School of Pharmacy

Development and Evaluation of Novel Biodegradable Nanoparticles for Vaccine Delivery

Sameer Sharma

This thesis is presented for the Degree of
Doctor of Philosophy
of
Curtin University of Technology

September 2010

DECLARATION

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature:

Date:

ACKNOWLEDGEMENT

First of all, I wish to express my deepest gratitude to my supervisor, **Dr Yan Chen** for her guidance, encouragement and patience throughout the journey. I am thankful to my co-supervisors **Associate Professor Heather AE Benson** and **Associate Professor TKS Mukkur** for their personal and professional support. My supervisors' acceptance and faith in my ideas and their enormous support made my research an enjoyable experience.

I express my sincere gratitude to the Department of Education, Employment and Workplace Relations (DEEWR), Australia and School of Pharmacy, Curtin University of Technology for providing me with an International Postgraduate Research Scholarship (IPRS) for undertaking the Doctor of Philosophy.

I express my sincere thanks to Dr. Paul Rigby and Mr. John Murphy (CMCA, UWA, Perth) for their support and cooperation in confocal microscopy studies. I am thankful to Dr. Rob Hart and Ms. Elaine Miller (Department of Applied Science, Curtin University) for providing me with training and assisting me with FIBSEM and TEM studies. I also acknowledge the support and help of Dr. Simon Fox and Ms. Erin Bolitho in the cell-culture work undertaken as part of this thesis.

I am thankful to Prof. Jeffery Hughes, Prof. Michael Garlepp and Prof. V B Sunderland for their support. I also acknowledge the cooperation of all academic, technical and administrative staff of School of Pharmacy.

I wish to thanks Mr. Adrian Paxman (School of Biomedical Sciences, Curtin University) for his guidance and help with the SDS-PAGE technique. I also acknowledge the support of Mr. Michael Boddy, Mr. Jorge Martinez, Ms. Angela Samec and Mr. Michael Stack. I would also like to convey thanks to Oki, Nirav, Ibrahim, Alex and Paul for their cooperation and friendly assistance in the lab work.

I am very lucky to have very close friends to take the stress out of life. I am thankful to Divyamaan, Pradeep, Vikas, Dhanushka, Yusuf, Basavraj, Chirag and Amit

for all their love and support. I would like to acknowledge the support of my lab mate Louise Whittal for creating an enjoyable environment in the lab.

I am especially thankful to my wife for her patience, support and inspiration. I express my sincere gratitude to my father, mother, sisters, brother, teachers and friends for their moral support to complete this work. Finally, I would like to thanks all of those who helped me directly or indirectly in this thesis work.

Sameer Sharma

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

µg	Microgram
Ab	Antibody
ACK	Ammonium-Chloride-Potassium
Ag	Antigen
ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
BALT	Bronchus Associated Lymphoid Tissue
BCG	Bacillus Calmette-Guérin
BSA	Bovine Serum Albumin
CAIV-T	Cold adapted influenza vaccine-trivalent
CHO	Chinese Hamester Ovary
CLSM	Confocal Laser Scanning Microscopy
CO_2	Carbon dioxide
Con-A	Concanavalin A
CS	Chitosan
CS-DS	Chitosan – Dextran Sulfate
CT	Cholera Toxin
CTL	Cytotoxic Lymphocytes
DC	Dendritic Cells
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribo Nucleic Acid
DPT	Diphtheria Pertussis Tetanus
DS	Dextran Sulfate
ELISA	Enzyme Linked Immunosorbent Assay
FCS	Fetal Calf Serum
FHA	Filamentous Hemagglutinin
FIBSEM	Field Emission Ion Beam Scanning Electron Microscopy
FITC	Fluorescein Isothiocyanate
FT-IR	Fourier Transform - Infrared

GALT	Gut Associated Lymphoid Tissue
HRP	Horseradish Peroxidase
HLB	Hydrophilic-lipophilic balance
IFN	Interferon
IgA	Immunoglobulin-A
IgG	Immunoglobulin-G
IL	Interleukin
IN	Intra-nasal
IP	Intra-peritoneal
IRIV	Immunopotentiating Reconstituted Influenza Virsomes
ISCOM	Immunostimulating Complex
KCl	Potassium Chloride
LT	Heat Labile Toxin
LUV	Large Unilamellar Vesicles
MCC	Mono-N-carboxymethyl Chitosan
M cell	Membranous or Microfold cell
MHC	Major Histocompatibility Complex
mL	Millilitre
MPL	Monophosphoryl lipid
mV	Milli-Volt
NaCl	Sodium Chloride
NALT	Nasal Associated Lymphoid Tissue
NaOH	Sodium Hydroxide
ng	Nanogram
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffer Saline
PGA	Poly-(glutamic acid)
PLA	Poly-(lactic acid)
PLG	Poly-(lactide-co-glycolide)
PRN	Pertactin
PTX	Pertussis Toxin

