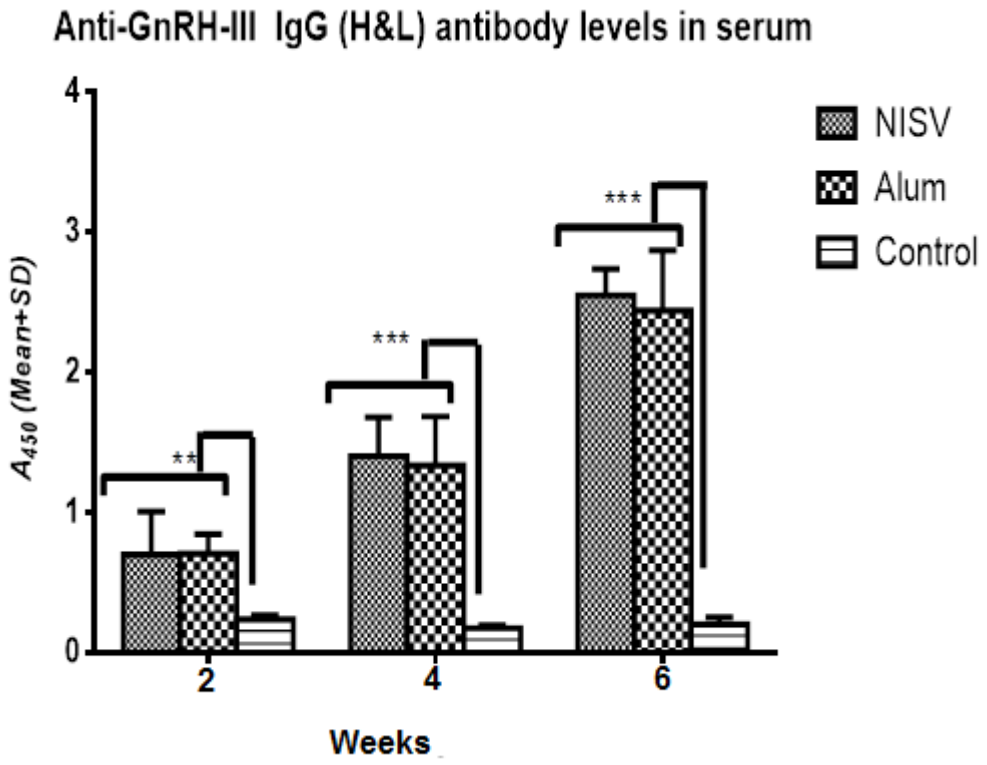


Figure 3.8 Anti-GnRH-III antibody response from male mice (n = 5) immunised subcutaneously Ova-GnRH-III. Each point represents the mean *A*450 ±SD readings, measured by indirect ELISA. *(p<0.05), **(p<0.01), ***(p<0.001) indicates significance level against the Control group.

3.3.4 Determination of testosterone serum level

Using a quantitative ELISA assay, the serum testosterone levels from mice immunised against TT-GnRH and OVA-GnRH conjugates were determined in serum samples collected at the study end. The standards index A/A_0 x 100 values were plotted against each concentration in a log-logit chart and the sample concentrations were determined from this chart (Figure 3.9). Samples from mice immunised orally were excluded from the assay due to the lack of antibody response observed. Serum from 3 mice per group were analysed, when applying statistic mean values from these sera, there was a marked decrease in testosterone levels between the treatment groups and the control group. Mice immunised with TT-GnRH conjugated showed a significant ($P<0.05$) decrease in testosterone levels for animals in the TT-GnRH-II Group and ($P<0.0001$) for Groups TT-GnRH-I and TT-GnRH-III (Figure 3.10). The sera testosterone concentration values were 1.5±0.1, 8.3±0.9 and 2.7±1.3ng/ml for TT-GnRH-I, TT-GnRH-II and TT-GnRH-III Groups, respectively (Figure 3.10). Sera from mice immunised by the alum mixed OVA-GnRH-I and OVA-GnRH-III showed significant ($P<0.0001$) lower testosterone values (0.7±0.2 and 1.9±0.4ng/ml, respectively, Figure 3.11). Similarly, groups immunised by NISV loaded with both conjugates showed reduced testosterone levels (2.0±1.3 and 1.9±0.8ng/ml, for OVA-GnRH-I and OVA-GnRH-III respectively, Figure 3.11) which was significant ($P<0.0001$) compared to control groups. However, individual serum testosterone levels varied within the same study group in NISV and control groups.

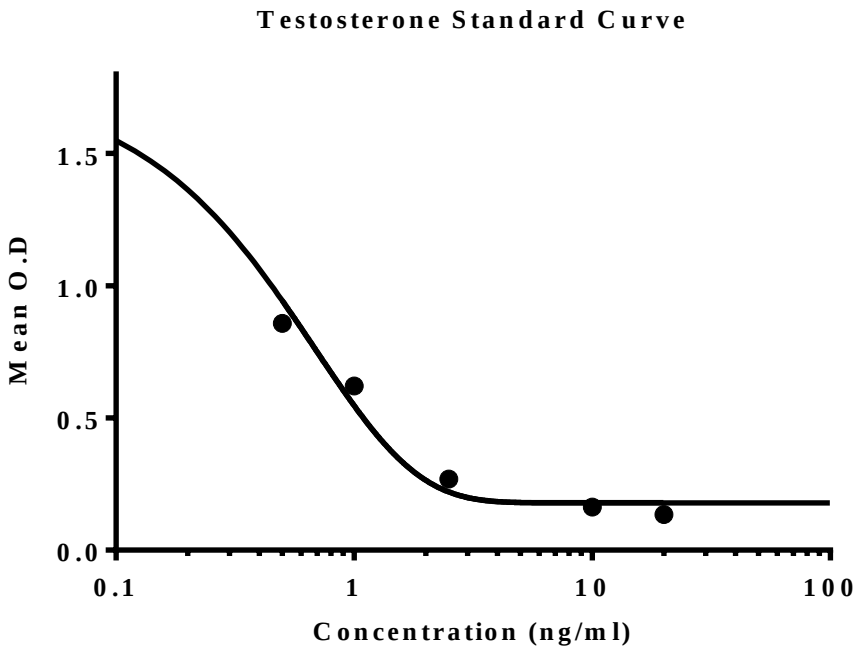


Figure 3.9: Chart for estimation of serum testosterone concentration using standards index plotted on log-logit chart.

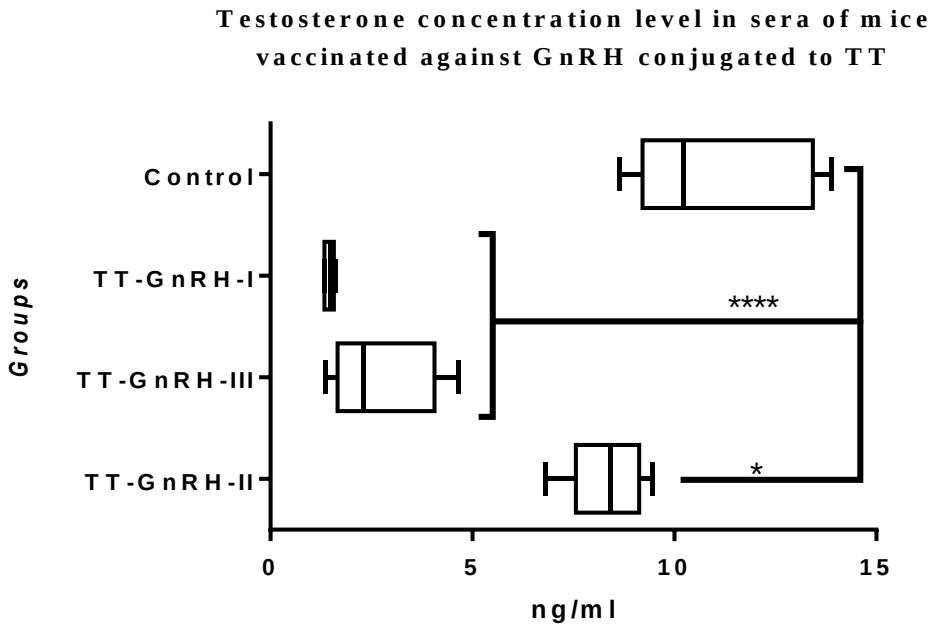


Figure 3.10 Testosterone level in sera of mice immunised against TT-GnRH conjugates adsorbed to Imject Alum administration via the subcutaneous route. *(p<0.05) and **** (p<0.0001) indicates the significance level between the groups.

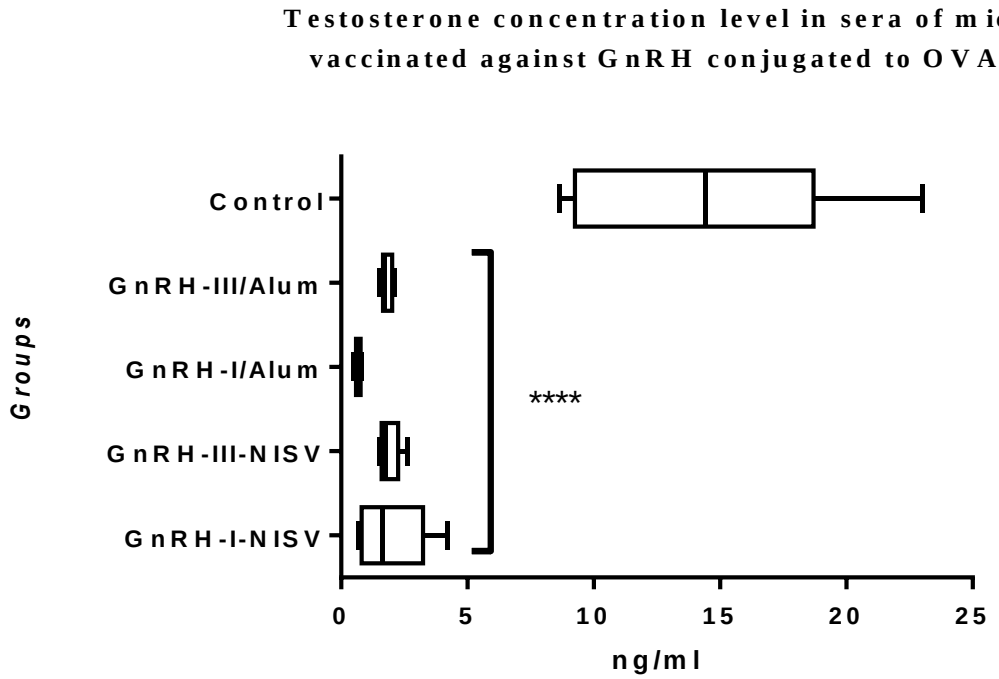


Figure 3.11 Testosterone level in sera of mice immunised against OVA-GnRH conjugates via subcutaneous route. **** (p<0.0001) indicates significance level against the Control group.

3.3.5 Cytokine detection by RT-PCR

3.3.5.1 Detection of IFN-γ in testes tissue by RT-PCR: proof of concept

RNA extracted from testis tissue samples from experiment 1 were used as a template for cDNA. The results obtained from primary RT-PCR have shown a distinct variability in intensity of the PCR bands between the studied groups (Figure 3.12). The correct band size was obtained from all the groups with variations in intensity which indicates the corresponding variation in quantity (the higher the intensity the higher the quantity).The highest bands intensity were observed in testes tissue samples from GnRH-III conjugated group.

3.3.5.2 Multiplex PCR results

Using all primer sets in a multiplex PCR allowed the detection of the different cytokines released at the same time. PCR bands corresponding to the expected PCR amplification product from all primers listed in Table 3.4 were detected by UV visualisation of ethidium bromide stained 1.5% agarose gels (Figure 3.13). A clear band at approximately 247bp corresponding to the IL4 primer set was detected from Group 3 (GnRH III vaccinated) and Group 1 (GnRH I vaccinated) samples. Other weak PCR bands at 345bp, 445bp and 550bp (corresponding to IL 6, IFN-γ and TNF-β, respectively) were also detected in some samples in Groups 1 and 3.

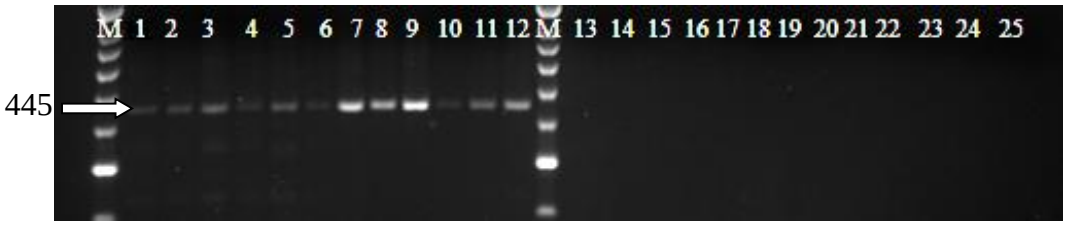


Figure 3.12: Gel electrophoresis for PCR products. 15µl of PCR products were loaded into each well of a 1.5 agar gel contains ethidium bromide. The gel was electrophoresed for approximately 1 hour at 60V and then visualised under UV. Band of 445bp represent PCR amplification of IFN-γ primers. M= DNA Marker. Wells 1-3= TT-GnRH-I, wells 4-6=TT-GnRH-II, wells 7-9= TT-GnRH-III, wells 10-12= Control. Wells from 13-25 are –ve control for RT step.

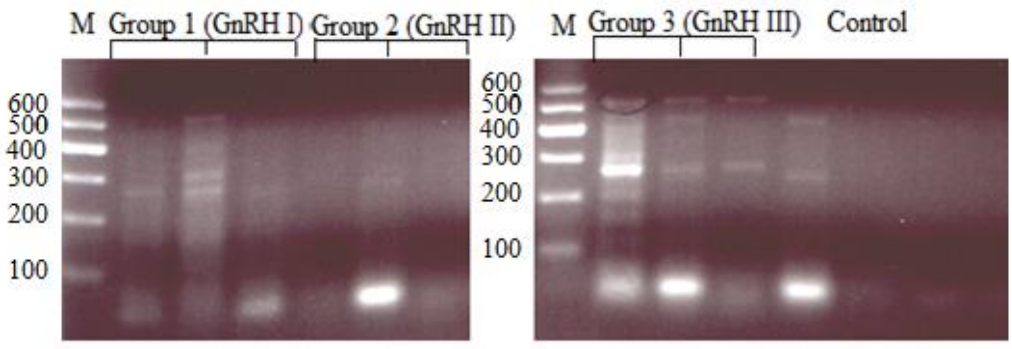


Figure 3.13: Ethidium bromide stained 1.5% agar gel of multiplex PCR products of RT-RNA processed samples obtained from mice testicular tissues from each study group. M= 100 bp DNA marker. PCR bands at 247bp, 345bp, 445bp and 550bp (corresponding to IL-4, IL-6, IFN-γ and TNF-β, respectively)

3.4 Discussion

3.4.1 Anti-GnRH ELISA assays

In the first experiment, different tetanus toxoid conjugated GnRH isoforms (GnRH-I, II and III) were evaluated in mice that had been immunised via the subcutaneous route for capability to produce antibodies to neutralise the native GnRH hormone circulating in the body. The primary results were in agreement with that obtained by Khan *et al.* (2007b). Sera from mice immunised against TT-GnRH-I or TT-GnRH-III gave very low cross reaction to other GnRH immunogens. While, sera from mice immunised against TT-GnRH-II strongly reacted with both GnRH-I and GnRH-III immunogens, which may indicate that immunisation with GnRH-II immunogen stimulates production of non-specific antibodies. However, in the second experiment to prove the concept that oral delivery of GnRH-vaccine using bilosomes can initiate systemic and mucosal response antibody titres could not be detected. Basically, lipid vesicles and NISVs are considered adjuvants (Walker *et al.*, 1996; Gregoriadis *et al.*, 2000) and therefore can be used to enhance the immunogenicity of poor or non-immunogenic antigens such as GnRH molecules (Ferro and Stimson, 1998). In this study, mice immunised with OVA-GnRH loaded into NISV generated stronger IgG responses than using alum as an adjuvant as shown by the ELISA results. However, oral administration of 50μg per dose of OVA-GnRH loaded bilosomes failed to produce any systemic responses in vaccinated mice.

3.4.2 Serum testosterone level.

The lower testosterone levels in sera of mice immunised against TT-GnRH indicate that systemic antibody against GnRH-I and GnRH-III prevented native GnRH released from the hypothalamus from activating of GnRHR to produce LH and FSH hormones, required for stimulation of the gonads to produce testosterone hormone. Similar results were reported by Ferro *et al.,* (2002) who studied the efficacy of conjugation of carrier protein to N- or C-terminal sites on GnRH-I. The study found that conjugation through the N-terminal proved to be a highly effective means of causing immunocastration in terms of decreased gonadotrophin and testosterone concentrations (Ferro *et al.*, 2002).

In contrast, TT-GnRH-II immunised mice, despite the high sera antibody titres seen, showed a much lower degree of reduction in testosterone levels when compared to control groups. There is a strong hypothesis that the GnRH-II gene has been silenced in the mouse (Pawson *et al.,* 2003), which may be the reason for the lower levels of testosterone release in groups immunised against GnRH-II conjugates in this research study. The GnRH-II receptor gene disruption or slicing is also reported in other species including man, chimp and rat (Morgan *et al.,* 2003; Pawson *et al.,* 2003), cow (Millar, 2003) and sheep (Gault *et al.,* 2003).

Although there was a marked decrease and almost total suppression of testosterone levels in s.c. immunised groups with NISV loaded or alum mixed immunogens, an inter-group variation was detected in the NISV immunised groups which was probably due to the variations in vesicle sizes and consequently the antigen entrapment efficiency. In the other hand, the observed high testosterone level in the Control group could be due limitations in the sensitivity of the assay, where

samples that contain higher than the standards range may be not estimated correctly. This could overcome by diluting the serum samples prior to the assay procedure.

3.4.3 Cytokine detection by RT-PCR

In this study the detection of cytokine release in the target organ was determined by RT-PCR. Samples from the GnRH-III vaccinated group showed release of all the different cytokines assessed (IL 4, IL 6, IFN-γ and TNF-β) with a noticeable internal variation in band intensity between individual samples in the same group. The same results were observed in the GnRH-I immunised group, while the group immunised with GnRH-II and the control group showed poor band signals or no bands at all. The variation in the number of bands or intensity within the same group indicates the variation of individual mouse response to immunisation. This may also explain the variation of testosterone levels within the same group. Testicular cytokines and growth factors, such as IL-1, IL-6, IFN-γ, TNF-β, LIF and SCF have been shown to affect both germ cell proliferation and Leydig and Sertoli cell functions and secretion. Cytokines and growth factors are produced by immune cells and in the interstitial and seminiferous tubular compartments by various testicular cells, including Sertoli, Leydig, peritubular cells, spermatogonia, differentiated spermatogonia and even spermatozoa (Huleihel and Lunenfeld, 2004). Corresponding cytokine and growth factor receptors have been demonstrated on some testicular cells. These cytokines also control the secretion of the gonadotropins and testosterone in the testis (Orava *et al.,* 1989; Verajankorva *et al.,* 2001). Cytokines, including IL-6, have been demonstrated in the seminal plasma of fertile and infertile human males (Huleihel *et al.,* 1996). It was established that Sertoli cells

secrete IL-6 *in vitro*, with low levels of testosterone and FSH (Syed *et al.*, 1993; Syed *et al.*, 1995). The results in this research study demonstrate that up-regulation of mRNA of these cytokines and growth factors from testis tissue in Groups TT-GnRH-I and TT-GnRH-III correlate with the lower levels of testosterone observed in mice from these 2 groups. However, the RT-PCR technique used in this study was semi-quantitative and the observed ethidium bromide stained DNA is only an indicator of the initial mRNA production of the corresponding cytokine. A recent study used real time-PCR for quantification of basal cytokine expression in the liver, spleen, respiratory, reproductive and intestinal tract of hens (Kolesarova *et al.*, 2011). The application of reverse transcriptase real time-PCR technique, which is a quantitative method, will allow accurate determination of the amount of mRNA and relative levels of gene expression in each sample tested (Gibson *et al.*, 1996; Liu, 2002).

The results described in this chapter showed that parenteral administration of immunogen loaded in NISV has stimulated the immune system regardless of the immunogen or the carrier protein. While no immune response obtained from orally immunised groups. Therefore, the administration of NISV via alternative mucosal routes need to be investigated which is described in the next chapter.