PTXd	Detoxified Pertussis Toxin or Pertussis Toxoid
RSD	Relative Standard Deviation
SC	Subcutaneous
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SE	Standard Error
SEM	Scanning Electron Microscopy
SIgA	Secretory Immunoglobulin-A
SMBV	Synthetic Biomimetic Supramolecular Biovector
SUV	Small Unilamellar Vesicle
TEM	Transmission Electron Microscopy
Th1	Helper T-cell type 1
Th2	Helper T-cell type 2
TLR	Toll Like Receptor
TMC	N-trimethyl Chitosan
TPP	Tri-polyphosphate
TRITC	Tetramethyl Rhodamine Iso-Thiocyanate
UEA	Ulex Europaeus
VLP	Virus Like Particles
WHO	World Health Organisation

ABSTRACT

The trend in new vaccine development is directed towards the use of antigens with better immunogenicity/risk ratio and away from the use of inactivated or attenuated pathogens. However, in many instances, purified antigens are poorly immunogenic particularly in the form of a simple solution. Various particulate delivery systems with or without immunological adjuvants have been investigated in recent past to enhance the immune responses against purified antigens but very few of the investigated particulate vaccine delivery systems have been successful in overcoming regulatory and/or commercial hurdles. The principle objective of this research project was to develop and characterize a biodegradable nanoparticulate vaccine formulation for induction of enhanced antibody and/or cell-mediated immune responses against a model protein antigen.

Many of the immunological adjuvants explored in the past were pathogen derived such as heat labile toxin of enterotoxigenic *Escherichia coli,* the B-subunit of cholera toxin or the Freund's complete adjuvant, which could become a possible risk inducing factor. Immunoglobulin-A (IgA) is an endogenous antibody recently reported to have an immunomodulatory effect after being taken up by lymphoid tissue probably through IgA specific receptors on M cells in mice and humans. The development and evaluation of a chitosan-dextran sulfate (CS-DS) nanoparticulate vaccine delivery system, with or without incorporation of IgA as an immunological adjuvant with M-cell targeting potential, was undertaken in this thesis work to serve the need and importance of research in the field of delivery of newer vaccines (proteins, nucleic acids, polysaccharides and synthetic peptides).

In this investigation, CS-DS nanoparticles were prepared using a complex coacervation (polyelectrolyte complexation) technique. The optimized nanoparticle formulation was loaded with a model protein antigen (pertussis toxin) and/or IgA. This formulation system was evaluated for *in-vitro* (particle size, zeta potential, electron microscopy, entrapment efficiency, release profile and stability) and *in-vivo* immunological parameters.

CS-DS blank nanoparticles of size and zeta potential in the range of 150 to 400 nm and -40 to +60 mV respectively, were obtained. The ratio of chitosan to dextran sulfate, order of mixing and pH of nanoparticle suspension were identified as important formulation factors governing size and zeta potential of nanoparticles, which in turn affect stability and entrapment efficiency of the formulation. An optimized blank CS-DS nanoparticle formulation with particle size 314.7 ± 9.2 nm, zeta potential +53.2 ± 4.4 mV and prepared with CS to DS weight ratio of 3:1 was used to load pertussis toxin (PTX) and/or IgA. An entrapment efficiency of higher than70%) was obtained for PTX and IgA in CS-DS nanoparticles and all loaded nanoparticle formulations showed less than 15% of initial release followed by no significant release of IgA or PTX in release studies.

The *in-vivo* uptake study of IgA-loaded CS-DS nanoparticles in nasal epithelia of Balb/c mice indicated the preferential uptake of nanoparticles in nasal associated lymphoid tissue (NALT) probably by M-cells. The *in-vivo* comparative immunological evaluation of detoxified pertussis toxin (PTXd)-loaded CS-DS nanoparticle formulations by subcutaneous (a parenteral route) versus intranasal (a mucosal route) administration highlighted the significance of route of administration. The PTXd-loaded CS-DS nanoparticle formulations were found to induce higher immune responses when administered through subcutaneous route compared to intranasal route. The immunological evaluation of developed formulations in female Balb/c mice groups showed that CS-DS nanoparticles formulations induced significantly higher serum IgG and IgG1 titers ($p < 0.05$) compared to conventional alum adjuvanted PTXd formulation, administered by the subcutaneous route. However, the IgG immune response induced by PTXd-loaded CS-DS nanoparticle formulations by intranasal administration were not significantly different ($p < 0.05$) compared to solution the PTXd solution formulation. The difference in level of immune response induced by different routes of administration highlighted the role of antigen-dose optimization for particular administration route, development of specific delivery devices for mucosal administration of vaccines especially for animal studies and the need of individualistic formulation research for different antigens.

This study indicated the potential of CS-DS nanoparticles as a simple and effective particulate delivery system with in-built immunological adjuvant property for acellular protein antigens. The study also indicated the potential relevance of IgA as a novel immunological adjuvant for particulate vaccine delivery systems. The research embodied in this thesis highlights the need for further research in the field of vaccine delivery systems with greater emphasis on defining a correlation between pharmaceutical factors and *in-vivo* immunological responses.

CHAPTER 1

LITERATURE REVIEW AND GENERAL INTRODUCTION

1.1. VACCINE DELIVERY SYSTEMS

Vaccination has become the most effective strategy for prevention, control and eradication of infectious diseases. The research related to vaccines is often focussed on the identification and application of novel antigens. In the last few decades, advances in pharmaceutical delivery systems, the emergence of purified subunit antigens, emergence of new infectious diseases such as AIDS and the evolution of antibiotic resistance in microorganisms have resulted in the development of various novel vaccination strategies. One of these novel vaccination strategies is the use of pharmaceutical vaccine delivery systems for manipulation and enhancement of immune responses.

Vaccine delivery systems have become more important with the continuous expansion of immunization programs as increasing the number of 'stand alone' vaccines is problematic. Firstly, participation in the vaccine program may decrease when children need to receive more injections. Secondly, more injections pose a higher threat of infections in developing countries during immunization. Lastly, 'stand alone' vaccines can significantly increase the cost of immunization programs. Development of combination vaccines containing two or more antigens solves the problem of the number of injections to some extent, but incorporation of a large number of antigens in a simple formulation can be risky and increases the risk of immunological and/or pharmaceutical interferences.[1] The use of an appropriate delivery system may reduce or even overcome these problems.

Delivery systems are also of great importance for the delivery of vaccines via routes other than the parenteral routes currently in practice for most infectious diseases. The delivery of an antigen to the mucosal surface can be achieved by several routes including oral, rectal, intranasal, pulmonary, or vaginal. Organized lympho-epithelial structures associated with the gut, upper respiratory tract and nasopharyngeal tissue are termed gut associated lymphoid tissue (GALT), bronchus-associated lymphoid tissue (BALT) and nasopharynx associated lymphoid tissues (NALT) respectively. These are the prime inductive sites for mucosal immunity.[2-4] Mucosal immunization is an attractive alternative to parenteral administration of vaccines as it may offer advantages

in the immunity generated and provide a non-invasive needle-free option for vaccine delivery. However, the advantages offered by mucosal routes are usually negated by possible degradation of antigens by enzymes or acid, rapid clearance or elimination of antigens due to ciliary or peristaltic movement and inefficient permeation of antigens across mucosa.[5] Therefore, although mucosal sites are potentially immunologically active, specialized delivery systems with a higher dose of antigen are necessary for induction of effective immune responses.

Clearly the success of vaccination is not only dependent on the nature of vaccine's immunogenic components (epitopes), but also on the delivery system.[6, 7] Various particulate delivery systems and fewer non-particulate systems have been reported in literature as carriers for antigens with many also showing immunostimulant effects.

1.1.1. Particulate Delivery Systems

The overall objective for pharmaceutical researchers has been to develop particulate nonliving delivery systems that have dimensions comparable to pathogens and may be efficiently taken up by antigen presenting cells (APCs), thereby generating and enhancing immune responses. Delivery systems promote the interaction of both antigens and adjuvants with the key cells of the innate immune system such as APCs including macrophages, dendritic cells and B cells, while facilitating generation of memory B- and T-cells in the adaptive immune effector arm of the immune response.[8] Delivery systems, especially particulate ones, facilitate presentation of an ordered and repetitive array of B-cell epitopes, which result in more efficient B-cell activation through their antigen receptors.[9, 10] Although it is thought that the primary function of particulate delivery systems is to promote the antigen depot and enhanced uptake by APCs, it is still possible that the delivery system may have direct immunostimulatory/adjuvant effects on APCs.

The adjuvant effect of particulate delivery systems has been suggested to be largely a consequence of their uptake into dendritic cells and macrophages following parenteral administration.[11] In the case of mucosal delivery of vaccines,

particulate structures potentially facilitate the specialized uptake by M cells present in organised lympho-epithelial structures. M cells can internalize the particles, microbes or antigen by various mechanisms such as clathrin-mediated endocytosis, fluid-phase pinocytosis, actin-dependent phagocytosis, and macropinocytotic engulfment involving disruption of the apical cytoskeletal organization.[12-15] The antigen can then be actively transported to APCs. Moreover, particulate delivery systems can increase the retention time of antigens in local lymph nodes.[16] The adjuvanticity of particulate delivery systems has also been ascribed by some to their ability to protect the antigen within the biological environment.[17]

The potential benefits of particulate delivery systems in vaccination have resulted in the use of some conventional particulate drug delivery systems (e.g. microparticles, liposomes) for vaccine delivery along with the development of various novel particulate vaccine delivery systems.