Chapter four: Comparison of non-pathogenic antigen vaccination via different mucosal routes

4.1 Introduction

Immunisation via the oral route using bilosomes as described in Chapter 3 did not produce a detectable systemic immune response in mice. The low antigen dose (50µg/dose) given by the oral route may have contributed to the failure of detecting anti-GnRH specific antibodies in the serum, although other antigens derived from pathogens (such as tetanus toxoid and influenza haemagglatunin) have been shown to produce antibody responses at this dose level (Mann *et al.,* 2006; Mann *et al.,* 2009b). This chapter therefore describes further investigation of the possibility of inducing local and systemic immune responses against non-pathogenic immunogens loaded in bilosomes following oral immunisation with higher antigen doses (100µg/dose). In addition, the LNP formulations were evaluated for vaccine delivery with other mucosal immunisation routes (nasal and vaginal). To achieve this, the GnRH isoforms were modified for conjugation to keyhole limpet haemocyanin (KLH) and entrapped in NISV for administration in female mice (via nasal, vaginal and subcutaneous routes). The carrier protein was changed as TT was not available. In order to increase mucoadhesivity of the LNP in the nasal and vaginal sites, they were suspended in 2% (w/v) xanthan gum (Chiou *et al.,* 2009).

The modifications in LNP formulation and preparation for mucosal administration, including resuspension in xanthan gum solution, required further characterisation of size, ZP and morphological structure. Techniques such as scanning probe microscopy (SPM) has long been recognised as a useful tool for measuring mechanical properties of materials, and until recently it has been impossible to achieve truly quantitative material property mapping in high resolution and to nanoscale. Atomic force microscopy (AFM) is one type of SPM, and with the

new mode PeakForce^{TM} Quantitative Nanomechanical Mapping (QNM^{TM}) allowed measuring the Young's modulus of materials with high spatial resolution and surface sensitivity, by probing at nanoscale across a topographic image, such as Surface Roughness, Adhesion, DMT Modulus; to obtain the Young's Modulus, the retract curve is fitted using the Derjaguin–Muller–Toporov model, which is called the DMT Modulus (Young *et al.*, 2011). The surface Deformation (defined as the penetration of the tip into the surface at the peak force, after subtracting cantilever compliance, and may include both elastic and plastic contributions) and Dissipation (given by the force times the velocity integrated over one period of the vibration; for pure elastic deformation there is no hysteresis between the repulsive parts of the loading-unloading curve, corresponding to very low Dissipation) (Maugis, 2000). This information provides more details on surface elasticity and antigen-vesicle interaction. . The reason for using these techniques was also to see if it was possible to throw some light on the location of the antigens relative to the bilayer and the effect on surface properties of the different vesicles in the presence and absence of DOC and xanthum gum.

Aims of this chapter

1- To evaluate LNPs as a vaccine delivery system via different mucosal routes using KLH-GnRH-I and III conjugates as immunogens where oral groups received higher doses.

2- To compare systemic and local specific immune responses by assessment of IgG in serum and IgA in lung and intestinal washes, following immunisation with KLH-GnRH conjugates.

3- To compare the effect of immunisation with KLH-GnRH conjugates on endocrine hormone release in males and female mice.

4.2 Methods

4.2.1 Preaparation of mcKLH (Mariculture KLH) conjugated immunogens

Peptide conjugation to mcKLH was carried out according to the manufacturer's instructions. Two mg of GnRH peptide was dissolved in 200µl of conjugation buffer (PBS; 0.1M sodium phosphate, 0.15M NaCl,pH 7.2) and added to 2mg activated mcKLH reconstituted in 200µl MilliQ water, immediately mixed and allowed to react for 2h at room temperature. The conjugate was desalted by dialysis as described by Ferro *et al*., (1996).

4.2.2 Vesicles preparation

LNPs (EB and EN) were prepared by the MW method as described in section 2.2.1.2. After addition of the antigens (KLH-GnRH-I and III conjugates), the mixture was further homogenised for 1min at 8000rpm and the vesicles named loaded NISV (LN) or loaded bilosomes (LB). The prepared emulsions were stored at 4°C until used for immunisation. For preparation of nasal administration, equal volumes of NISV and 0.2% (w/v) xanthan gum solution were mixed immediately before administration (LN+Xn).

4.2.3 Vesicle characterisation

4.2.3.1 Sizing and ZP

LNP sizing and ZP measurements were carried out as described in section 2.2.4.

4.2.3.2 Scanning Electron Microscopy (SEM)

SEM was performed in the Department of Physics, University of Strathclyde by Dr Paul Edwards. Samples of LNP diluted 1:50 in 0.025M carbonate buffer (2µL) were dried on a silicon substrate and placed under vacuum. Secondary electron images were collected using a FEI Quanta 250 field emission variable pressure scanning electron microscope equipped with an Everhart-Thornley type detector and running FEI software. An accelerating voltage of 5kV was applied to each sample in high vacuum mode.

4.2.3.3 PeakForce QNM Atomic Force Microscopy (AFM)

For AFM experiments, the samples were diluted 50 times with 0.025M carbonate buffer, pH 9.7 and 5µl of the lipid suspensions were deposited onto a freshly cleaved mica surface (G250-2 Mica sheets 1" x 1" x 0.006"; Agar Scientific Ltd, Essex, UK), and left to dry for 1h before AFM imaging. The images were obtained by scanning the mica surface in air under ambient conditions using a scanning probe microscope (Digital Instruments, Santa Barbara, CA, USA; Bruker Nanoscope analysis software Version 1.40), operating using PeakForce-QNM. The AFM measurements were obtained using ScanAsyst-air probes, for which the spring constant (0.58 N/m; Nominal 0.4 N/m) and deflection sensitivity had been calibrated, but not the tip radius (the nominal value used was 2nm). Surface roughness (Ra) values were determined by entering surface scanning data into a digital levelling algorithm (Bruker Image Analysis Nanoscope Analysis software V 1.40). AFM images were collected from two different samples and at random spot surface sampling (at least seven areas).

4.2.4 Immunisation and sampling

All animal experimentation was conducted in accordance to UK Home Office Legislation. In-house bred female or male BALB/c mice, 6-8 weeks old, were randomised, divided into groups of five and ear coded (n=5), food and water were supplied *ad libitum*. Mice were immunised on days 1, 15, 29 and 43 of the study. The protocols and delivery systems used are summarised in Table 4.1. A control group receiving no immunisation was included in each study. For nasal administration, 50µg equivalent of GnRH conjugate in 25µl of NISV was slowly pipetted in one or both of the nares. For subcutaneous administration, 50µg equivalent of peptide in 100µl of NISV was administered subcutaneously. Only prior to oral immunisation, mice were starved for 2h, but allowed access to water; 200µl of bilosomes containing 100µg of immunogen was intragastrically administered by gavage. Tail bleeds were collected 5 days after each immunisation in 1.5ml microfuge tubes. After centrifugation at 1000g for 20min, serum was collected in fresh microfuge tubes and stored at -20°C until antibody levels were determined by enzyme linked immunosorbent assay (ELISA). At the study end, blood was collected by cardiac puncture and serum obtained as described above. For IgA antibody level determination, tracheal-lung washes were carried out by infusion and aspiration of 1ml of PBS several times in the trachea. Similarly 5cm parts of the small intestine were collected and washed five times with 2ml of PBS and then 1ml of the suspension transferred into microfuge tubes and centrifuged as above. The supernatant was collected and stored at -20°C until assessed by ELISA.

4.2.5 Measurement of specific antibody levels

The ELISA was carried out as described in section 3.2.3 except for intestinal and lung washes (0.1ml, diluted 1:10 in PBS), were incubated per well (carried out in triplicate for each sample) and the secondary antibody step carried out by addition of 1:3000 dilution of IgG (H + L), IgG1, IgG2a or IgA goat anti-mouse (Southern Biotech, UK).

4.2.6 Determination of serum testosterone level in male mice

Serum testosterone levels were estimated as described in section 3.3.4.

4.2.7 Determination of Oestradiol 2 hormone level in female mice

Briefly; serum samples from female mice, collected at the end of the experiment, and standards were incubated in appropriate microtitre plate wells pre-coated with an antibody specific to oestradiol with a HRP-conjugated oestradiol and antibody preparation specific for oestradiol (Cusabio Biotech, China). Then a TMB substrate solution was added to each well. The enzyme-substrate reaction was terminated by the addition of a 10 % (v/v) sulphuric acid solution and the colour change measured spectrophotometrically at wavelength 450 nm. The concentration of oestrogen in the samples was then determined by comparing the O.D. of the samples to the standard curve.

4.2.8 Statistical analysis

Statistical significance and differences were compared against the control group, determined by one-way ANOVA with Dunnett's post-test performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, USA).

Groups	Immunogen	Route	Gender	Delivery system
1-2	KLH-GnRH-I	Nasal	M/F	LN+Xn
3-4	KLH-GnRH-III	Nasal	M/F	LN+Xn
5-6	KLH-GnRH-I	Oral	M/F	Bilosome
7-8	KLH-GnRH-III	Oral	M/F	Bilosome
9-10	KLH-GnRH-I	SC	M/F	LN
11-12	KLH-GnRH-III	SC	M/F	LN
13	KLH-GnRH-I	Vaginal	F	LN+Xn
14	KLH-GnRH-III	Vaginal	F	LN+Xn
15	Control (no immunisation)			

Table 4.1 Immunisation protocols of *in vivo* experiments. Mice were immunised on days 1, 15, 29 and 43 of the study. Nasal and vaginal administrations contained 50µg equivalent of GnRH peptide in 25µl NISV, suspended in 0.2% (w/v) xanthan gum. Subcutaneous administrations contained 50µg equivalent of peptide in 100µl NISV. Oral administrations consisted of 200µl bilosomes containing 100µg equivalent of peptide.
Abbreviations: LN; Immunogen Loaded NISV, Xn; Xanthan Gum, SC; Subcutaneous, M; male, F; female.

4.3 Results

4.3.1 NISV and bilosomes are similar in terms of size and ZP

Measurements, using different techniques (DLS, AFM and SEM) of the LNP revealed that they were in the nanometre size range, with negative surface charges (Table 4.2). The average particle size and ZP indicate that there is an increase in size between empty LNP (average 115±12nm and 134±15nm for EB and EN, respectively) and immunogen loaded LNP (average 130±14nm, 155±5nm and 170±3nm for LB, LN and LN+Xn, respectively).

The ZP values (ζ-potential) of LNP were negative and ranged from -34 to -52mV (Table 4.2). SEM images revealed the morphological appearance to be nearly spherical in shape, with a dark spot in the centre, an apparent smooth surface and in the size range of about 120-250nm (Figures 4.1A-C).

4.3.2 Surface analysis of NISV and bilosomes with and without protein

The mechanical values for the individual nanoparticles were calculated by AFM QNM mode, and the results are shown in Table 4.3 and in Figure 4.2. The particles were spherical as expected, without significant differences in the diameter between antigen containing and empty LNP. The surface roughness was lower when the LNP were loaded, while the presence of Xn caused the particles to be 9 times rougher. There were slight differences in the DMT Modulus between the empty and loaded LNP, whereas Deformation increased in the presence of Xn and Dissipation values were higher for the NISV compared with the bilosomes, although in the presence of Xn Dissipation was reduced.

Lipid Particles	Size (nm)±SD			PDI	ζ (mV) ±SD
	DLS	SEM	AFM		
EB	115 ±12	139 ±8	105 ± 18	0.19	-52 ±1
LB	130 ±14	158 ±31	93 ± 8	0.25	-34 ±2
EN	134 ±15	144 ±24	129 ± 20	0.21	-41 ±3
LN	156 ±5	221 ±14	131 ± 5	0.11	-47 ±2
LN+Xn	170 ±3	132 ±16	99 ± 5	0.27	-37 ±3

Table 4.2 Lipid particles analysis of size (measured by Dynamic Light Scattering (DLS), Scanning Electron Microscopy (SEM), and Atomic Force Microscopy (AFM)), Polydispersity index (PDI) and zeta potential (ζ), values represent mean ± SD (n=3).

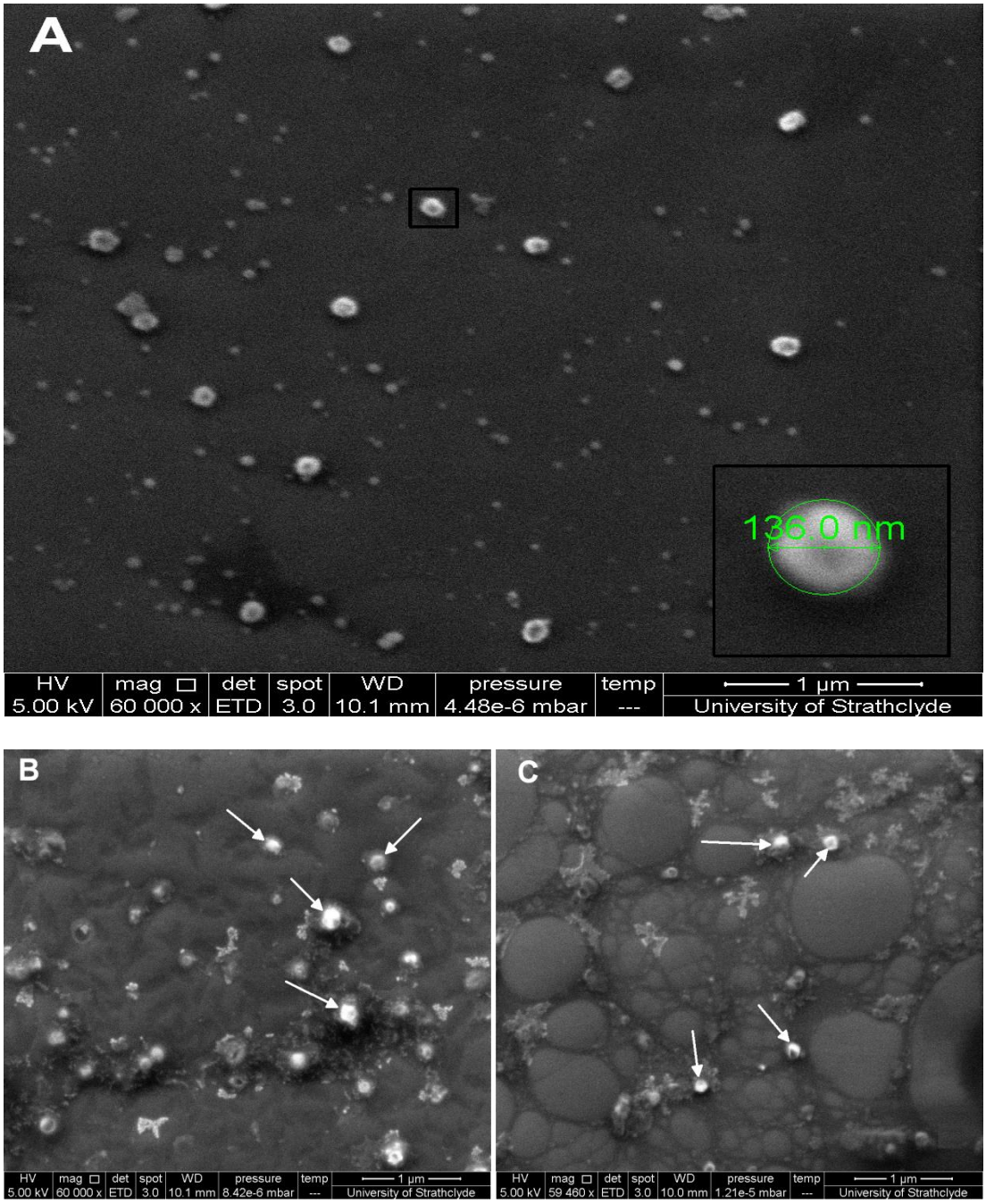


Figure 4.1 Scanning electron micrograph (SEM) images of LNP (A) representative SEM of bilosomes with entrapped GnRH-I conjugate (magnification x60,000, insert x240,000), (B) SEM showing a comparison of protein loaded NISV (arrows) and (C) in the presence of xanthan gum (magnification x60,000).

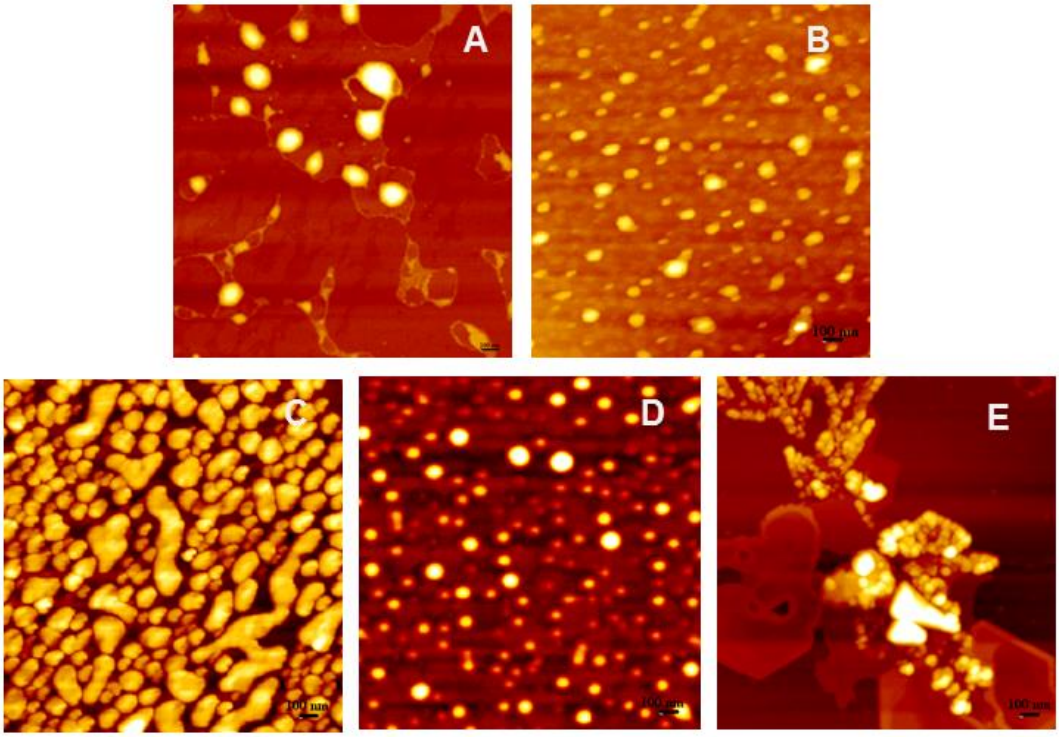


Figure 4.2 Representative AFM images of LNP; EB (A), LB (B), EN (C), LN (D) and LN+Xn (E); all images are 2µm x 2µm, with a 100nm scale bar.

Mechanical properties	EB	LB	EN	LN	LN+Xn
Surface Roughness / nm	3.2 ± 0.6	1.8 ± 0.4	2.1 ± 0.2	1.0 ± 0.1	9.4 ± 0.7
Force / nN	1.1 ± 0.4	4.3 ± 0.5	1.5 ± 0.6	2.8 ± 0.5	1.5 ± 0.1
DMT modulus / Mpa	45 ± 10	33 ± 8	52 ± 7	29 ± 7	38 ± 9
Deformation / pm	275 ± 20	245 ± 12	211 ± 28	492 ± 14	682 ± 19
Dissipation / eV	233 ± 18	245 ± 22	1290 ± 60	1134 ± 40	474 ± 8

Table 4.3 Mechanical properties of lipids as been measured by AFM; n= 20 random particles.

4.3.3 Specific anti-GnRH antibody detection

4.3.3.1 Antibody IgG (whole and subclass) responses

Levels of specific anti-GnRH-I and anti-GnRH-III in sera of immunised female mice varied between the study groups. Both immunogens loaded in LN and administered by s.c. and nasal routes showed significant ($p<0.001$) titres compared to the control, vaginal and oral groups (Figure 4.3). Anti-GnRH-I and anti-GnRH-III IgG (whole and subclasses) antibody levels expressed as the mean *A*450 of triplicate readings ±SD are listed in Table 4.3. IgG1 subclass was the major component of the IgG response observed in both s.c. and nasal groups, while the oral and vaginal groups did not show any significant difference from the control group (Figure 4.4 and Figure 4.5). IgG1 and IgG2a antibody subclasses produced against both immunogens by s.c. and nasal groups were both comparable in these groups (Table 4.3).

Specific anti-GnRH-I and anti-GnRH-III in sera from male mice showed very close similarity in nasal, s.c. and oral groups to that observed from female mice with the same IgG (whole and subclass) profiles (Table 4.3 and Figures 4.6-4.8).

4.3.3.2 Antibody IgA responses

Determination of IgA antibodies produced against both GnRH isoforms were observed in lung and intestinal washes collected from mice at experiment termination. Table 4.4 lists IgA antibody levels in lung and intestinal washes expressed as the mean *A*450 of triplicate readings ±SD. Mice (both male and female) immunised via the nasal route showed IgA release in the lung washes to GnRH-I and GnRH-III conjugates which was significant ($p<0.01$-0.001) when compared to

control groups. However, only female mice immunised against the GnRH-III conjugate via the vaginal route showed significant ($p<0.01$) IgA release in lung washes compared with control the control group. Whereas, oral and s.c groups did not show any significant IgA when compared with the control group (Figures 4.9-4.10 and Figures 4.13-4.14). On the other hand, ELISAs performed on intestinal washes did not show any significant difference between male groups ($p>0.05$). However, female mice immunised via the nasal route showed slightly significant ($p<0.05$) antibody responses to both immunogens when compared with the control group. While, orally immunised groups did not show any significant difference except in female mice immunised against GnRH-I conjugate (Figures 4.11-4.12 and Figures 4.15-4.16).

Female Groups						
Anti-GnRH-I	**IgG**		**IgG1**		**IgG2a**	
	Mean±SD	*P* vs. Control	Mean±SD	*P* vs. Control	Mean±SD	*P* vs. Control
LN/SC	3.06±0.1	<0.001	3.4±0.05	<0.001	1.03±0.28	<0.001
LN-Xn/Nasal	1.80±0.7	<0.001	3.9±0.33	<0.001	0.63±0.15	<0.001
LN-Xn/ Vaginal	0.47±0.1	>0.05	0.27±0.02	>0.05	0.07±0.02	>0.05
LB/Oral	0.39±0.1	>0.05	0.16±0.06	>0.05	0.06±0.001	>0.05
Anti-GnRH-III						
LN/SC	3.16±0.1	<0.001	3.4±0.05	<0.001	1.6±0.3	<0.001
LN-Xn/Nasal	2.11±0.8	<0.001	2.5±0.27	<0.001	0.4±0.1	<0.001
LN-Xn/ Vaginal	0.2±0.08	>0.05	0.17±0.07	>0.05	0.07±0.01	>0.05
LB/Oral	0.24±0.2	>0.05	0.21±0.02	>0.05	0.07±0.01	>0.05
Control	0.19±0.08	-	0.14±0.03	-	0.05±0.02	-
Male Groups						
Anti-GnRH-I						
LN/SC	3.13±0.3	<0.001	2.7±0.15	<0.001	0.5±0.42	<0.01
LN-Xn/Nasal	2.12±1.0	<0.001	1.8±0.27	<0.001	0.4±0.25	<0.01
LB/Oral	0.35±0.2	>0.05	0.13±0.03	>0.05	0.08±0.01	>0.05
Anti-GnRH-III						
LN/SC	3.01±0.2	<0.001	2.4±0.12	<0.001	0.7±0.23	<0.001
LN-Xn/Nasal	1.94±0.7	<0.001	1.5±0.23	<0.001	0.53±0.14	<0.001
LB/Oral	0.29±0.2	>0.05	0.11±0.03	>0.05	0.06±0.03	>0.05
Control	0.21±0.1	-	0.10±0.02	-	0.05±0.04	-

Table 4.3 Antibody IgG (whole and subclasses) of sera from all *in vivo* studies represented as O.D. at *A*450 mean ± S.D. with the relevant *p* values.

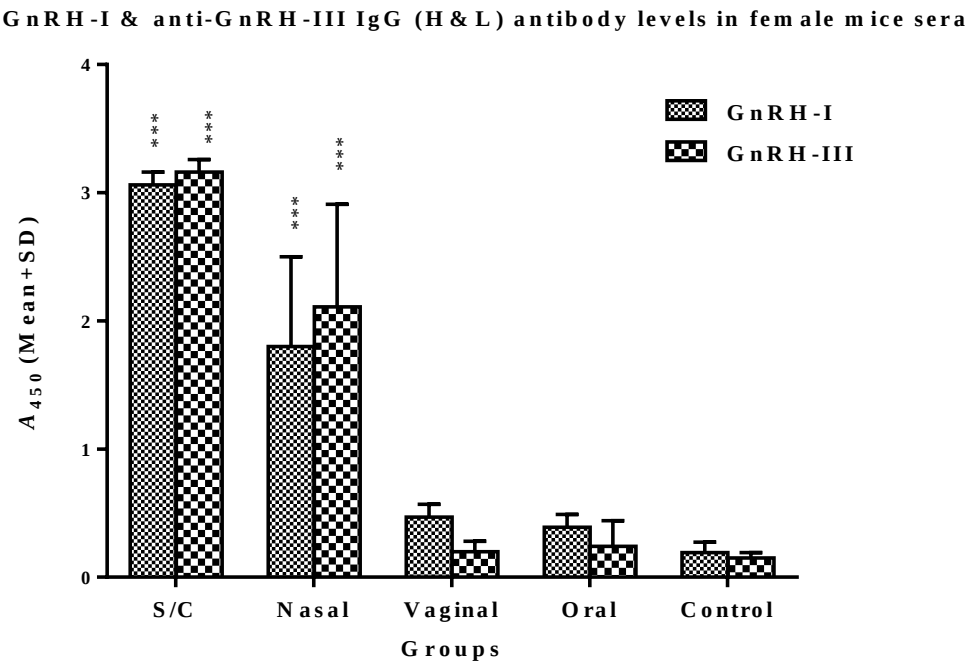


Figure 4.3 Anti-GnRH-I and anti-GnRH-III whole IgG antibody levels in sera from female mice. Bars represent Mean±SD O.D reading (n=5). *** ($p<0.001$) indicates significance level versus the control group.

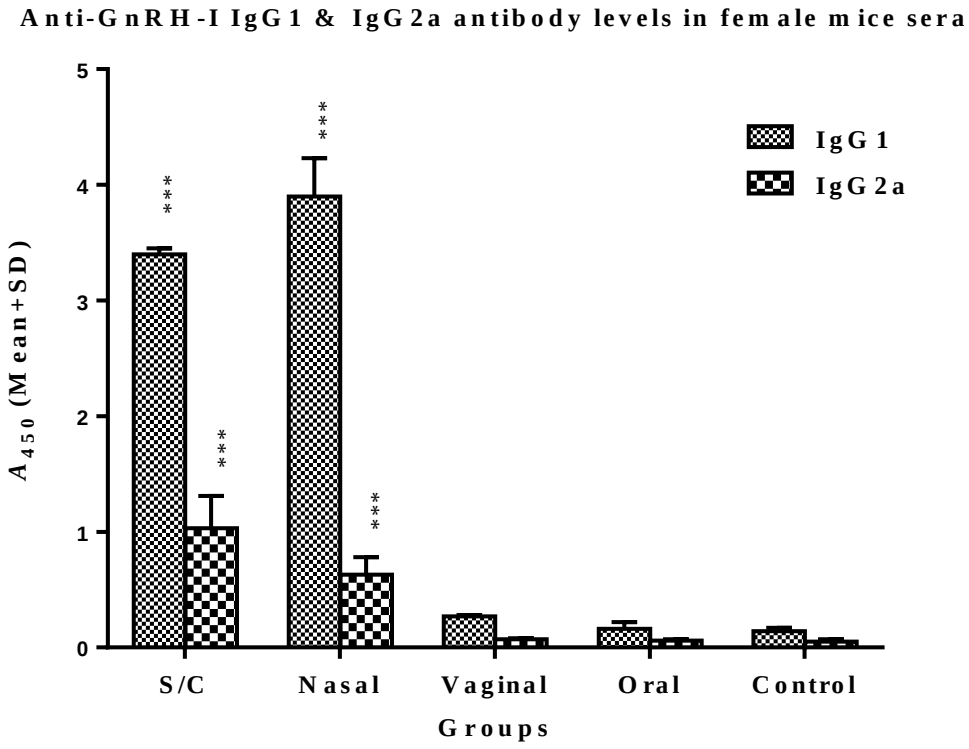


Figure 4.4 Anti-GnRH-I IgG1 and IgG2a antibody levels in sera from female mice. Bars represent Mean±SD O.D reading (n=5). *** ($p<0.001$) indicates significance level versus the control group.

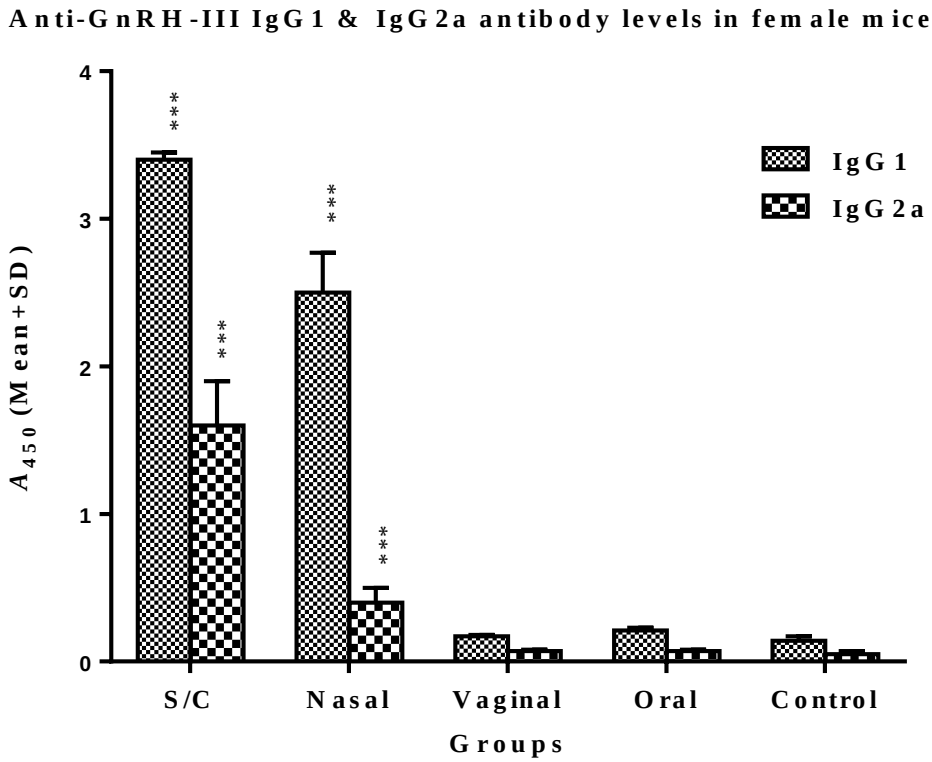


Figure 4.5 Anti-GnRH-III IgG1 and IgG2a antibody subclasses levels in sera. Bars represent Mean±SD O.D reading for ELISA performed on sera from female mice. *** ($p<0.001$) indicates groups significant value versus control group.

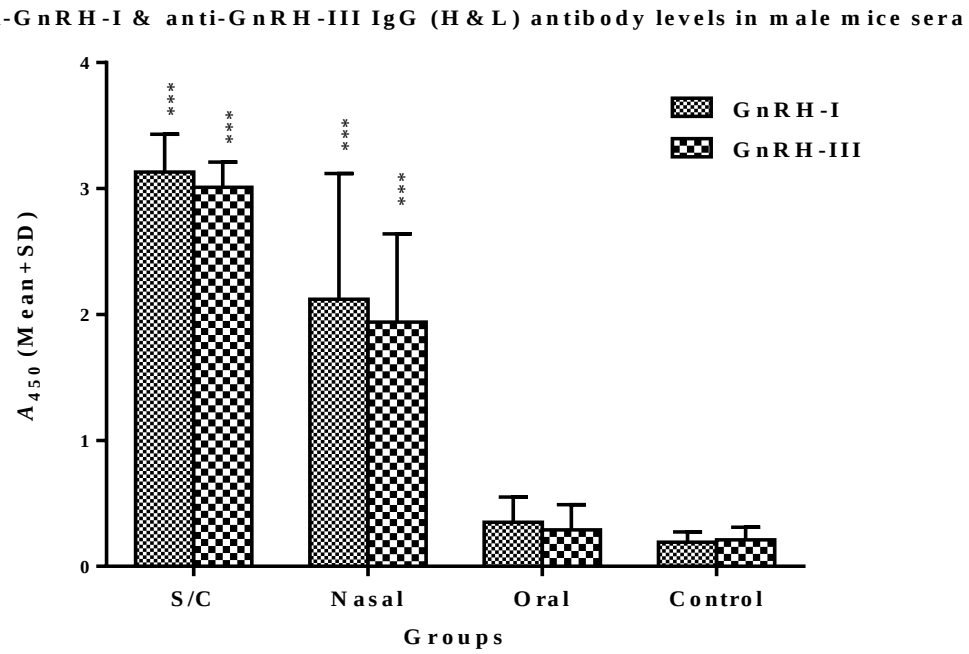


Figure 4.6 Anti-GnRH-I and anti-GnRH-III whole IgG antibody levels in sera from male mice. Bars represent Mean±SD O.D reading (n=5). *** ($p<0.001$) indicates significance level versus the control group.

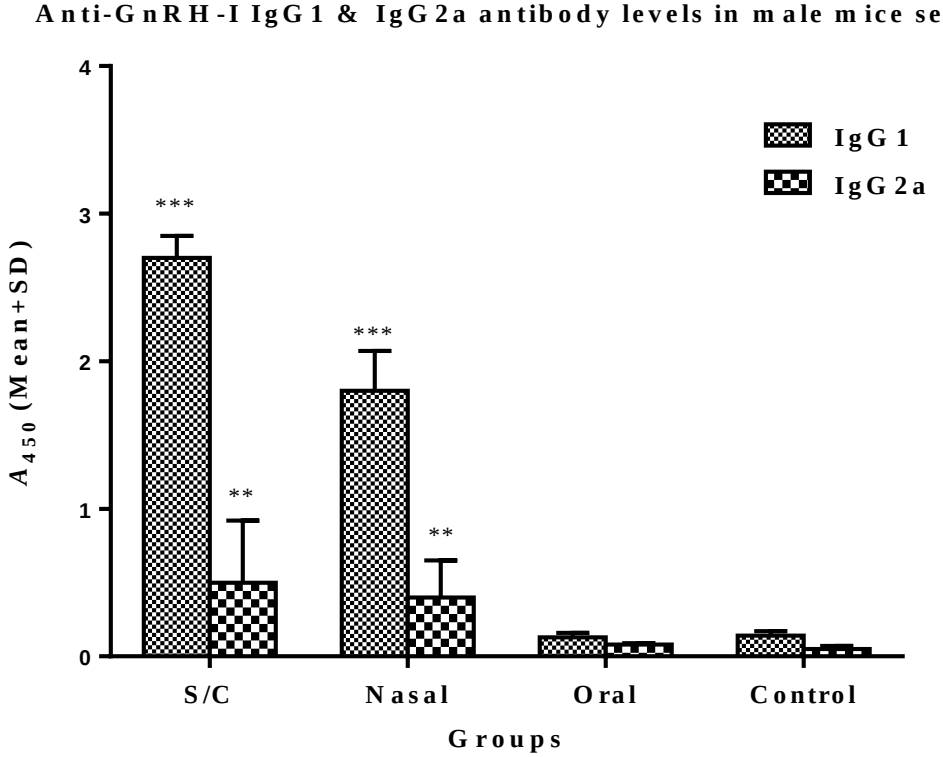


Figure 4.7 Anti-GnRH-I IgG1 and IgG2a antibody levels in sera from male mice. Bars represent Mean±SD O.D reading (n=5). ** ($p<0.01$) and *** ($p<0.001$) indicates significance level versus control group.

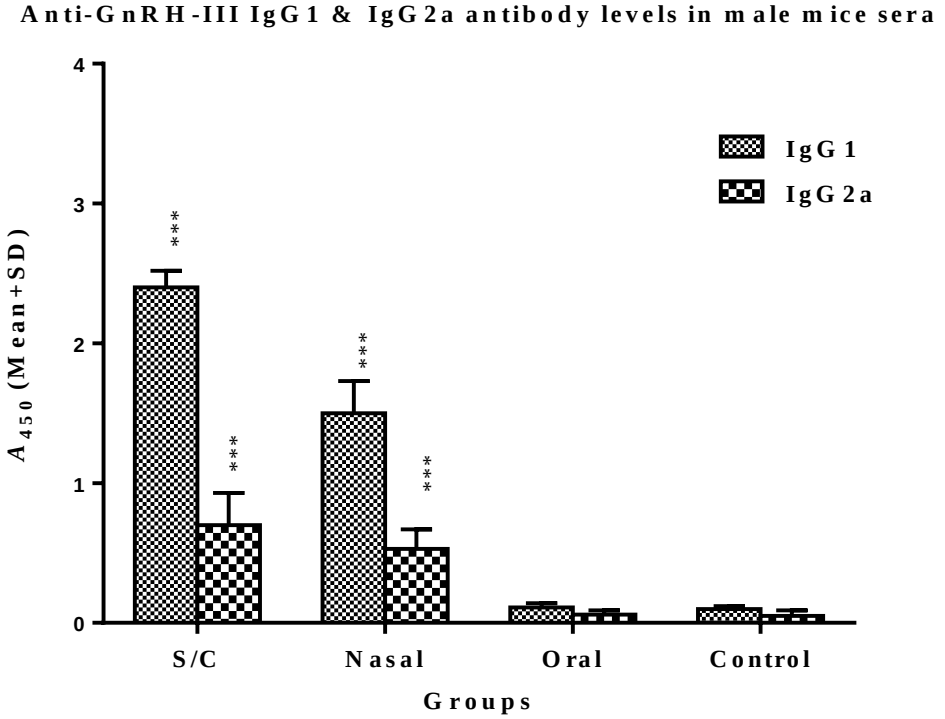


Figure 4.8 Anti-GnRH-III IgG1 and IgG2a antibody levels in sera from male mice. Bars represent Mean±SD O.D reading (n=5). *** ($p<0.001$) indicates significance level versus control group.

Female Groups				
Anti-GnRH-I	**Lung wash**		**Intestinal wash**	
	Mean±SD	*P* vs. Control	Mean±SD	*P* vs. Control
LN/SC	0.25±0. 04	>0.05	1.2±0.34	>0.05
LN-Xn/Nasal	1.72±1.53	<0.01	1.8±0.86	<0.05
LN-Xn/ Vaginal	0.32±0.07	>0.05	0.88±0.23	>0.05
LB/Oral	0.55±0.26	>0.05	1.7±0.61	<0.05
Anti-GnRH-III				
LN/SC	0.33±0.07	>0.05	0.55±0.25	>0.05
LN-Xn/Nasal	1.2±0.91	<0.01	1.35±1.05	<0.05
LN-Xn/ Vaginal	1.1±0.47	<0.01	1.08±0.40	>0.05
LB/Oral	0.55±0.12	>0.05	0.95±0.30	>0.05
Control	0.34±0.2	-	0.68±0.37	-
Male Groups				
Anti-GnRH-I				
LN/SC	0.38±0.2	>0.05	1.3±0.27	>0.05
LN-Xn/Nasal	2.03±1.3	<0.001	1.75±0.89	>0.05
LB/Oral	0.73±0.6	>0.05	1.41±0.73	>0.05
Anti-GnRH-III				
LN/SC	0.44±0.21	>0.05	0.74±0.38	>0.05
LN-Xn/Nasal	1.5±1.2	<0.01	1.26±0.94	>0.05
LB/Oral	0.42±0.11	>0.05	0.93±0.38	>0.05
Control	0.34±0.2	-	0.88±0.22	-

Table 4.4 IgA antibodies of lung and intestinal washes from female and male mice groups of mucosal *in vivo* experiment. Values represented as O.D. at *A*450 mean ± S.D. with the relevant *p* values.

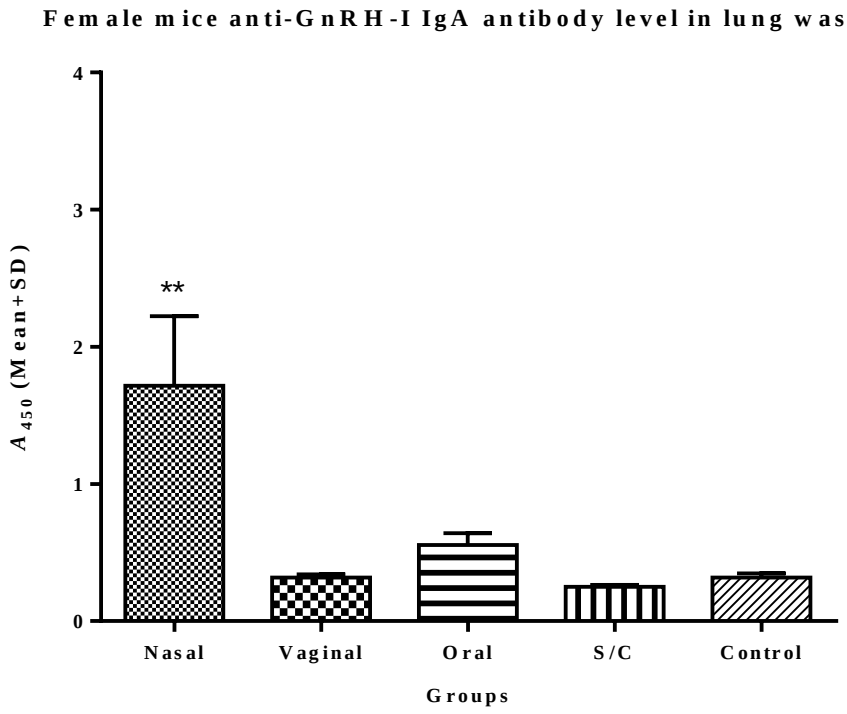


Figure 4.9 Anti-GnRH-I IgA antibody levels in lung washes from female mice. Bars represent Mean±SD O.D reading (n=5). ** ($p<0.01$) indicates significance level versus control group.