1.1.1.1. Microparticles and Nanoparticles

Microparticles (1-100 μm) and nanoparticles (< 1 μm) have been studied by various groups[18-21] as vaccine delivery systems via almost all routes of administration. It is generally accepted that particles in the nano-size range are a more efficient carrier with immunostimulant properties, when compared to larger size particles,[18, 21, 22] but no definite particle size limits have been established from the current literature, as particles of different size ranges may follow different endocytic pathways and be preferentially taken up by different phagocytic cells.[23] If uptake and processing of different sized particles occurs via different mechanisms, this may bias immune responses to generate a specific type of immunity. Particles in the size range of 20–200 nm are usually taken up by receptor-mediated endocytosis and elicit a cellular biased response, whereas particles with the size between 0.5 μm and 5.0 μm are predominantly taken up by phagocytosis and/or macropinocytosis and elicit a humoral response.[23, 24] However, the effect of particle size on the immune responses after vaccination has not been consistent therefore the use of a particular particle size for delivery system must be trailed on a case-by-case basis in combination with variations

such as route of administration, schedule of delivery, preparation method and the nature of the antigen and main excipients in the delivery system.

The availability of multiple natural and synthetic materials and versatile preparation procedures make polymeric micro- and nano-particles potential vaccine delivery systems which warrant further research evaluation.[25]

1.1.1.1.1. Synthetic Polymers

Poly-(lactide co-glycolides) (PLG) and poly-(lactic acid) (PLA) are the principal synthetic polymers investigated for incorporation of antigens, primarily because of their biodegradable and biocompatible nature.[18, 26] These polyester polymers have also been extensively used as controlled release vehicles and suture material. The method most commonly used for the preparation of polyester based particulate vaccines is the double emulsification-solvent evaporation method using dichloromethane or ethyl acetate as the polymer solvent.[27, 28] The aqueous antigen solution is emulsified into the polymer solution in organic solvent by sonication followed by homogenization of the first emulsion in poly-vinyl alcohol (PVA) or sodium cholate solution. Other methods, such as nanoprecipitation and simple emulsification (o/w) have also been reported for entrapment of antigen.[29] However there are some problems commonly encountered during the encapsulation of antigens in synthetic polymers such as exposure of antigens to high shear, organic solvents, cavitation and localized elevated temperature, leading to antigen denaturation.[30] Degradation of antigens may also occur following encapsulation and during the release from PLG particles when the polymer breaks down and releases an acidic component.[31] To overcome some of these problems, alternative approaches such as adsorption of antigen on empty polymeric particles and use of more hydrophilic derivatives of synthetic polymers (e.g. PLA-PEG) for incorporation of antigens have been investigated.[32, 33] Jabbal-Gill et al.[34] reported an interesting strategy for adsorption of antigens to the surface of polymeric lamellar substrate particles (PLSP) that were produced by a non-solvent

induced precipitation of PLA or another suitable semi-crystalline polymer. Potential advantages of this technique include lack of antigen exposure to organic solvents, high shear stresses and low pH. The adsorption of antigen onto PLSP is dependent on antigen characteristics such as hydrophobicity, surface charge and conformation, as well as pH of the suspension and incubation period. Intranasal or parenteral administration of PLSP adsorbed antigens, induced a significantly higher systemic immune response in comparison to corresponding solution formulations administered at the same dose. The angular lamellar morphology of PLSP and good antigen stability during manufacturing are thought to be additional features of PLSP compared to spherical or plain PLA particles.[35-37] Cationic PLG microparticles with adsorbed DNA enhanced the protective efficacy of a DNA-based tuberculosis vaccine and increased antibody and cellular immune responses to hepatitis B antigen.[38, 39] The adjuvant effect of microparticles and nanoparticles has been suggested largely to be a consequence of their uptake into dendritic cells and macrophages or M-cells following parenteral and mucosal administration respectively.[11] In addition, the acidic moieties of lactic or glycolic acid monomers liberated during erosion of the PLG or PLA polymers can assist in acidification of the endosomes of antigen presenting cells that will trigger antigen presentation via the MHC class-II pathway.[24, 40] PLG microparticles have been shown to be effective for the induction of cytotoxic T lymphocytes in addition to systemic and secretory mucosal immunity.[27, 41, 42] The amount and sequence of the antigen that initiates the response may also influence the differentiation of $CD4^+$ T cells into distinct effector subsets, that is, Th1 or Th2 cell responses.[43, 44] Hence, when stability of the encapsulated antigen is compromised, this might result in the Th1 or Th2 skewing of the immune response, since the relative accessibility of different epitopes might change.[24]

Various other synthetic polymers have also been investigated as carrier materials for vaccine candidates including sodium polystyrene sulphonate, polystyrene benzyltrimethylammonium chloride,

polyphosphazenes, polycaprolactone, polymethyl methacrylate and polydimethylsiloxane.[42, 45]

One interesting multi-functional polymer which has been investigated for vaccine delivery is polyglutamic acid (PGA). PGA is a highly anionic polymer used to form antigen-loaded nanoparticles for induction of antigen specific immune responses.[46, 47] While α-PGA is generally synthesized by an established chemical synthesis, various strains of *Bacilli* have been suggested for the microbial production of γ-PGA.[48, 49] The γ-D-PGA capsule has been recognized as one of the important virulence factors of *B. anthracis* and is essential for establishment of a lethal anthrax infection.[50] While not acting as an antigen on their own, γ-PGA nanoparticles can act as a vaccine delivery system with in-built immunostimulant effect for other antigens incorporated in a vaccine formulation.[51, 52]