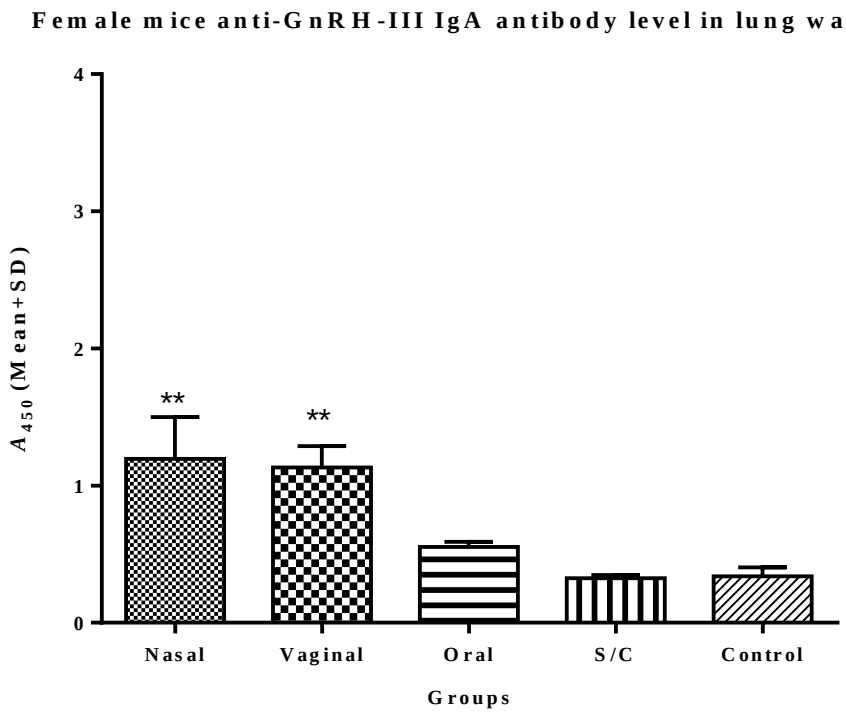


Figure 4.10 Anti-GnRH-III IgA antibody levels in lung washes from female mice. Bars represent Mean±SD O.D reading (n=5). ** ($p<0.01$) indicates significance level versus control group.

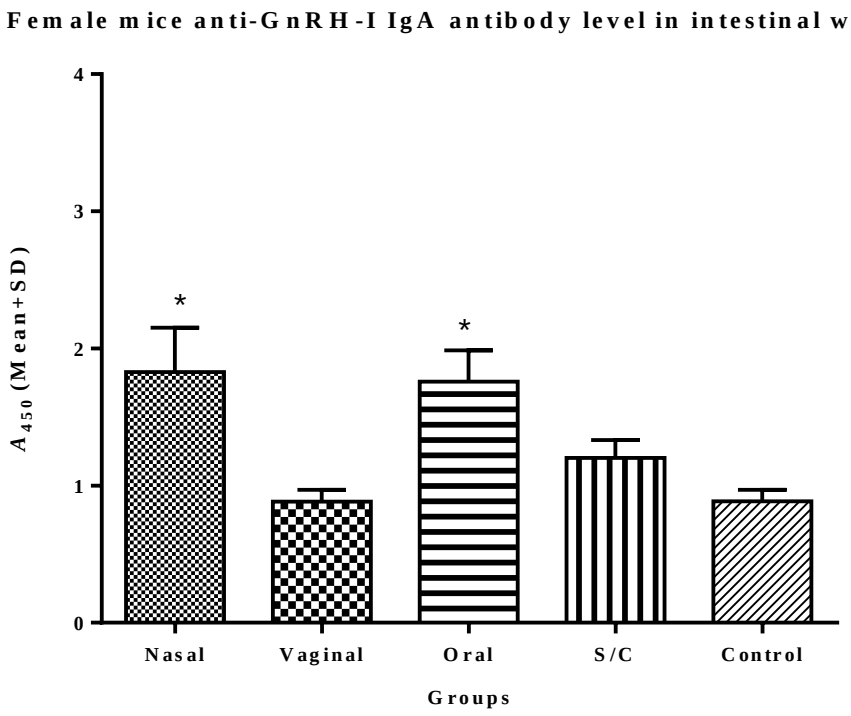


Figure 4.11 Anti-GnRH-I IgA antibody levels in intestinal washes from female mice. Bars represent Mean±SD O.D reading (n=5). * ($p<0.05$) indicates significance level versus control group.

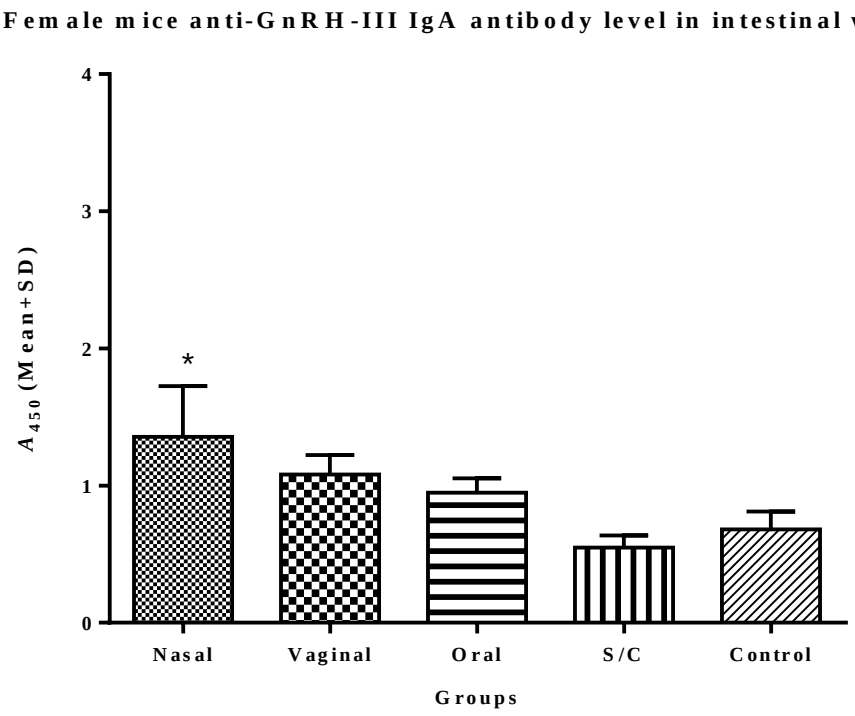


Figure 4.12 Anti-GnRH-III IgA antibody levels in intestinal washes from female mice. Bars represent Mean±SD O.D reading (n=5). * ($p<0.05$) indicates significance level versus control group.

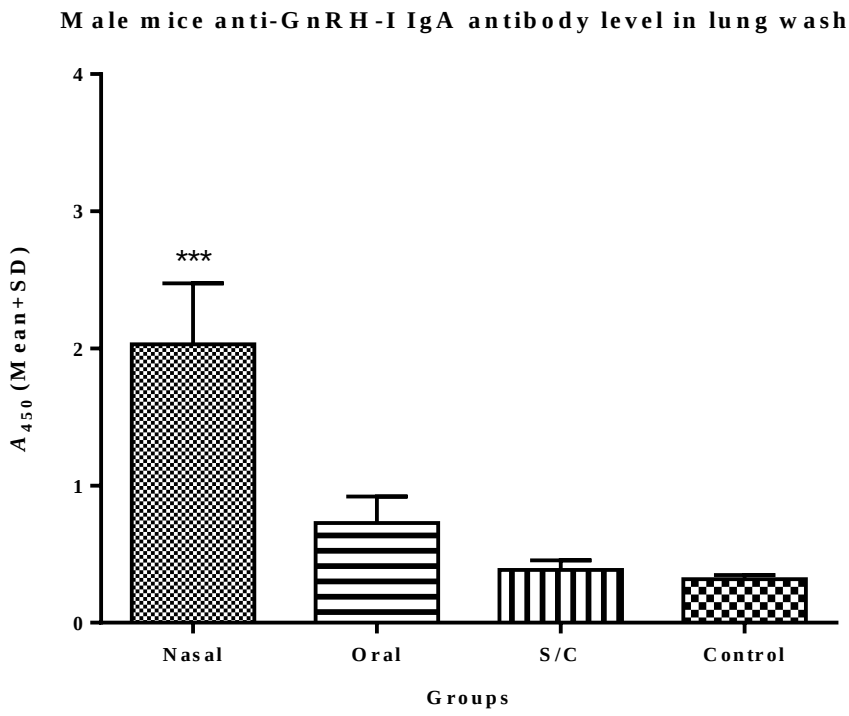


Figure 4.13 Anti-GnRH-I IgA antibody levels in lung washes from male mice. Bars represent Mean±SD O.D reading (n=5). *** ($p<0.001$) indicates significance level versus control group.

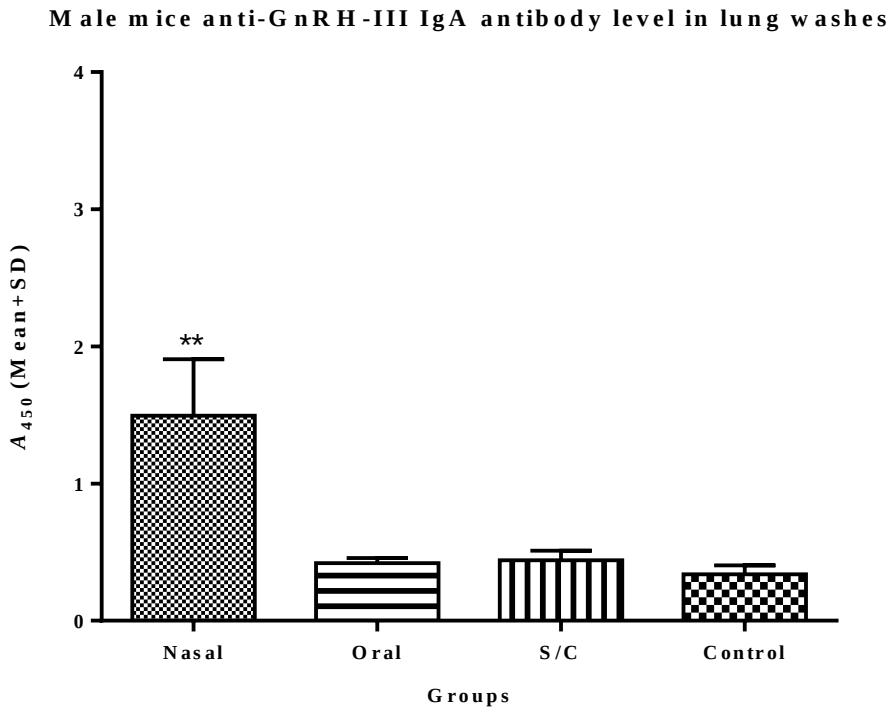


Figure 4.14 Anti-GnRH-III IgA antibody levels in lung washes from male mice. Bars represent Mean±SD O.D reading (n=5). ** ($p<0.01$) indicates significance level versus control group.

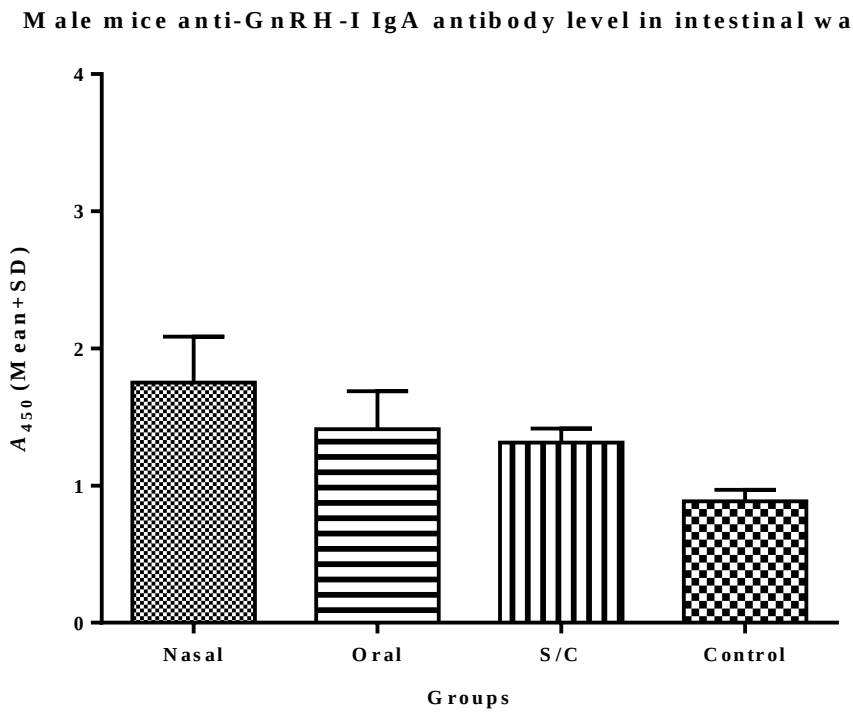


Figure 4.15 Anti-GnRH-I IgA antibody levels in intestinal washes from male mice. Bars represent Mean±SD O.D reading (n=5).

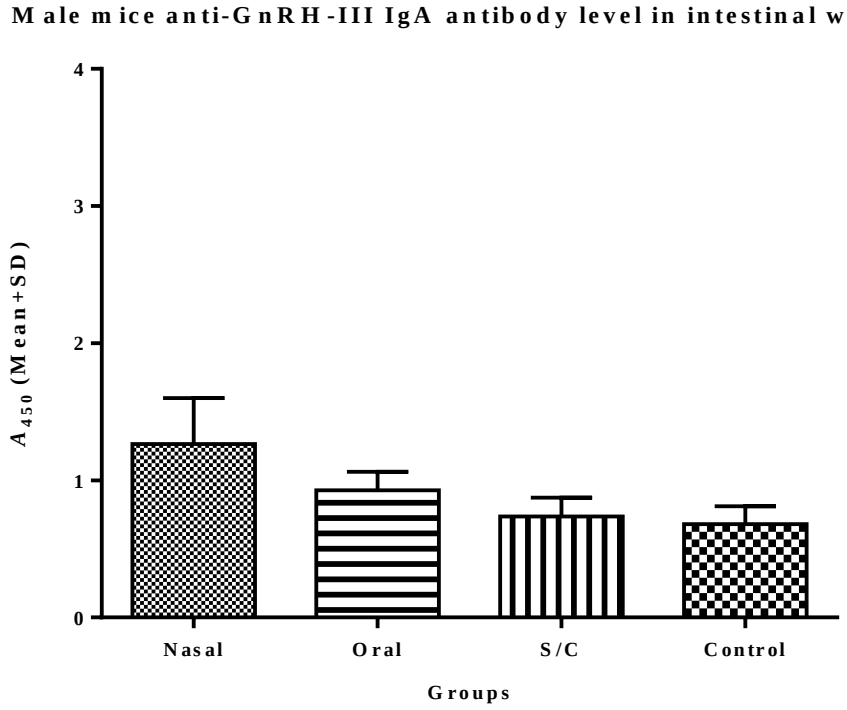


Figure 4.16 Anti-GnRH-III IgA antibody levels in intestinal washes from male mice. Bars represent Mean±SD O.D reading (n=5).

4.3.3.3 Effects on hormones levels in serum

Serum oestradiol concentrations were determined in samples from GnRH-I immunised female and control groups (Table 4.5). There was a non-significant variation in oestradiol 2 hormone levels in serum (Figure 4.17). However, oral and vaginal groups showed intergroup variation reflected as higher SD values when compared to other groups. Similarly, groups immunised via nasal, vaginal and s.c routes with GnRH-III conjugates showed non-significant decrease in oestradiol hormone concentration in serum of nasal, vaginal and s.c groups (Figure 4.17).

In male groups, testosterone hormone levels in serum from mice immunised against GnRH-I and GnRH-III conjugates showed a significant decrease in all treatment groups when compared with the control group (Table 4.5). Groups immunised against both immunogens via nasal and s.c routes showed very low testosterone hormone levels in serum which were highly significant ($p<0.0001$) when compared to the control group (Figure 4.18 and Figure 4.19). While, groups immunised against both immunogens via the oral route were also significant ($p<0.01$) compared with the control group. There were also significant differences between nasal and s.c groups when compared with oral groups. However, no significant differences were observed between nasal and s.c groups immunised with any of the conjugates (Figure 4.18 and Figure 4.19).

Hormone	Oestradiol (pg/ml)		Testosterone (ng/ml)	
Group	**GnRH-I**	**GnRH-III**	**GnRH-I**	**GnRH-III**
Control	51.7±1.2	56.3±1.0	14.6±5.5	14.2±1.5
Nasal	43.8±1.5	46.1±2.1	0.6±0.1	0.64±0.11
Oral	54.4±11.1	49.6±4.0	7.4±1.5	7.3±1.3
SC	43.3±1.0	42.6±6.1	1.8±0.3	1.9±0.4
Vaginal	43.6±8.5	45.5±13.6		

Table 4.5 Concentration of oestradiol and testosterone hormones in sera of female and male mice immunised against GnRH-I and GnRH-III conjugated to KLH via various routes represented as Mean±SD. Control group received no immunisation.

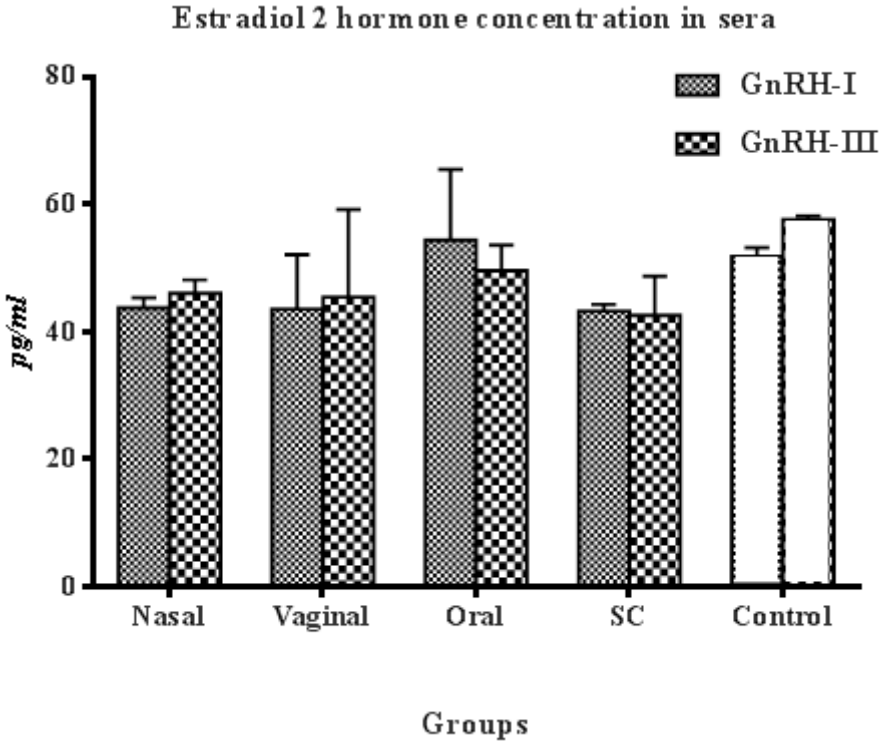


Figure 4.17 Serum oestradiol 2 hormone concentrations in female mice. Bars represent Mean±SD O.D reading (n=5).

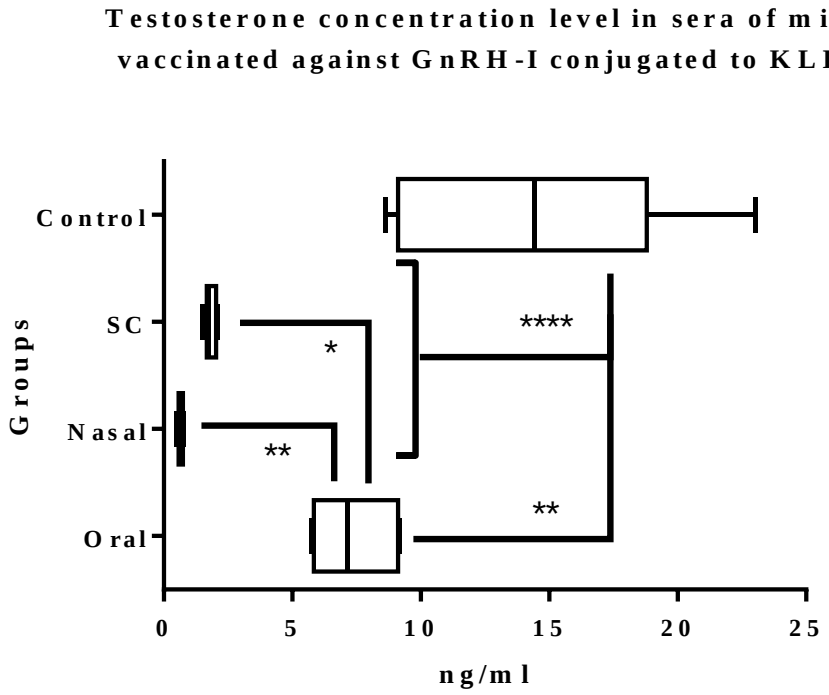


Figure 4.18 Testosterone hormone concentrations in male mice sera immunised against GnRH-I conjugated to KLH. Bars represent Mean±SD O.D reading (n=5).

* ($p<0.05$), ** ($p<0.01$) and **** ($p<0.0001$) indicates significance level. Lines link compared groups.

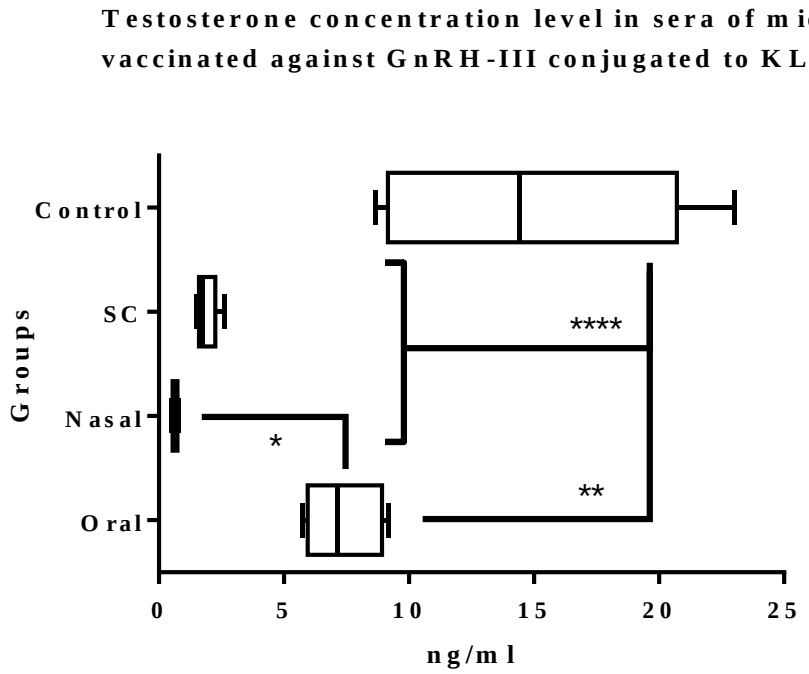


Figure 4.19 Testosterone hormone concentrations in male mice sera immunised against GnRH-III conjugated to KLH. Bars represent Mean±SD O.D reading (n=5). * ($p<0.05$), ** ($p<0.01$) and **** ($p<0.0001$) indicates significance level. Lines link compared groups.

4.4 Discussion

4.4.1 Vesicle characterisation

Established methods for characterising the vesicles were used in this study and confirmed previous measurements on size, ZP and loading capacity. The slight increase observed in LN+Xn size particles is most likely to be due to the properties of xanthan gum, but also may be due to limitations in DLS, single particles are less easily distinguished from agglomerates. The bilosomes showed smaller diameters compared with the NISV; these differences can be attributed to the effect of the addition of DOC. The negative ζ-potential values observed in all LNP are due to the charge of the DCP. This study used new techniques to study the physical characteristics of the vesicles produced to provide new insights into the appearance of the LNP. High resolution SEM was used, which revealed that the LNP were not just spherical-like, but they also had a dark centre. The significance of this is unknown, and it may be an artefact of the SEM processing procedure caused by high energy electron impact into the surface of the vesicles, which can be avoided by coating the vesicles with gold.

In order to study the surface properties in more detail, PeakForce QNM AFM was employed. All the vesicles are spherically shaped and appear flattened as a result of their adsorption on the mica surface, without any significant difference in the diameter between the loaded and unloaded LNP. The surface roughness determined from the images revealed that it was half that before loading, which means that the protein covers the particles and makes a smoother surface, with the exception of in

the presence of Xn which increased roughness. This is probably because Xn is a polymeric material and does not cover the whole surface homogenously. Furthermore, the individual vesicles appear to be located in different planes, which may be caused by rearrangements of the lipids during imaging, causing a reduction in image resolution (Serro *et al.,* 2012). The DMT Modulus of the empty vesicles is slightly larger than the loaded. The (force of) Adhesion of the loaded vesicles was approximately 3 times larger, and without any substantial difference in the Deformation, with the exception of the LN+Xn which were double the value than before, since the Xn changes the elastic/plastic properties of the surface. This was not unexpected, as Xn is known to alter properties of liposomes (Chiou *et al.,* 2009), and it is possible that changes in viscosity may have an influence. The Energy Dissipation did not show differences between the loaded and unloaded bilosomes, however the NISV showed the dissipation increased by 5 times. In a recent study, the effect of DOC on lipid membranes has been explored and this may provide an explanation for the observations made in the present study (Kiselev *et al.,* 2013). The mechanical data clearly shows that GnRH conjugates influence lipid membrane stiffness significantly, and lipids derived from either bilosomes or NISV without GnRH loading show similar mechanical properties, whereas loading the vesicles results in a noticeable reduction in vesicle membrane stiffness. This shows that the immunogen has an effect on the membrane flexibility and is a part of the membrane, rather than found in the aqueous core or just decorating the outer surface.

The AFM, DLS, and SEM values show that protein loading has no significant effect on the overall size of the LNP except in the presence of Xn. Moreover, all three techniques (AFM, DLS, SEM) show similar values, with the exception of in the

presence of Xn where in this case DLS showed a larger value since DLS cannot distinguish between single and several particles stuck together. Therefore, the AFM values for the particular samples are more accurate and provide complementary information and indicate that the roughness of the surface and mechanical properties of the LNP were altered by protein loading and in the presence of Xn.

4.4.2 Antibody response to KLH-GnRH immunisation

GnRH vaccines have been successfully developed for parenteral immunisation in both animals and humans (Talwar *et al.,* 1992; Miller *et al.,* 2008), yet the prevailing trend in vaccine products is towards mucosal administration. Previous studies established the potential of oral delivery using bilosomes for antigens derived from pathogenic organisms (Mann *et al.,* 2004; Mann *et al.,* 2006; Mann *et al.,* 2009b). In this study, the objective was to evaluate the use of bilosomes for a non-infective target (GnRH-I and GnRH-III isoforms). In order to generate antibodies against these "self-peptides", analogues were designed and conjugated to the carrier protein KLH. In the present study, comparable whole IgG results for the s.c. and nasal routes were obtained. A similar pattern was observed between the s.c and nasal groups in terms of pre-dominant IgG1 titres, indicating a Th2 response. Induction of a Th2 response was previously reported in rats immunised via parenteral routes with a tetanus toxoid-GnRH-I conjugate (Ferro *et al.,* 2004a). In addition, antigen entrapped in small lipid vesicles are known to induce a Th2 response as a result of how trafficking to late endosomes of antigen presenting cells with nanoparticles in this size range is achieved (Brewer *et al.,* 2004). Administration via the nasal route showed significant induction of an IgA response in female and male

groups in the nasal washes and with a lower degree in intestinal washes only in female mice. Although, it is recognised that Xn can be used to enhance immunogenicity (Chiou *et al.,* 2009). However, administration via the vaginal route did not show any significant induction of IgA response except in the group immunised with GnRH-III conjugates, which could indicate that the response is antigen-based. The antibody response to the conjugates entrapped in bilosomes and administered orally was very low in terms of whole IgG, IgG subclasses and IgA levels, but indicates that this platform technology may be limited to certain antigens and that a self-peptide of non-pathogenic origin such as GnRH is not a suitable immunogen. Several research attempts have been carried out with respect to GnRH-based oral vaccines (Kirkpatrick *et al.,* 2011), however no oral-active products have been forthcoming and this seems to support the results in this study for both GnRH-I and GnRH-III. This put forward the importance of further studies to understand how antigen characteristics and the chemical composition of the LNP evoke particular responses when delivered mucosally. Delivery via the oral route would have provided extensive applications in animal products for controlling fertility via the use of baits, but is not an essential mode of delivery for human vaccines. Indeed, nasal vaccines are now readily accepted as an alternative to injectable products (Rose *et al.*, 2012).

4.4.3 Effect of immunisation against GnRH analogues on hormone levels

In this study, a marked decrease in testosterone hormone levels in serum was noted in male mice immunised with both GnRH analogues via nasal and s.c routes. This is in agreement to previous studies in a rodent model immunised via parenteral

administration where an effective induction of high antibody titres, resulted in significant suppression of testosterone production and spermatogenesis (Khan *et al.,* 2007b). The carrier protein used in the Khan study (Khan *et al.,* 2007b), was tetanus toxoid administered with Imject® Alum in male mice. Despite that, immunisation via the oral route with GnRH conjugates loaded into bilosome did not induce systemic or local specific antibodies, yet a significant decrease in testosterone level was achieved. This suggests that GnRH conjugates have a role in blockage and desensitisation of GnRHR receptors following repeated administration of conjugated peptides (Tolkach *et al.,* 2013). In contrast, in female mice, the observed the reduction in oestradiol hormone levels were less ornot achieved which probably due to the amount of anti-GnRH produced not enough to totally neutralise native GnRH in the circulation. It is also possible that the oestradiol was of adrenal origin as sexual steroids are also produced by the adrenal cortex in response to adrenocorticotropic hormone (Imboden *et al.*, 2006). The reduction in hormone levels was achieved in a 6 weeks period which is considered a short period to gain a sustainable reduction of oestradiol hormone. Studies on mice vaccinated with GnRH-BSA conjugate required up to 12 weeks to reach highly significant reduction in gonadal hormones (Ganaie and Shrivastava, 2010). In another study, using mares as the animal model, vaccination against GnRH for more than 100 weeks did not significantly influence oestradiol-17 concentration in serum (Imboden *et al.*, 2006).

Further studies are required to investigate the female hormone changes due to immunoneutralisation of GnRH. The side effects encountered during manipulation of female mice genital area leading to a false pregnancy symptoms did not allow the evaluation of local IgA antibody responses in the vaginal discharges. In order to

eliminate such interference, large animal models such as ewes could be used in those studies and accompanied with pregnancy rate determination to evaluate the antifertility effects.

Immunisation against small self-hormone such as GnRH was not achieved via the oral administration of bilosomes which possibly immunogen specific, given that bilosomes have been previously studied for immunisation against pathogen-derived immunogenic epitopes. Therefore, further investigation on the use of bilosome as a delivery system for vaccination against pathogen-derived antigens is described in the next chapter.

Chapter five: Vaccination via different mucosal routes using pathogen-derived antigen

5.1 Introduction

Results obtained from mucosal immunisation with LNP loaded with a model non-pathogenic immunogen (GnRH) revealed that only the nasal route produced systemic and local immune responses compared with the vaginal and oral routes. The aim of this chapter was to investigate the LNP as a vaccine delivery system using pathogen-derived immunogens in vaccination using different mucosal administration routes. Haemagglutinin (HA) derived from influenza A H1N1 virus, New Caledonian strain has previously been used to evaluate the adjuvant properties of bilosomes in the application of an oral influenza vaccine (Bennett *et al.,* 2009). This antigen initiated IgG and IgA antibody production in serum and biological fluids. Therefore, in this Chapter, this antigen was used to compare mucosal immunisation of mice using different types of LNP by different administration routes and to evaluate mucosal and systemic immune responses.

A collaboration study with Finlay Institute (Havana, Cuba) resulted in a challenge experiment using a pathogen-derived immunogen – in this case TT was formulated into LNP modified for different mucosal routes. The immunised animals were then challenged with the pathogen toxin and the survival rates were evaluated.

Aims of this chapter

1- Immunisation of mice against HA antigen loaded in different LNP vesicles via different mucosal routes. Evaluating the systemic and mucosal antibody response in immunised animals.

2- Investigate to the specificity of the antibodies produced upon mucosal immunisation using LNP deliver system by toxin challenge trial in mice.

5.2 Methods

5.2.1 Vesicle preparation for evaluation of influenza mucosal vaccination

LNPs (EB and EN) were prepared by the MW method as described in section 2.2.1.2. New Caledonian haemaglutinin, N/Cal HA, (kindly supplied by Solvay Pharmaceutical, Netherlands) was entrapped in the prepared LNP before being further homogenised for 1min at 8000rpm and the vesicles named loaded loaded bilosomes (LB) or NISV (LN), respectively. The prepared emulsions were stored at 4°C until used for immunisation. For preparation of nasal and vaginal administrations, equal volumes of NISV and 0.2% (w/v) xanthan gum solution were mixed immediately before administration (LN+Xn).

5.2.2 Vesicles preparation for challenging experiments

LNPs (EB and EN) were prepared by the MW method as described in section 2.2.1.2 and then lyophilised as described in section 2.1.3. The lyophilised formulations were sent to the Finlay Institute (Havana, Cuba) where the challenge experiments were carried out by Dr Reinaldo Acevedo (Research and Development vice-presidency of Finlay Institute).

5.2.3 Animal experiments

5.2.3.1 Mucosal vaccination against N/Cal HA

In vivo experiments were designed to compare the efficacy of immunisation using the N/Cal HA loaded in LNP by nasal (LN+Xn), oral (LB), vaginal (LN +Xn) and subcutaneous (LN) routes. 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 with food and water available *ad libitum*. The immunisation schedule started with immunisation at day 0 followed by 2 booster immunisations with a 2 week interval period. The N/Cal HA dose for each group is listed in Table 5.1. Tail bleeds were collected on days 5, 20, and 35 and centrifuged at 13,000rpm in 1.5ml microfuge tubes for 20min. Serum was transferred into fresh 0.5 ml micro-centrifuge tubes (Fischer, UK), and stored at -20°C until IgG levels were determined by ELISA. The study was terminated on day 35. For determination of IgA levels, post-mortem lung and intestinal washes were carried out as described in section 4.2.4.

Group	Group	Route	LNP	N/Cal HA (µg)
1	**SC**	SC	LN	50
2	**Nasal**	Nasal	LN+Xn	50
3	**Bilo-Oral**	Oral	Bilosome	100
4	**Oral-plain**	Oral	None	100
5	**Oral+Xn**	Oral	None*Xn	100
6	**Vaginal**	Vaginal	LN+Xn	50
7	**Control**	Control	NA	

Table 5.1 Immunisation protocols of *in vivo* experiments. Mice were immunised on days 0, 14, 28 and 42 of the study. Nasal and vaginal administrations contained 50µg equivalent of N/Cal HA in 25µl NISV, suspended in 0.2% (w/v) xanthan gum. Subcutaneous administrations contained 50µg of N/Cal HA in 100µl NISV. Oral administrations consisted of 100µg N/Cal HA in 200µl of bilosomes, in 200µl of 0.1 (w/v) Xn (Oral-Xn) or in 200µl of PBS (Oral-plain). Abbreviations: LN; Immunogen Loaded NISV, Xn; Xanthan Gum, SC; Subcutaneous.

5.2.3.2 Evaluation of mucosal tetanus toxoid immunisation by lethal challenge model with tetanus toxin

Lyophilised LNP were reconstituted with the required amounts of TT vaccine in order to formulate LN and LB. The mixtures were vortexed for 5 min at high speed to ensure the process of protein entrapment into the lipid vesicles. LN were used as such, for s.c administration and for nasal administration LN+Xn was prepared to form a mixture containing a final concentration of 0.1% (w/v) xanthan gum solution. In addition to LB for oral immunisation, LB+Xn was also prepared by mixing LB with xanthan gum solution to a final concentration of 0.1% (w/v). The control group received no immunisation. The protocol and groups of the challenge experiment are listed in Table 5.2.

All animal immunisation, ELISA and toxin challenging were performed in Finlay Institute, Havana, Cuba. OF1 Outbred mice were used in this experiments as recommended by the vaccine quality control department in the Finlay Institute. Mice were randomly divided into groups of 10 (n=10) and immunised on day 0. All mice received the booster immunisation as shown in Table 5.2. The positive control group (Group 7) immunised on day 21 with the recommended dose of TT vaccine (vax-TET®, Finlay, Cuba). A negative control group (Group 8) received no immunisation. Tail bleeds were collected on day 42 from all groups including the control group for evaluation of IgG against TT in the serum. On day 49 all mice groups including the control group were injected with LD_{50} tetanus toxin for challenge evaluation. Survivors and deaths in mice were recorded for the next 4 days post-challenge.

Group	Immunogen	Route	Dose (µg)	Dose (ml)	Schedule	Sample	Challenge
1	**TT alone**	Oral	200	0.2	Days 0, 7, 14, and 21	Sera at day 42 for IgG ELISA	Tetanus toxin LD_{50} injected on day 49
2	**TT in LB**						
3	**TT in LB+Xn**						
5	**TT alone**	Nasal	50	0.02			
6	**TT in LN+Xn**						
7	**TT+Alum**	SC	0.16	0.5	Day 21		
8	**Control**	No immunisation					

Table 5.2 Schedule and groups for *in vivo* challenge experiment with tetanus toxin. Each group contains 10 OF-1 mice, Control group received no immunisation. Abbreviations: TT; tetanus toxoid, LB; TT loaded bilosomes, LB+Xn; TT loaded bilosome in 0.1% xanthan gum solution, LN+Xn; TT loaded NISV in 0.1% xanthan gum solution, TT+Alum; tetanus toxoid adsorbed onto an aluminum hydroxide gel (vax-TET®, Finlay institute, Cuba).

5.2.4 Measurement of specific antibody levels

For evaluation of specific IgG and IgA antibody against HA antigen, ELISAs were carried out on plates coated with 0.2µg/well N/Cal HA antigen. All procedures were as per sections 3.2.3 and 4.2.4.

Evaluation of anti-TT IgG antibody in sera by direct ELISA using polystyrene 96-well plates was carried out as routinely performed in Immunology Department, Finlay Institute, Cuba. Briefly, plates were coated with TT (100µl per well) at 5µg/ml in carbonate buffer (0.1mol/l, pH 9.6) at 4°C overnight, and blocked

with 1% (w/v) BSA in PBS (0.15mol/l, pH 7.3, blocking solution) for 1h at room temperature. Serum samples were diluted 1:100 in blocking solution and incubated for 1h at 37°C. Anti-mouse IgG peroxidase-conjugated antibodies (Sigma) were added (100µl per well) at 1:2500 dilution in blocking solution and incubated for 1h at 37°C. Bound antibodies were detected with 100µl per well of the substrate–chromogen mixture (o-phenylenediamine and H_2O_2 in citrate–phosphate buffer, pH 5). The reaction was stopped by adding 50µl of H_2SO_4 at 2mol/l and the optical density at 492 nm was measured in a microplate reader (Titertek, Multiskan Plus; Labsystem). All incubation steps were followed by three washes with PBS containing 0.05% (v/v) Tween-20.

5.2.5 Statistical analysis

Statistical significance and differences were compared against the control group, determined by one-way ANOVA with Dunnett's post-test performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, USA).

5.3 Results

5.3.1 Systemic and local immune response *against N/Cal HA*

All immunised groups produced IgG antibody against N/Cal HA. The IgG levels were highly significant ($p<0.0001$ for groups s.c., nasal and HA antigen with Xn given orally (group denoted Oral+Xn), $p<001$ for the group given bilosome loaded with HA antigen orally (denoted Bilo-Oral), and <0.05 for the group given HA antigen alone orally, denoted Oral-plain) with exception of the group immunised via the vaginal route Table 5.2 and Figure 5.1. However, the highest IgG levels were observed in groups immunised via s.c. and nasal routes with A_{450} Mean±SD values of 3.55±0.1 and 3.51±0.1 for s.c. and nasal groups, respectively. Even though groups immunised via oral routes showed high levels of serum IgG, the inter-group variation was also high as noted from the high SD values (Table 5.3). In contrast, all immunised groups showed significant production of IgA antibody in intestinal washes. However, groups immunised via oral routes had the highest titres compared with the nasal followed by the s.c groups and lastly the vaginal group (Figure 5.2). The inter-group variation was very low in all groups (Table 5.3). On the other hand, only groups immunised via s.c., nasal and by bilosome orally showed significant IgA antibody levels in lung washes (Figure 5.3). However, the SD of these groups was very high indicating a high level of mouse-to-mouse variation in immune response (Table 5.3).