1.1.1.1.2. Natural and Semi-synthetic polymers

Alternative biocompatible polymers have been widely evaluated for particulate delivery systems for antigens because of their aqueous solubility, biodegradable and bioadhesive properties. Much of the work undertaken with biopolymers has focused on their potential for mucosal delivery of vaccines. Chitosan and its derivatives have been used for vaccine delivery both in particulate and solution dosage forms.[53-55] Chitosan and its derivatives form nanoparticles by a precipitation/coacervation method using a precipitating agent. Chitosan has very low toxicity and is soluble in aqueous acidic environments. In solution, the amine functional groups of chitosan are protonated which allows chitosan salts to bind strongly to negatively charged materials such as cell surfaces and mucus. This binding delays the clearance of chitosan formulations from the mucosal sites.[56] The range of properties of chitosan and chitosan derivatives has resulted in a number of recent studies of chitosan and its derivatives in the delivery of peptides, proteins, drugs, genes and vaccines.[57-61]

Alginate is another biodegradable natural polysaccharide which has been widely studied through both the mucosal and parenteral route as delivery system component for vaccines. It is an algae derived anionic polysaccharide which can be used to prepare micro- or nano-spheres by ionotropic gelation using divalent or trivalent cations. Ajdary et al. [62] reported that oral vaccination of Balb/c mice with BCG vaccine encapsulated in alginate microspheres resulted in higher proliferative and delayed-type hypersensitivity (DTH) responses and IFN-γ production in comparison with results of mice immunized orally with BCG alone. Following systemic infection with BCG, mice vaccinated with encapsulated BCG had lower mean bacterial counts compared to those vaccinated orally with BCG alone.[62] Rebelatto et al.[63] reported high levels of anti-pig serum albumin (anti-PSA) IgG antibodies in serum and nasal secretions of Holstein female calves after intranasal vaccination with PSA encapsulated in alginate microparticles compared to the corresponding solution formulation, but no significant increase of PSA specific IgA. On the other hand, Tafaghodi et al.[64] reported that intranasal immunization with alginate microspheres encapsulated with tetanus toxoid, results in strong systemic IgG and mucosal IgA response in rabbits. In a few recent studies researchers have reported that alginate coated chitosan nanoparticle are an effective subcutaneous or oral delivery system for hepatitis B surface antigen with in-built adjuvant properties.[65, 66]

Many other natural and semi-synthetic polymers have been proposed to prepare microspheres for antigen delivery e.g. polyacryl starch, gelatin, cross-linked chondroitin-4-sulfate, modified collagen derivatives, and hyaluronic acid derivatives.[67-69]

1.1.1.2. Vesicular Particulate Delivery Systems

The interest in vesicular systems for vaccines has given rise to various potential novel vaccine delivery systems in the last two decades.

1.1.1.2.1. Liposomes

Liposomes are artificial closed vesicle structures composed of lipid bilayers separated by aqueous compartments. They are prepared from phospholipids similar to those naturally occurring in cell membranes and therefore are generally regarded as biocompatible. Liposomes can be prepared by suspending a mixture of desiccated lipids in an aqueous buffer. The lipid mixture should usually contain a large proportion of a neutral lipid such as phosphatidylcholine, and lipids used as either tracers or protein anchors, and the suspension is made by either sonication or extrusion through polycarbonate filters of defined pore size.[70] Liposomes are conventionally divided into three types mainly based on the size and number of bilayers present in the liposome. Concentric layers of phospholipids with aqueous phases in-between form multilamellar vesicles (MLV) with an average size of 1-50 μm. Liposomes with a single bilayer in a size range of 100 – 500nm and 10-100nm are called as large unilamellar vesicles (LUV) and small unilamellar vesicles (SUV) respectively.[71] As a consequence of the presence of both hydrophilic and lipophilic structures, a wide spectrum of antigens can be entrapped into or adsorbed on liposomes. Although most work with liposomes has involved systemic administration of vesicles, various studies in the last few decades have shown their usefulness for mucosal immunization. Liposomes have been identified as effective immunological adjuvant and delivery systems but formulation parameters such as surface charge, antigen density, site of antigen incorporation in liposomes, liposomes size and rigidity of bilayers need to be optimized for individual cases of antigens.[72-74] The enhanced immune response obtained with administration of a liposome-antigen formulation is probably due to increased uptake and presentation of antigen, by both the particulate nature of liposomes as well as their ability to fuse with the epithelial cells of the mucosa.[71] Liposomes are preferentially endocytosed by macrophages due to their ability to accommodate multiple copies of antigenic epitopes and this may also contribute to the adjuvant effect of liposomes.[75, 76] The adjuvanticity of

liposomes has also been ascribed to their ability to protect the antigen within the biological environment and effects on the intracellular processing of antigen following uptake.[25]