Group	IgG (H&L)		Lung-IgA		Intestinal-IgA	
	Mean±SD	***P* vs. Control**	**Mean±SD**	***P* vs. Control**	**Mean±SD**	***P* vs. Control**
SC	3.55±0.1	<0.0001	1.5±1.4	<0.01	0.8±0.07	<0.0001
Nasal	3.51±0.1	<0.0001	1.6±1.5	<0.01	0.86±0.02	<0.0001
Oral+Xn	2.29±1.4	<0.0001	1.7±1.5	<0.01	1.3±0.07	<0.0001
Bilo-Oral	1.24±1.1	<0.001	0.6±0.5	ns	1.31±0.16	<0.0001
Oral-plain	0.93±0.6	<0.05	0.46±0.3	ns	0.97±0.04	<0.0001
Vaginal	0.31±0.19	ns	0.3±0.1	ns	0.33±0.02	< 0.001
Control	0.09±0.05	-	0.14±0.07	-	0.08±0.04	-

Table 5.3 IgA levels in lung and intestinal washes and IgG levels in sera from *in vivo* studies represented as O.D. at A450 mean ± S.D. with the relevant *p* values. Abbreviations: Bilo-Oral; mice immunised with bilosome via oral route, Oral-plain; mice immunised N/Cal (HA) diluted in PBS via oral route, and Oral+Xn; mice immunised with N/Cal (HA) in 0.1% (w/v) xanthan gum solution via oral route.

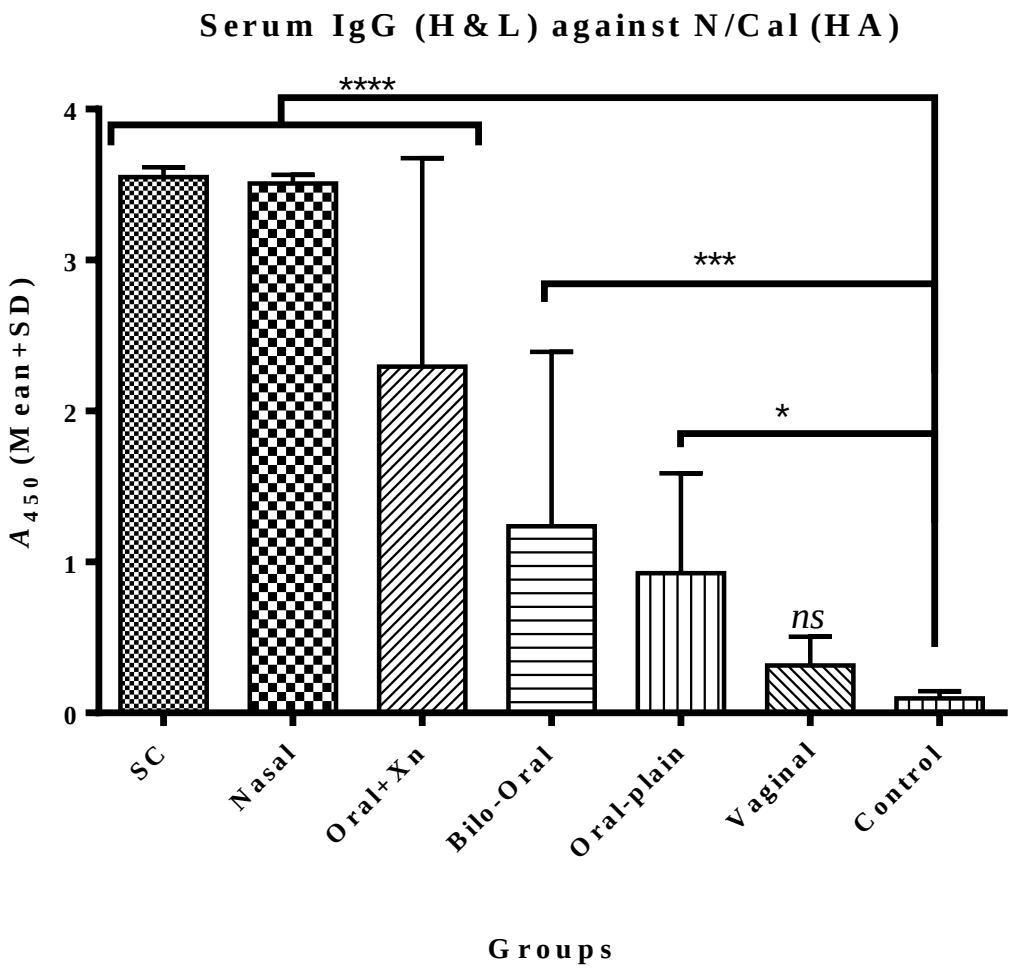


Figure 5.1 Specific IgG levels against N/Cal (HA) in sera *in vivo* experiment. Bars represent Mean±SD O.D reading (n=5).

Abbreviations: Bilo-Oral; mice immunised with bilosome via oral route, Oral-plain; mice immunised N/Cal (HA) diluted in PBS via oral route, and Oral+Xn; mice immunised with N/Cal (HA) in 0.1% (w/v) xanthan gum solution via oral route.

* ($p<0.05$), *** ($p<0.001$) and **** ($p<0.0001$) indicates significance level versus control group. *ns*; not significant.

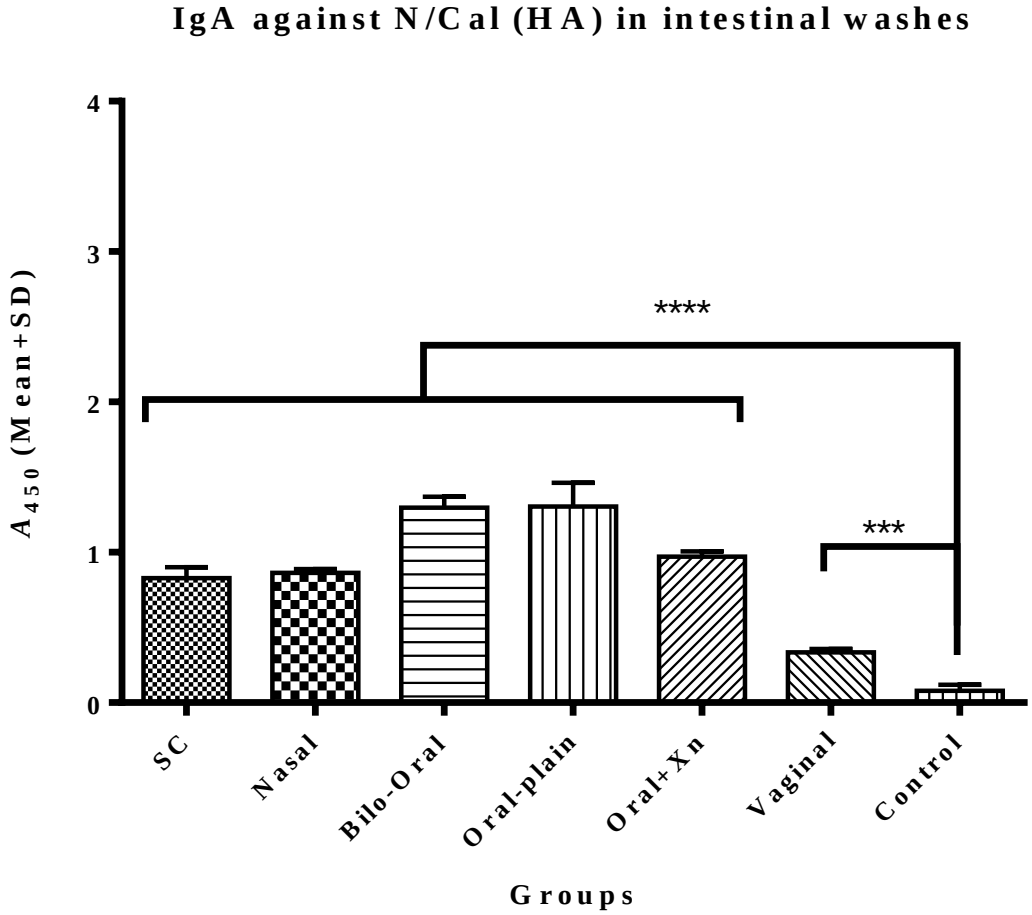


Figure 5.2 Specific IgA levels against N/Cal (HA) in intestinal washes from *in vivo* experiments. Bars represent Mean±SD O.D reading (n=5).

Abbreviations: Bilo-Oral; mice immunised with bilosome via oral route, Oral-plain; mice immunised N/Cal (HA) diluted in PBS via oral route, and Oral+Xn; mice immunised with N/Cal (HA) in 0.1% (w/v) xanthan gum solution via oral route.

*** ($p<0.001$) and **** ($p<0.0001$) indicates significance level versus control group.

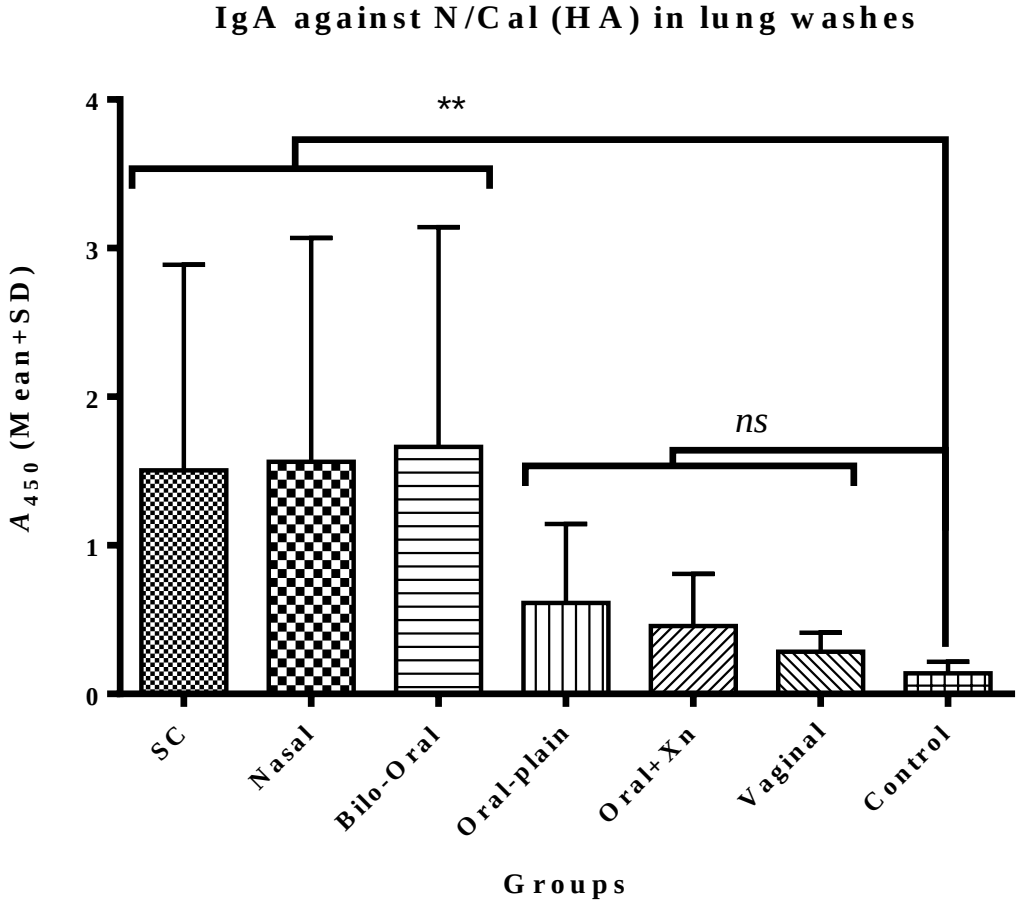


Figure 5.3 Specific IgA levels against N/Cal (HA) in lung washes from *in vivo* experiments. Bars represent Mean±SD O.D reading (n=5).

Abbreviations: Bilo-Oral; mice immunised with bilosome via oral route, Oral-plain; mice immunised N/Cal (HA) diluted in PBS via oral route, and Oral+Xn; mice immunised with N/Cal (HA) in 0.1% (w/v) xanthan gum solution via oral route.

** ($p<0.01$) indicates significance level versus control group. *ns*; not significant.

5.3.2 Antibody response and survivors rate for in vivo TT challenge experiment.

ELISAs were carried out at the Finlay Institute, Cuba. The O.D. at 492mn of specific IgG antibody was determined in sera of vaccinated groups (Figure 5.4). Groups immunised via the oral route showed the highest level of IgG which was significant ($p<0.0001$) compared to the positive control, LN+Xn and control groups. Similarly, groups immunised via the nasal route were significantly higher ($p<0001$) compared with the control group. Table 5.4 lists the IgG OD levels and significant degree between the experimental groups.

The initial challenge study was carried out by injection of LD_{50} tetanus toxin into mice. The deaths were recorded for the next 4 days. Table 5.5 shows the death and final percentage of survivors in the study groups. All animals in the orally immunised groups survived the challenge with no deaths after 4 days. Similarly, 100% of animals in the positive group survived, compared with 80% of animals in the nasally immunised groups and 0% of the negative control group.

In a pilot toxin-challenge trial to determine dose response and LNP:immunogen ratio compared to standard vaccine. The optimal LNP:immunogen ratio was 10:1 formulation with the immunogen dose equivalent to the standard vaccine dose Figure 5.5.

Group	TT alone / orally	TT in LB	TT in LB+Xn	TT alone / nasally	TT in LN+Xn	TT+ Alum	Control
Mean±SD	1.1±0.17	0.99±0.2	1.2±0.13	0.81±18	0.55±0.21	0.41±0.1	0.15±0.04
TT alone / orally	NA	NS	NS	<0.05	<0.0001	<0.0001	<0.0001
TT in LB	NS	NA	NS	NS	<0.0001	<0.0001	<0.0001
TT in LB+Xn	NS	NS	NA	<0.001	<0.0001	<0.0001	<0.0001
TT alone / nasally	<0.05	NS	<0.001	NA	<0.05	<0.0001	<0.0001
TT in LN+Xn	<0.0001	<0.0001	<0.0001	<0.05	NA	NS	<0.0001
TT+Alum	<0.0001	<0.0001	<0.0001	<0.0001	NS	NA	<0.05
Control	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.05	NA

Table 5.4 Mean±SD of anti-TT IgG ELISA reading at 492nm (n=10), and the significant level between groups using ANOVA with Tukey's multiple comparisons test.

Abbreviations: TT; tetanus toxoid, LB; TT loaded bilosomes, LB+Xn; TT loaded bilosome in 0.1% (w/v) xanthan gum solution, LN+Xn; TT loaded NISV in 0.1% (w/v) xanthan gum solution, TT+Alum; tetanus toxoid adsorbed onto an aluminum hydroxide gel (vax-TET®, Finlay Institute, Cuba).

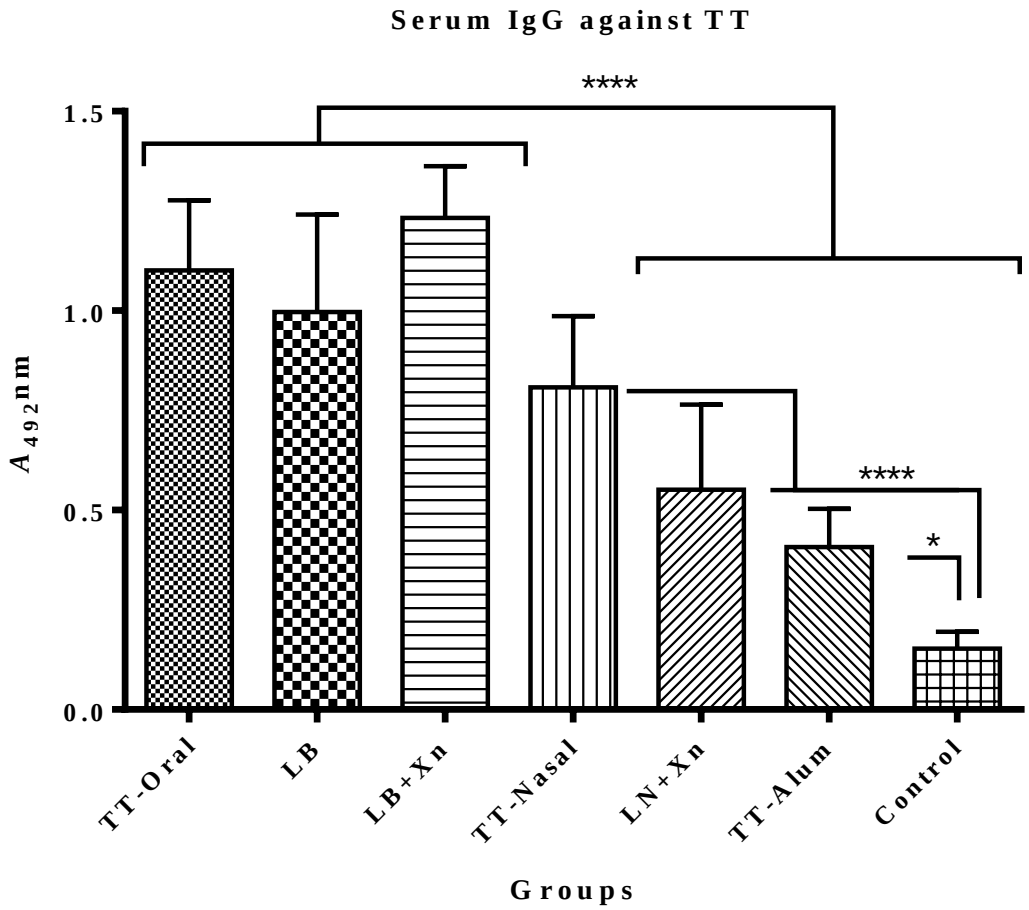


Figure 5.4 Anti-TT IgG antibodies in sera of mice immunised against TT on days 0, 7, 14 and 21. Bars represented as Mean±SEM and n=10.

Abbreviations: TT; tetanus toxoid, LB; TT loaded bilosomes, LB+Xn; TT loaded bilosome in 0.1% (w/v) xanthan gum solution, LN+Xn; TT loaded NISV in 0.1% (w/v) xanthan gum solution, TT+Alum; tetanus toxoid adsorbed onto an aluminum hydroxide gel (vax-TET®, Finlay Institute, Cuba).

Group	Route	Dose µg	Animal (N)	Day 1	Day 2	Day 3	Day 4	% Survivors
TT alone	Oral	200	9	0	0	0	0	100
TT in LB			7	0	0	0	0	100
TT in LB+Xn			7	0	0	0	0	100
TT alone	Nasal	50	10	1	1	0	0	80
TT in LN+Xn			10	1	1	0	0	80
TT+Alum	SC	0.16	10	0	0	0	0	100
Control	No immunisation		10	5	5	-	-	0

Table 5.5 Deaths and survival percentage after challenge with tetanus toxin (International standard).
Abbreviations: TT; tetanus toxoid, LB; TT loaded bilosomes, LB+Xn; TT loaded bilosome in 0.1% (w/v) xanthan gum solution, LN+Xn; TT loaded NISV in 0.1% (w/v) xanthan gum solution, TT+Alum; tetanus toxoid adsorbed onto an aluminum hydroxide gel (vax-TET®, Finlay Institute, Cuba).

Groups				**% Survivors**				
Oral	**LNP:Imm**	**Dose (LF)**	**N**	**Day 0**	**Day 1**	**Day 2**	**Day 3**	**Day 4**
LB+Xn	100:1	0.05	10	100	100	100	100	90
LB+Xn	1:1	0.1	10	100	100	100	100	90
LB+Xn	1:1	0.05	11	100	100	100	100	81
LB+Xn	1:1	0.025	10	100	70	70	40	10
LB+Xn	1:1	0.0125	10	100	0	0	0	0
LB+Xn	10:1	0.1	10	100	100	100	100	100
LB+Xn	10:1	0.05	10	100	100	100	100	100
LB+Xn	10:1	0.025	10	100	100	100	100	100
LB+Xn	10:1	0.0125	10	100	100	100	100	100
vax-TET	**Dose (LF)**							
A	0.1		20	100	95	95	95	95
B	0.05		20	100	95	95	85	80
C	0.025		20	100	95	65	30	20
D	0.0125		20	100	50	20	5	0
Placebo	-		10	100	0	0	0	0

Table 5.6: Survival percentage for *in vivo* experiment using various LNP:immunogen ratios and doses.
Abbreviations: LB+Xn; loaded bilosome in 0.1% (w/v) xanthan gum solution, LF; flocculation value (0.05 LF= 0.16µg). LNP; lipid nanoparticles, Imm; immunogen protein.

5.4 Discussion

5.4.1 Immune response against N/Cal HA mucosal immunisation

Immunisation using LNP as a delivery vaccine system for N/Cal HA antigen showed comparable immune responses in groups immunised via the nasal route compared with the group immunised via the s.c. route. Nasal vaccination with LN+Xn showed high serum IgG and local IgA production. Recently, xanthan gum has been studied in a nasally delivered bioadhesive liposome using an inactivated H5N3 virus as a model antigen (Chiou *et al.*, 2009). The study showed that mixing liposomes with 0.3% (w/v) Xn solution elicited higher mucosal s-IgA and serum IgG after two vaccinations.