Although conventional liposomes have been reported as a promising delivery system for vaccines; instability on storage, sensitivity to host enzymes and rapid removal from the blood by the reticulo-endothelial systems limit their potential.[77, 78] In the last few decades, scientists have reported various modifications in liposome delivery system to improve stability and further enhance the efficiency of liposomes as a vaccine delivery system. Microencapsulated liposome systems (MELs) have been investigated as a potential immunisation carrier for recombinant HBsAg.[79] MELs were prepared by first entrapping the HBsAg particles within liposomes composed of phosphatidylcholine:cholesterol (1:1 molar ratio), which were subsequently encapsulated in alginate-poly(L-lysine) hydrogel microspheres. The study reported significant anti-HBsAg titres in mice subcutaneously administered with HBsAg in MELs, and indicated that MELs were relatively more efficient than conventional liposomes or alum in eliciting higher and prolonged antibody levels. The superior adjuvanticity of MELs is related to the increased residence of HBsAg in MELs at injection sites and the sustained delivery of the liposomal antigen from these systems.[80] In the case of conventional liposomes, it is suggested that, a large fraction of the liposomes are degraded by phospholipases, resulting in liberation of the naked HBsAg particle to the surroundings, which is less efficient in eliciting the humoral immune responses.[79]

Selective combination of the lipid components used in liposome formulation can achieve controlled stability of the liposomal membrane and selective release of encapsulated antigens in specific environmental conditions.[25] One approach involves the use of pH sensitive lipids such as phosphatidyl-β-oleoyl-γ-palmitoyl ethanolamine. The liposomal membrane containing these lipids can destabilize at the slightly acidic pH of endosomes,

resulting in their fusion with the endosomal membrane and the release of peptide and protein antigens.[81, 82] These type of liposomes have been reported to promote cell-mediated immune responses.[83]

1.1.1.2.2. Proteoliposomes

Proteoliposomes are nano-vesicles containing major outer membrane proteins and lipopolysaccharides representing selected pathogen associated molecular patterns. The term was formerly used to designate multi-molecular vesicles formed of highly hydrophobic outer membrane proteins of meningococci and other Neisseria species.[84] In the literature the terms ‘outer membrane vesicle’, ‘proteosome’ and ‘proteoliposome’ have been used interchangeably.[77, 84] Proteoliposomes exert an adjuvant effect over antigens and have demonstrated an ability to induce potently enhanced immune responses against antigen with a modulation towards Th1 response. The proposed mechanism of action is that proteoliposomes contains several pathogen-associated molecular patterns (PAMPs) which are delivered as danger signals to immune competent cells (dendritic cells and macrophages) along with the antigen incorporated within.[85] The mucosal application of proteoliposomes has been restricted to the nasal route, perhaps due to low stability of preteoliposomes in aqueous solutions with varying pH.[77, 86] Monovalent [87] and trivalent [88] influenza A/H1N1-proteosome (no LPS) vaccines administered IN produced high antibody titers in serum as well as in nasal secretions, suggesting that IN delivery of proteosome based vaccines may be able to produce both systemic and mucosal immunity. Furthermore, preclinical studies have shown that such a vaccine is capable of protecting mice upon challenge with the infective pathogen.[89-91]

The proteoliposome technology has been successfully utilized in some commercial products such as VA-MENGOC-BC™ vaccine and Protollin™ mucosal adjuvant.[92, 93]

1.1.1.2.3. Virosomes

Virosomes are small unilamellar vesicles, containing viral envelope glycoprteins embedded into the membrane.[78, 94] Using preformed liposomes and hemagglutinin and neuraminidase, purified from influenza virus, Almeida et al.[95] were first to report successful generation of membrane vesicles with spike proteins protruding from the vesicle surface. Due to the resemblance to native influenza virus, the system was named Virosomes.[96] The majority of methods employed to reconstitute influenza viral membranes are based on three steps: (a) detergent solubilisation of the viral membrane, (b) sedimentation of the internal viral proteins and the viral RNA genome by ultracentrifugation, and (c) reconstitution of the phospholipids and membrane proteins into a biological membrane by selective removal of the detergent.[96] Immunopotentiating reconstituted influenza virsomes (IRIVs) are spherical unilamellar vesicles with an average diameter of about 150 nm. They are reconstituted empty influenza virus envelopes and have been evaluated as a vehicle for trivalent inactivated influenza vaccine in humans and many animal models by the intranasal route.[97, 98] Figure 1.1 depicts the schematic structure of an IRIV.