However, when comparing IgG antibody, groups immunised via the oral route (with bilosome or Xn alone) a very high inter-group variation was observed as noted from the high SD value. This probably indicates that there may be an oral tolerance against the antigen in some of the animals or degradation of the immunogen which rendered the antigen non-immunogenic and this could account for these variations. This inter-group variation was also seen in the group immunised with the antigen alone orally, which confirms that the change is occurring in the immunogen itself. However, among the orally immunised groups, the group immunised with immunogen added to Xn solution showed a higher IgG antibody response compared with those obtained after nasal and s.c. immunisation. This result suggested that the presence of the mucoadhesive agent in orally administered vaccine allowed the antigen to remain for a longer period in contact with mucosal surfaces in the GALT to enable sampling by APCs or M cells in order to stimulate T and B lymphocytes (Pearse and Drane, 2005;

Stewart and Devlin, 2006). While, the group immunised via the vaginal route showed low production of IgG and IgA, which was not significant when compared with the control group. In a recent study, vaginal immunisation of progesterone-treated BALB/c mice with recombinant influenza A virus elicited humoral and local antibody responses (Garulli *et al.*, 2004). The study suggested that the use of influenza virus for vaginal immunisation as the viruses are strong inducers of cellular immune responses. Specifically, the ability of influenza viruses to infect DCs and to replicate and produce viral genes at high levels permits APCs to initiate an appropriate immune response. However, in this research study the N/Cal HA protein was used as an antigen, which is not a complete virus and has no capability to replicate in host cells.

5.4.2 In vivo challenge to tetanus toxin

The percentage of animals that survived when challenged with an international standard of tetanus toxin was in agreement with the level of IgG determined in the serum. Even though, there was no significant difference between orally immunised groups, there were significant differences between orally and nasally immunised groups and also to the positive control group. On the other hand, TT alone administered nasally was significantly different from the Alum vaccine. In a previous study, the coadministration of cholera toxin as an adjuvant with TT via oral route induced peak serum IgG and IgA anti-TT responses (Jackson *et al.*, 1993). This raises the question, do LB, LB+Xn and LN+Xn have any adjuvant effect to enhance the vaccine?

In this study, a high dose was administered orally in comparison to nasally and s.c immunised groups. As a result, further study was carried out to investigate lower doses and different LNP:immunogen ratios. The results obtained from those studies were focused on survival percentages after challenge (Table 5.6). However, the evaluation of antibody responses was not carried out in this experiment. The results showed that 10:1 LNP:immunogen ratio gives the best protection (100%) against the toxin challenge compared to the equivalent immunogen amount used in international standard candidate. Therefore, this result suggests that the use of LB+Xn in a 10:1 ratio formulation has a strong adjuvant effect which, would be recognised if the antibody response had been evaluated.

In this chapter, mucosal administration of pathogen-derived antigens with and without LNP was investigated. The results obtained showed that administration of these antigens alone by oral route produced systemic and local immune response. The antibodies produced were specific and protected the animals against toxin challenge. Therefore, it seems that bilosome has no adjuvant effect to these antigens and it could be the loaded antigens liberated from bilosome vesicles in the GIT and has been sampled by APCs in the GALT. The next chapter investigate the fate of bilosome in GIT.

Chapter six: Bilosome fate in the GIT

6.1 Introduction

Oral administration of LNP containing bile salts has been studied by many groups for vaccine development (Singh *et al.,* 2004; Mann *et al.,* 2006; Shukla *et al.,* 2011; Premanand *et al.,* 2013)or for drug delivery (Ayogu *et al.,* 2009). In this research the use of the bilosome as a delivery system for immunisation in mice has been evaluated using non-pathogenic peptide formulations and proteins from pathogenic sources. Systemic and local antibody responses developed after oral immunisation by both types of antigens. However, the systemic responses were not comparable to vaccination via nasal or s.c. routes despite the use of higher antigen concentrations in the bilosome formulations. On the other hand, the challenge experiment showed that immunisation against TT using bilosomes via the oral route protected the animals from death by 100%. At this point, one explanation that may apply here, is that bilosomes helped to protect the antigens from the harsh environment in the stomach, especially due to exposure to low pH (the effect of stomach HCl) and degradation by enzymes present in the gastric fluids. Furthermore, when bilosomes reach the small intestine, they will encounter further enzymatic degradation that could affect the lipid structure of the bilosome and through degradation reduce the amount sampled by M cells or DCs in the intestinal mucosa. To investigate the fate of bilosomes in the GIT, bilosomes loaded with BSA were treated with simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) in an *in vitro* experiment. The hallmark of digestion of proteins by digestive enzymes is the cleavage of proteins into smaller units of peptides and single amino acids. The simulation of BSA protein digestion by pepsin or trypsin using ExPASy PeptideMass online tool produce specific peptide/amino acid profiles. The degradation of BSA via

the proteolytic effect of enzymes in simulated fluids was assessed by quantification of small peptides resulted from the complete digestion using a liquid chromatography/mass spectrometry (LC/MS) method. In addition, in order to track this degradation an *in vivo* experiment involving oral administration of bilosomes loaded with gold nanoclusters of a BSA conjugate (BSA-AuNCs) in rats followed by fluorescent microscopy using two-photon excitation of frozen sections from rat intestine was carried out to visualise the protein uptake/distribution by the mucosa epithelial cells.

Aims in this chapter

1- To study the effect of incubation of bilosomes with simulated gastric intestinal milieu.

2- To study the cellular uptake of bilosome in the GALT.

6.2 Methods

6.2.1 Vesicles preparation

Empty bilosomes were prepared as described in section 2.2.1.2 and lyophilised as per section 2.2.1.3

6.2.2 Study of effect of GIT fluids on BSA entrapped in bilosome (in vitro)

6.2.2.1 Preparation of SGF and SIF

SGF and SIF were prepared according to the United States Pharmacopeia (USP 24) (United States pharmacopeial, 1999). SGF was prepared with 2g of NaCl and 7ml of HCl, diluted to 1l with water and pH adjusted to 1.2 using 1.0M HCl. After that 320mg of porcine pepsin (enzymatic activity 800-2500units/mg, Sigma-Aldrich Ltd., UK) was added to 100ml of the HCl solution. SIF was prepared with 0.68g of K_2HPO_4, 19ml of 0.2N NaOH and diluted to 100ml with water and pH being maintained at 6.8. Then 1g of porcine pancreatin (Sigma-Aldrich Ltd., UK) was dissolved in 100ml of the prepared solution to produce enzyme containing SIF.

6.2.2.2 Incubation of BSA loaded bilosome in simulated fluids

BSA was entrapped in bilosomes by dissolving 100mg of BSA (Sigma-Aldrich Ltd., UK) in 2ml ultrapure water and adding to 160mg of lyophilised bilosome. The mixture were vortexed for 5min and further stirred for 2h at 800rpm. Then 0.5ml of the formulation was suspended in 5ml 0.025M carbonate buffer pH 9.7 and centrifuged at 35,000rpm for 1h to remove any unentrapped BSA. The pellet was resuspended in 0.5ml of 0.025M carbonate buffer pH 9.7 and incubated with

2ml SGF or SIF for 3h in a water bath at 37°C. The treated bilosome mixture was then centrifuged at 13,000rpm for 10min and the supernatant was filtered and stored at -80°C until used in LC/MS analysis.

6.2.2.3 LC/MS procedure

A method previously described by Bratty *et al*., (2011) was adopted for LC/MS analysis of samples. Briefly, LC/MS data were acquired using a Finnigan LTQ Orbitrap instrument (Thermo Fisher Scientific, Hemel Hempstead, UK) set at 30,000 resolution. Sample analysis was carried out under positive ion mode. The mass scanning range was m/z 50-1200, while the capillary temperature was 200°C and the sheath and auxiliary gas flow rates were 30 and 10 arbitrary units, respectively. The LC/MS system (controlled by Xcalibur version 2.0, Thermo Fisher Corporation) was run in binary gradient mode. Solvent A was 0.1% (v/v) formic acid in HPLC grade water and solvent B was 0.1% (v/v) formic acid in acetonitrile. A flow rate of 300µl/min was used and the injection volume was 10µl. The gradient used was as follows: 90% B at (0 min), 50% B at (16 min), 20% B at (18 min), 20% B at (28 min) and 90% B at (36 min). Samples were kept in a vial tray which was set at a constant temperature of 3°C. Mass measurement was externally calibrated according to the manufacturer's instructions just before commencing the experiment, and was internally calibrated by lock masses (positive ion mode m/z 83.06037 and m/z 195.08625, due to acetonitrile dimer and caffeine, respectively, and negative ion mode 91.00368 due to formic acid dimer).

Data files were processed using Sieve 1.3 (Thermo Fisher,Hemel Hempstead UK). The parameters used in Sieve were: time range 4–35 min, mass range 50–1200

amu, frame width 0.02 amu and Rt width 2.5 min. The detected peaks aligned and screened against the mass values of peptides and amino acids obtained from the simulation enzymatic digestion produced by ExPASy PeptideMass online tool. Representative graphs of detected masses in the treated and non-treated samples were created using MZmine, version 2.9.1 (Pluskal *et al.,* 2010).

6.2.3 In vivo intestinal distribution/uptake of bilosome

6.2.3.1 Preparation of BSA-AuNCs

BSA-AuNCs (kindly gifted by Dr Yu Chen, Physics Department, University of Strathclyde) were synthesized via a modified Xie method (Xie *et al.*, 2009b; Chenhao *et al.*, 2012). Typically, aqueous $HAuCl_4$ solution (5ml, 10mM) was added to BSA solution (5ml, 50mg/ml) with vigorous and continuous stirring for 2min at 37°C. NaOH solution (0.5ml, 1M) was then added, changing the pH value of the solution to ~12, which is essential for successful synthesis. The solution was vigorously stirred at 37°C for ~6h and subsequently stored at 37°C for 24h. The colour of the solution eventually changed to dark orange (Figure 6.1).

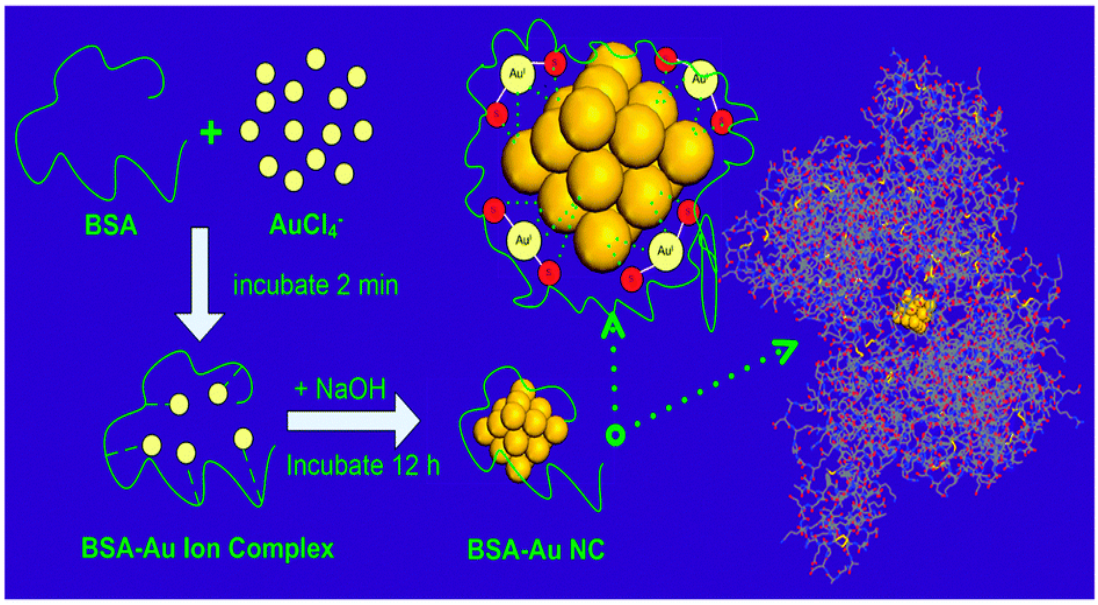


Figure 6.1 Schematic illustration of the formation process of BSA-AuNCs (Adopted from (Xie *et al.*, 2009a).

6.2.3.2 Preparation of BSA-AuNCs loaded bilosome

BSA-AuNCs were entrapped in bilosomes by resuspending 160mg of lyophilised bilosomes in 2ml of 10% (v/v) BSA-AuNCs solution diluted in MilliQ water. The mixture was vortexed for 5min and further stirred for 30min at 800rpm. The mixture was stored at 4°C.

6.2.3.3 Rat administration and intestine frozen section slides procedure

Three ex-breeder female Sprague Dawley rats were starved for 2h prior to being orally administered by gavage 0.5ml water alone, 10% (v/v) unentrapped BSA-AuNCs or 10% (v/v) bilosome loaded with BSA-AuNCs . After 3h, the rats were sacrificed and approximately 5cm long sections of small intestine removed, washed briefly with 1x PBS to remove any intestinal contents. Intestinal tissue sections of 0.5cm long were vertically oriented in cryomatrix embedding medium (Shandon, Thermo-scientific, UK) and immediately frozen at -20°C. Thin frozen sections (20μm) were cut with a cryomicrotome at -24°C. The sections were carefully transferred onto glass slides and washed briefly with a few drops of water, then air dried before being counterstained by adding one drop of 4',6-diamidino-2-phenylindole (DAPI) containing mounting medium (Vectashield®, Vector Laboratories Ltd., UK). The slides were covered with coverslips and kept at 4°C until examination by microscopy was carried out.

6.2.3.4 Two-photon excitation microscopy

All fluorescent microscopy and imaging were carried out by Dr Yu Chen at the Physics Department, University of Strathclyde, Glasgow, UK. Briefly; prepared slides were mounted in a confocal microscope (LSM 510, Carl Zeiss) equipped with a time-correlated single photon counting (TCSPC) module (SPC-830, Becker & Hickl GmbH). Two wavelengths were used (800nm and 480nm for DAPI and AuNCs, respectively).

6.3 Results

6.3.1 Degradation of BSA after treatment in simulated gastric intestinal milieu

The detection of peptides and amino acid molecules in the supernatant of treated bilosomes was indicative of vesicle disruption and release of BSA. Fractions with masses in the range of 75-1200 were detected by LC/MS. Samples treated with SGF with pepsin showed very little fractions (7 out of 133 peptides) in the supernatant that could be due to incomplete digestion of BSA attached to the bilosome outer surface by pepsin enzyme (Table 6.1). However, samples treated with SIF with pancreatin enzyme showed most of the peptides obtained in the simulation process. About 24 peptides and 2 amino acids were detected by LC/MS in the supernatant of SIF with pancreatin enzyme (Table 6.1). Visualisation of the total ion chromatogram (TIC) of the 2 amino acids molecular masses (147.1129 m/z and 175.1190 for lysine and arginine amino acids, respectively) with retention times around 25min, in LC/MS raw data analysis showed that the highest amounts of both amino acids were in the SIF with pancreatin treated samples, Figure 6.2 and 6.3.

6.3.2 Multi-photon fluorescence microscopy of intestine frozen sections

Examination of frozen sections under multi-photon laser excitation revealed cellular uptake and widespread tissue distribution of AuNCs in samples taken from rats fed unentrapped AuNCs (Figure 6.5) and loaded in bilosome (Figure 6.6). However, there were some areas of oval vesicles containing dense amounts of AuNCs appeared inside the cells, which were identified as intact bilosome vesicles

(indicated by arrows in Figure 6.6). As was expected no signals were obtained from samples of the control rat (Figure 6.4).

Mass	Peptide sequence	RT	Mass	Peptide sequence	RT
Simulated intestinal fluid with pancreatin enzyme					
1177.559	ECCDKPLLEK	22.3	509.3194	HKPK	21.5
1050.492	EACFAVEGPK	20.4	508.2514	FGER	14.7
1015.488	SHCIAEVEK	21.1	475.2875	LSQK	13.6
1014.619	QTALVELLK	25.1	464.2173	DVCK	20.3
1002.583	LVVSTQTALA	21.7	462.2194	ADEK	14.4
922.488	AEFVEVTK	20.5	439.2299	YTR	20.1
886.4152	DDSPDLPK	22.2	432.2565	VGTR	17.6
818.4254	ATEEQLK	21.8	404.2503	SLGK	19.5
752.3573	NYQEAK	20.6	383.2401	VHK	27.1
649.3338	IETMR	21.9	347.2289	VTK	16.5
649.3338	CASIQK	21.9	303.1775	QR	26.6
609.2878	AFDEK	21	175.1189	R	25.8
545.3405	VASLR	20.5	147.1128	K	25.7
Simulated gastric fluid with pepsin enzyme					
728.4413	TRKVPQ	19.5	414.2711	VPKA	5.9
728.395	RRHPY	19.5	414.2711	GPKL	5.9
716.3209	DTHKSE	5.7	359.2653	KVL	5.8
436.219	NGVF	5.9			

Table 6.1: The LC/MS measured mass and retention time with expected peptide amino acids sequences of digested BSA from bilosome samples treated with simulated gastric and intestinal fluids for 3h at 37°C.

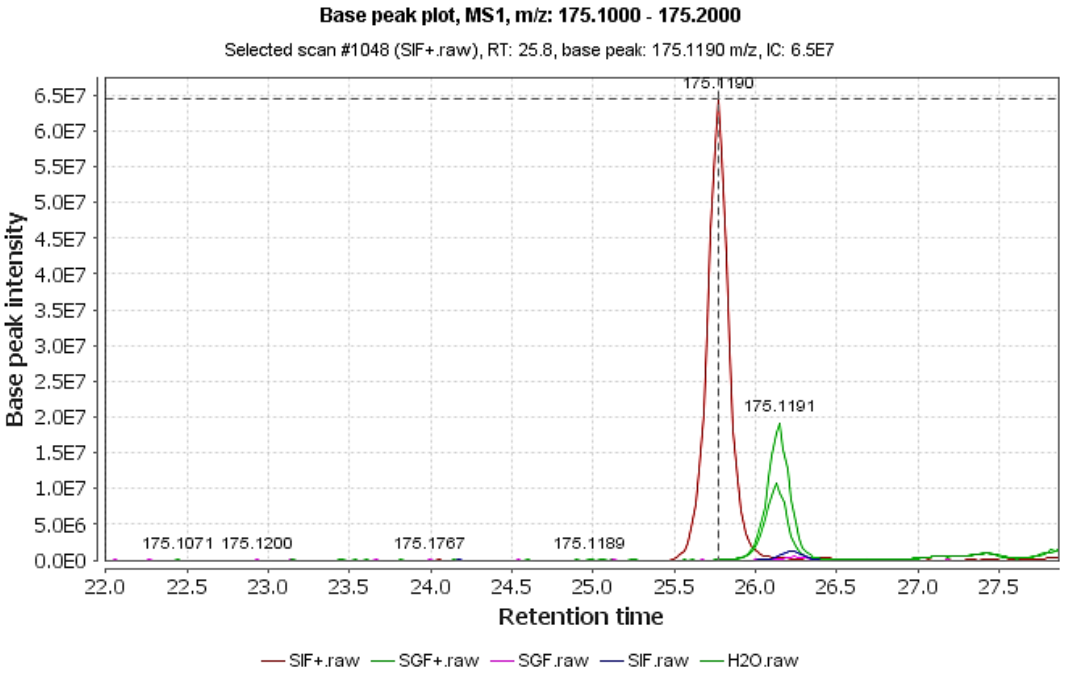


Figure 6.2: Total ion chromatogram (TIC) created by MZmine software showing the arginine base peak at 175.1190 m/z. Samples were incubated with H_2O, SGF or SIF for 3h at 37°C.

Abbreviations:
SGF+; bilosome sample treated with simulated gastric fluid. SGF; simulated gastric fluid alone as a reference. SIF+; bilosome treated with simulated intestinal fluid. SIF; simulated intestinal fluid alone as a reference.

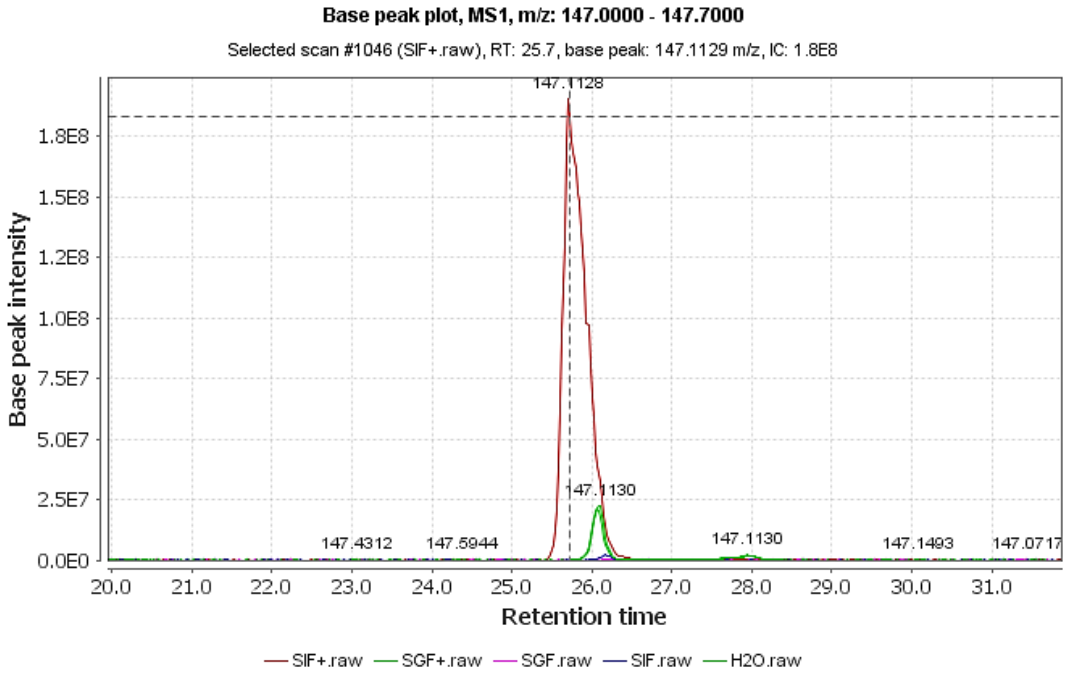


Figure 6.3: Total ion chromatogram (TIC) created by MZmine software showing the lysine base peak at 147.1128 m/z. Samples were incubated with H2O, SGF or SIF for 3hrs at 37°C.

Abbreviations:
SGF+; bilosome sample treated with simulated gastric fluid. SGF; simulated gastric fluid alone as a reference. SIF+; bilosome sample treated with simulated intestinal fluid. SIF; simulated intestinal fluid alone as a reference.

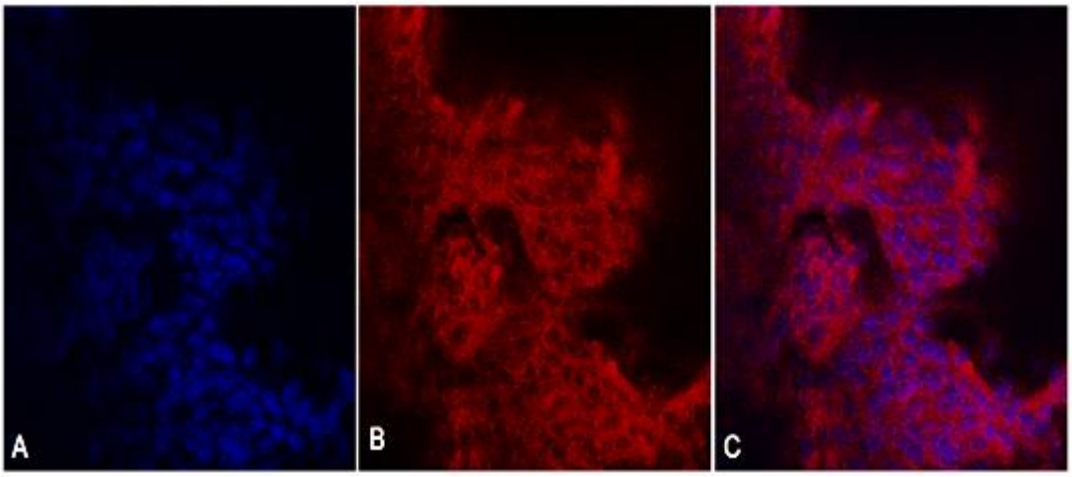


Figure 6.4: Multi-photon fluorescence microscopy slide images of frozen intestine sections from a control rat fed only water. A) channel 2 for laser excitation at 800nm to visualise DAPI DNA stain, showing the cell nucleus (blue). B) channel 1 for laser excitation at 488nm showing tissue auto-florescence (slightly red) and C) combined channel 1 and 2 images.

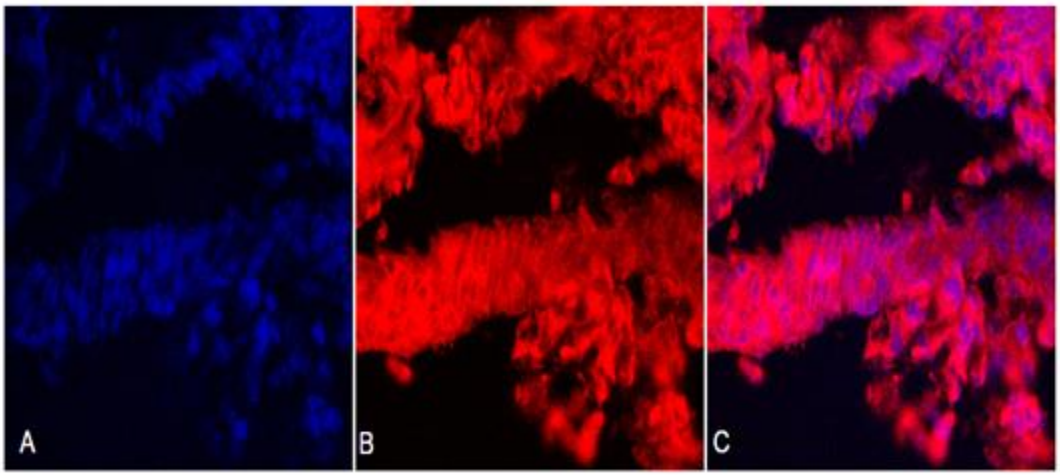


Figure 6.5: Multi-photon fluorescence microscopy slide images of frozen intestine sections from a rat fed BSA-AuNCs only. A) channel 2 for laser excitation at 800nm to visualise DAPI DNA stain, shows the cell nucleus (blue). B) channel 1 for laser excitation at 488nm to visualise BSA-AuNCs fluorescence (red). and C) combined channel 1 and 2 images.

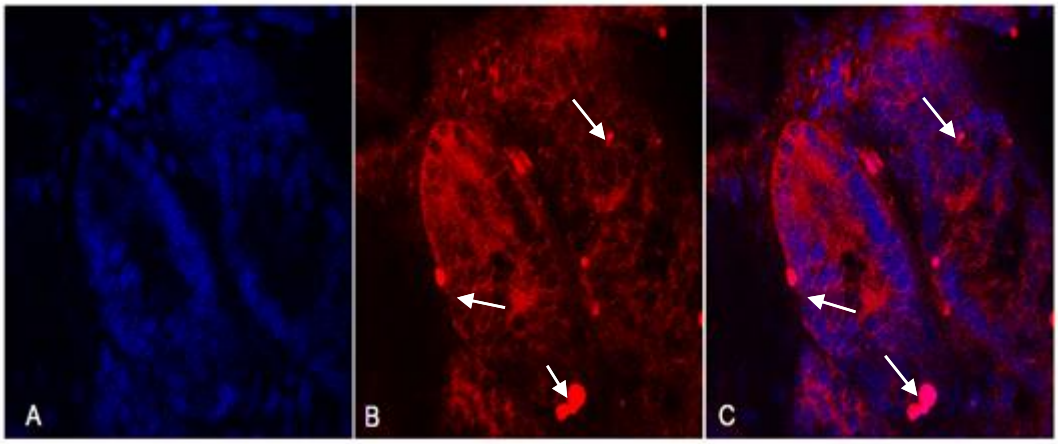


Figure 6.6: Multi-photon fluorescence microscopy slide images of frozen intestine sections from a rat fed bilosomes loaded with BSA-AuNCs. A) channel 2 for laser excitation at 800nm to visualise DAPI DNA stain, showing the cell nucleus (blue). B) channel 1 for laser excitation at 488nm to visualise BSA-AuNCs fluorescence (red). and C) combined channel 1 and 2 images. Bilosome vesicles are indicated by white arrows.

6.4 Discussion

Vaccines administered via the oral route encounter the risk of inactivation of the immunogenic protein due to extremes in pH and presence of proteolytic enzymes in the stomach and gut before it can provoke immune responses in the GALT. Although, the encapsulation of protein components of vaccine may provide protection against low pH in the stomach, this protection depends on the integrity of the encapsulating delivery system. In the intestinal lumen, the encapsulating material should promote direct contact of the immunogen with mucosal antigen sampling mechanisms through APCs, M cells and DCs. By this means, immunogenic proteins in the vaccine formulation will be further exposed to proteolytic enzymes in the gut.

In this study, the effect of simulated gastric and intestinal fluids on the ability of LNP to provide protection to the loaded protein were studied by looking at any signs of protein digestion. The hallmark of protein degradation is the digestion of proteins into smaller units. Complete tryptic digestion of BSA yeilds 78 smaller fraction (peptides) and further peptides can be formed in case of partial digestion (Walcher *et al.,* 2003). In addition, only 2 amino acids resulted from this digestion (Arginine and lysine). The detection of those amino acids after treatment of bilosomes loaded with BSA for 3h at 37°C with simulated fluids was very high in SIF samples. This indicates that some of the BSA encountered a protein digestion process by proteolytic enzymes present in the composition of the fluid (primarily pancreatin and trypsin). However, not all peptide fractions resulted from tryptic digestion of BSA were detected due to the mass scanning range of 50-1200 m/z. Larger peptide fraction can be detected by increasing the mass range up to 5000 m/z in future work (Walcher *et al.,* 2003). Previous studies suggested that protection of

protein antigens via the encapsulation in particulate delivery systems could be used to avoid degradation in the GIT (des Rieux *et al.,* 2006; Mann *et al.,* 2006; Garinot *et al.,* 2007). While these particulate delivery systems provided protection against the low pH in the stomach, there was no proof that it could resist degradation by pancreatic enzymes such as amylase (for carbohydrate digestion) and lipase (for lipid digestion). Given that bilosome vesicles can resist the low pH effect of SGF with and without pepsin *in vitro*, as observed in this study and other studies (Shukla *et al.,* 2011). In addition, an *in vivo* study carried out revealed that suppression of gastric acid has no effect on the immune responses after oral immunisation with bilosomes loaded with N/Cal HA (Bennett, 2010). These endorsed the opinion that gastric fluids has limited or no effect on the bilosome vesicles or the loaded antigen and any physical alteration in the bilosome structure likely to take place in the intestine. A recent study carried out on liposomes containing bile salts exposed to simulated media of various pH, bile salt and enzyme levels suggests that the liposomal integrity changed when the liposomes were subjected to bile salts by forming transient pores but not by a rupture or complete disintegration (Hu *et al.*, 2013). In this study, the lipid components in bilosome vesicles probably begin to be digested by lipase, rendering BSA exposed to proteases. However, may be this has less significant effect on bacterial or viral proteins due to sufficient immunogenic epitopes that can provoke an immune response. This was observed in the TT antibody response from all orally immunised groups as mentioned in Chapter 5. Unlike the case of using small non-pathogenic proteins or peptides (such as GnRH molecules), the exposure of these peptides to peptidase enzyme in the gut will change the structure and the ability to produce a stable immune response against such antigen. Previous *in vitro*

studies, reported that endopeptidases such as ACE, endopeptidase-24.15 and endopeptidase-24.18 are involved in the degradation of GnRH in mucosal homogenates from rabbit (Han *et al.*, 1995) and possum intestine (Wen *et al.*, 2002).

The fluorescence microscopy applied in this study may provide evidence that intestinal enzymes have affected bilosomes. This can be seen from the spread of AuNCs in the tissue similar to that seen in the samples given un-entrapped BSA-AuNCs. However, not all bilosomes lost their integrity, as there were a few spherical particles inside the epithelial cells, some of which accumulated in large numbers. A recent study applied fluorescent markers to study the uptake of bilosomes in the GALT particularly at PP sites (Shukla *et al.,* 2011). The study found that rhodamine entrapped in bilosomes was able to reach PPs, but there was no fluorescence in animals fed rhodamine alone. This could be due to collection of intestine samples from animals after 5h from oral administration (compared to 3h) and or the concentration of fluorescent agent used was too low. However, the images in the present study showed that bilosomes are inside the cells of the microvilli, and it probably accumulated at the PP.

7. Overall conclusions

7.1 Conclusions and future work

In this research, attempts to evaluate lipid-based nanoparticles as mucosal vaccine delivery system were carried out using non-pathogenic and pathogen-derived immunogens. In Chapter 2, the preparation methods and physical characterisation of LNP vesicles were studied. The use of MW method for preparation of LNP showed a reduction in processing time compared to traditional methods with the possibility for precise configuration of synthesis parameters such as temperature, pressure, lipid melting time and formulation volume. This method also does not require the use of chemical solvents, avoiding unnecessary exposure and or discard of such solvents.

Although there was an increase in average size of LNP prepared by the MW method, this could be controlled with an additional homogenisation step. However, the size range was in the limit to target APCs in the mucosal surfaces. There was no marked differences between the MW and MM methods in the vesicles electric charge, this is due to the DCP component. The negative ζ-potential value of the vesicles (-80mV to -140mV) allowed the formation of a relatively stable colloidal solution, where the LNP exhibits continuous movements due to Brownian motions. The EE% was not greatly affected by the preparation method used and ranged between 25-35% with bilosomes entrapping more protein in both MM and MW methods. This is probably due to the effect of DOC, however, in future work different DOC ratios in bilosomes should be examined with correlation to EE%.

While the ninhydrin assay for determination of protein concentration is compatible with solutions that contains lipid, the procedure is tedious and consumes time. It is therefore recommended that the BCA assay be used to determine the un-

entrapped protein concentration in the supernatant, then calculating the entrapped amount.

The lyophilisation of LNP has very low effect in EE% although there was an overall increase in vesicle size. It is presumed that vesicles are reconstructed into larger vesicles during rehydration of the lyophilised vesicles. Future work could include a water bath sonication step for 15s or membrane filtration to maintain relevant vesicle size range. However, in this study the use of cryoprotectant agents was not attempted. Cryoprotectants such as lactose or sucrose usually can be used for increasing the redispersibility index and reconstitution score. Future work should test various cryoprotectants with different concentrations that may also maintain the vesicle size.

The use of HPLC for LNP lipid content analysis in this research was initially carried out to determine the incorporation of bile acid in the lipid vesicle membrane. The HPLC method used successfully to determine different compound concentrations in the formulations. This method can be adapted for quality control purposes in manufacturing process to maintain batch to batch similarity. The position of incorporated bile acids in the LNP vesicles was not previously known. The results obtained in this thesis, suggests that bile acids might form ionic bonds with the phosphate moiety of the DCP. Therefore, it may reside between the lipid layers.

Previous studies demonstrated that DOC involved in the protection of bilosomes from the intestinal bile acids. In this research, only one lipid:DOC ratio was used in the preparation of the vesicles. Future work may include evaluation of a range of lipid:DOC ratios in correlation with vesicle stability.

In Chapter 3, synthetic GnRH isoform conjugates were evaluated for production of specific immune response via s.c route. GnRH-I and GnRH-III were selected for further work because of the initial results of cross reactivity of produced antibody against other isoforms, and the lower baseline of testosterone hormone levels observed in animals immunised against these 2 isoforms. Given that, GnRH is a self-protein, TT and OVA were used to provide an immunogenic epitope to the conjugate that enhanced recognition by T-cells and production of IgG antibodies, which neutralise native GnRH in the body. The immune response in animals given immunogen loaded NISV was comparable to those given alum adsorbed immunogens via the s.c route. Therefore, groups administered NISV via the s.c route were used as a positive control in the subsequent *in vivo* studies. However, administration of bilosomes via the oral route was disappointing and no anti-GnRH specific antibodies were observed. RT-PCR was used to provide evidence that T-cells initiated Th2 immune responses in the testes tissues by up-regulating IL-4 and IL-6 cytokines expression. However, this multiplex PCR depends on visualisation of the final products in agarose gel electrophoresis with a limited quantification ability. Future work may consider the use of real-time PCR for accurate quantification of PCR products and therefore cytokine expression.

In Chapter 4, introducing a different immunogen carrier protein (mcKLH) proved the concept of compatibility and suitability of synthetic GnRH isoforms for conjugation. The addition of a homogenisation step after MW synthesis reduced the average LNP vesicles sizes. This step could be optimised for production of LNP with specific sizes. Previous studies reported that the homogenistation period affects the bilosome vesicle average size. Therefore, future work could investigate the effect of

homogenisation period on different LNP average sizes to see if this has any effect on EE%.

AFM was used to investigate the surface properties of the LNP, to see if it was possible to determine the relative localisation of DOC and proteins in the vesicles. AFM showed that protein loaded vesicles have a smooth surface compared to empty vesicles. This may propose that part of the antigen/protein is adsorbed or conjugated to the vesicle membrane. Using fluorescent labelled lipids and proteins may allow the visualisation of the protein in the surface of the vesicles under confocal microscopy. AFM also showed that addition of Xn to NISV made the vesicles stickier. Therefore, NISV coated with Xn improved the mucoadhesive properties of the formulation and allowed particles to remain for longer periodwith contact with the mucosal surfaces. However, only NISV+Xn administered via the nasal route showed comparable immune response to the s.c route. NISV+Xn administered via the i.vag route and bilosomes administrated via the oral route failed to produce significant systemic antibodies. Whereas, GnRH-III immunogen loaded NISV+Xn administrated via the nasal and vaginal routes showed significant IgA. Which probably indicates that both immunogen and administration route mediate a specific response, as it did not appear when GnRH-I immunogen loaded NISV+Xn where administered via the vaginal route.

The oestradiol hormone assessment carried out in female mice did not show marked variation, which was probably due to the sensitivity of the assay used and the detection of oestradiol originating from adrenal glands. Future work in female mice should consider assessment of other reproductive hormones such as FSH and include fertility challenge.

In Chapter 5, various LNP loaded with established immunogenic HA protein from a pathogenic influenza strain was used as a vaccine. The systemic and mucosal immune responses were significantly higher in s.c and nasal groups as compared to with the control group. In addition, bilosome and oral administration of plain immunogen mixed with Xn showed IgG and local IgA antibody production. This indicate that these proteins can resist the effect of gastric harsh conditions and intestinal digestive enzymes. In the next experiment carried out, bilosomes were mixed in Xn solution to produce bilosome+Xn. The challenge against the toxin derived from pathogenic stains, allowed the evaluation of the efficiency of systemic antibodies produced in animals immunised with TT loaded in various LNP formulation administered via different routes, and defining the optimal lipid:antigen ratio as well.

Future studies should look at the cytokine expression and the adjuvant properties using those pathogen-derived immunogens with combination with different LNP formulations.

In Chapter 6, the fate of bilosomes in GIT was tracked by *in vitro* and *in vivo* experiments. Bilosome stability under the harsh conditions in the GIT was examined by incubation with simulated fluids. Protein degradation due to enzymes in the SIF was assessed by LC/MS. This may suggest that parts of the vesicle lipid membrane have been ruptured or opened due to the effect of lipase enzyme allowing the protein to leak out of the vesicles and be exposed to other proteinase enzymes. However, it seems from the multiphoton microscopy that not all vesicles are affected, which means this could be due to weak points in the vesicle structure. This can be noted from the variations in size and morphological shape of the vesicles. Future studies

could address the production of uniform bilosomes by manipulating the lipid component ratio and the incorporation of Xn to provide more protection to the loaded antigen.

7.2 Concluding remarks

The achievements from this study established that LNP in the form of NISV with and without a mucoadhesive showed adjuvant properties and is a potential candidate for enabling vaccine delivery via the nasal route. Further studies as addressed above, will be needed for optimisation of this LNP delivery system to enable effective and safe mucosal vaccination.

In this research, new techniques such as HPLC, LC/MS and AFM were involved in the analysis and characterisation of LNP vesicles. In addition, RT-PCR was applied for cytokine expression detection in specific tissues that have been targeted via immunisation. In terms of processing, MW prepared LNP has been successfully developed to incorporate various immunogens. Whereas, mechanistic studies involving the use of gold nanoparticles and fluorescent microscopy provided understanding of uptake and distribution at the cellular levels.

The LNP were tested for efficacy of vaccine delivery via different parenteral, oral and other alternative mucosal routes. A toxin challenge study was used for providing information about the specificity of the immune response produced.

In order to take LNP for further development, the following challenges need to be overcome; synthesis of small homogenised vesicles with a protective coating against GIT enzymes in order to be used successfully in oral vaccine delivery. Similarly, NISV can be fine-tuned in terms of size to be involved in spray

formulation for nasal delivery. The number of publications, including new Intellectual Property generated from this project indicates the level of interest in this field and in particular the drive for understanding of mucosal vaccination to enable the next generation of vaccines to be developed.

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