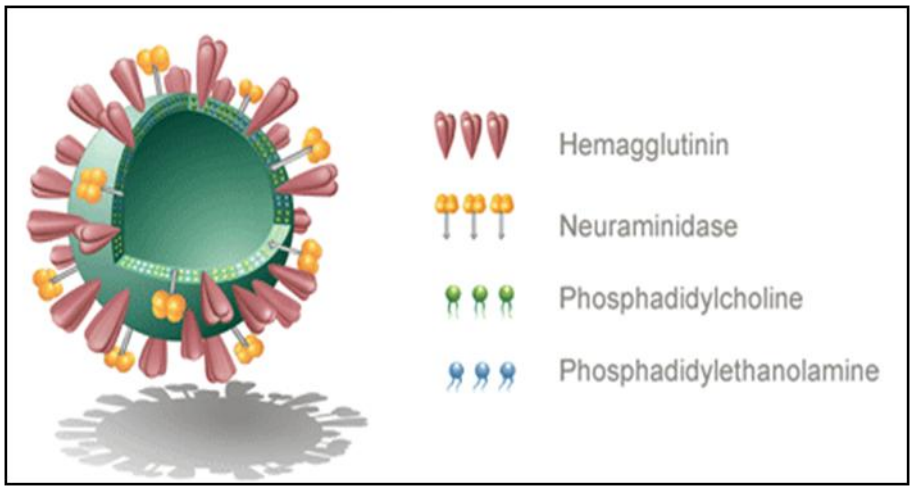


Figure 1.1. Graphical structural representation of an Immunopotentiating Reconstituted Influenza Virosome (IRIV).[99]

Since the first description of influenza virosomes, virus envelopes have been reconstituted from a diversity of viruses. The viral component in virosomes enhances their efficiency in APC binding and promotes cytosolic delivery that enhances antigen presentation over MHC class-I.[100] In addition, virosomes containing specific glycoproteins, such as those extracted from human parainfluenza viruses, also possess the ability to induce membrane fusion with cell surfaces.[101] This suggests that incorporation of the membrane fusion glycoprotein in the design of particulate delivery systems is a useful feature for increasing the uptake of the antigen incorporated in the delivery system by APCs.[78] Virosomes are already approved for human vaccination in two commercial products, Epaxal® with hepatitis A and Inflexal V® with influenza antigens.[102]

1.1.1.2.4. Transferosomes and Vesosomes

Transferosomes are highly deformable liposomes or vesicles developed mainly for transdermal delivery system of bioactives.[72] Transfersomes are composed of phospholipids such as phosphatidylcholine, but also contain surfactants, such as sodium cholate, deoxycholate, Span 80, Tween 80 and dipotassium glycyrrhizinate. The surfactant acts as an edge activator that destabilises the lipid bilayers and increases the deformability of the vesicle.[103, 104] The serum IgG antibody titre generated in response to administration of tetanus toxoid in transfersomes, niosomes and conventional liposomes to the shaved skin of rats, and also an alum-absorbed tetanus toxoid given intramuscularly, was compared in an immunological study. It was reported that two applications of tetanus toxoid in transfersomes 28 days apart could induce an immune response that was equivalent to that produced by intramuscular injection of alum-absorbed tetanus toxoid. In comparison, niosome and conventional liposome formulations induced weaker immune responses.[105] Transfersomes offer two main advantages, first, they are a non-invasive and second, they give rise to high titer values with respect to other vesicular carriers. The transfersomes if suitably designed may have suitable

immunoadjuvant action and could be targeted to the APCs also.[106] The transfersome structure offers the advantages of the liposome lipid bilayer while incorporating a surfactant that permits elasticity and deformation of the bilayer structure. This deformation allows the transfersome to squeeze through small spaces; thereby facilitating permeation through skin.[103] It is likely that this ability of transferosomes to permeate the stratum corneum, which is the major permeation barrier of the skin, is the mechanism by which these vesicles are able to deliver incorporated antigen to APCs but exact mechanism of action still needs to get investigated.

Vesosomes were initially developed as multicomponent or multifunctional bilayer vesicular drug delivery systems.[107] Vesosome is a multicompartmental aggregate of tethered vesicles encapsulated within a large bilayer.[80, 108] In the recent past, the vesosomes concept has been utilized for enhanced delivery of antigens through the transdermal route.[108] Mishra et al. [108] investigated the potential of fusogenic vesosomes (cationic liposomes within an outer largely neutral liposomal bilayer) for topical delivery of tetanus toxoid antigen. In the immunisation study, the vesosomal systems were shown to elicit a combined Th1 and Th2 immune response following topical administration, suggesting the effectiveness of vesosomal systems for the topical delivery of vaccines. The authors have reported that fusogenic vesosomes have potential to microinject entrapped antigen directly into the cytoplasm of APCs, via a fusion event at the plasma membrane. The fusion-mediated delivery of encapsulated antigen to the cytosol of APCs has been indicated as the reason for induction of class-I antigen presentation in addition to presentation with class-II MHC molecules, due to capture and endosomal processing of extracellular antigen encountered after the release or exocytic spill of antigen in the vicinity of APCs.[106, 108]

1.1.1.2.5. Archaeosomes and Exosomes

Archaeosomes are liposomes made from the polar ether lipids of *Archaea*. *Archaea* is a Domain of prokaryotes, which is phylogenetically more related to *Eukarya* than to *Bacteria*.[109, 110] Figure 1.2 represents the 2-dimensional structure of an archeosome.

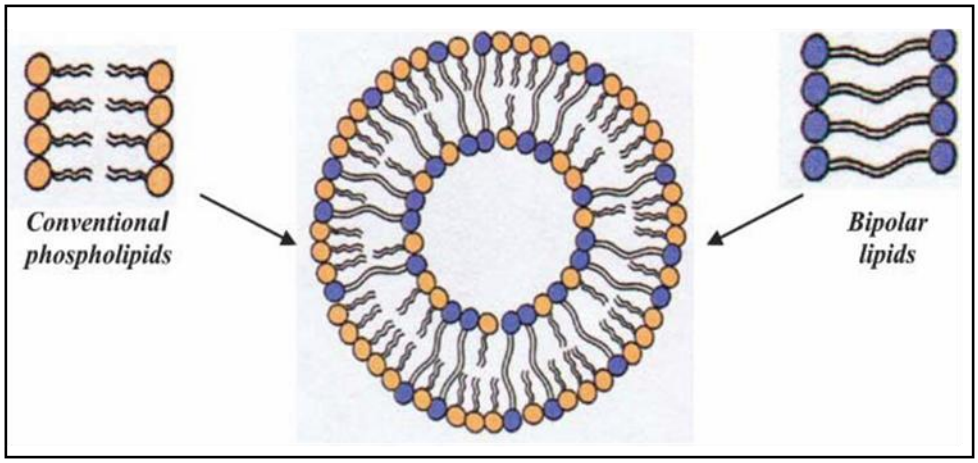


Figure 1.2. Schematic 2-dimensional presentation of structure of an archaeosome.[111]

These lipid structures provide formulary advantages, and contribute to the physico-chemical stability of the archaeosomes and their efficacy as self-adjuvanting vaccine delivery vesicles. The uptake of archaeosomes by phagocytic cells is several folds greater than that of classic liposomes. The immunologically important structural features of archaeosomes include: ether polar lipid structures leading to immunopotentiating interactions with the antigen-presenting cells (APCs), the ability to direct antigen cargo for MHC class I processing leading to potent induction of CD8+ T cell response, and the stability of archaeal lipid cores facilitating profound immune memory.[112] Archaeosomes are stable to

oxidative stress, high temperature and alkaline pH, and resist attacks of phospholipases, bile salts and serum proteins.[113] It appears that archaeosomes are taken up in APCs and the antigen is released into both cytosolic and phagolysosome compartments for processing, although the exact mechanism of antigen delivery has yet to be determined.[114] It has been suggested that archaeosomes are sufficiently degraded in vivo, because internalized as well as surface bound BSA was equally able to induce an antibody response. It has also been shown that incorporation of the antigens into archeosomes was a precondition to achieve high antigen-specific antibody titers, because immunizations with antigen only mixed with archeosomes elicited significantly lower antibody titers.[115] The potential of archaeosomes has also been reported for development of immunotherapy against cancer and infectious diseases in murine models.[116, 117]

One another vesicle type which has been recently investigated for potential use in therapeutic cancer vaccine development is known as Exosome. These are 50–100-nm vesicles secreted by APCs, such as dendritic cells and B-cells, as well as a whole range of other cell types, including T-cells, mast cells, tumor cells, and intestinal epithelia.[118-121] The biological role of exosomes *in vivo* has not been completely elucidated but some researchers have suggested that a circulating network of exosomes in animals is responsible for distal communication between cells.[122] This network can be suggested as mechanism for enhancement of the immune responses by exosomes. Indeed, Raposo et al.[119] reported that B cell-derived exosomes can directly activate CD4+ T cells. Exosomes from different cellular origins bear distinct proteins of the producing cell type.[123, 124] One of the most abundant protein families found in exosomes are tetraspanins, such as CD9, CD63, CD37, CD53, CD81, and CD82.[118, 123, 125] Exosomes released from dendritic cells have been named dexosomes. They have characteristic protein content, incorporating a high number of MHC class I and II molecules and appear to be therapeutically and immunologically relevant.[123, 126] Dexosomes pulsed with tumor-derived peptides elicited potent antitumor T-cell responses and

tumor regression in mice.[127] The rationale for the use of dexosomes in cancer therapy is mainly founded on protein content of dexosomes and the ability of dexosomes to activate distant dendritic cell (DC) by delivering key proteins.[126, 128] The manufacturing or preparation of exosomes for cancer immunotherapy purpose involves collection of monocytes from the patient followed by cultivation of monocytes in the presence of GM-CSF and IL-4 to let them differentiated in dendritic cells. After a specified time of culture, dexosomes released from dendritic cells are isolated and purified from the cell culture medium. Dexosomes could also be loaded with tumor cell derived antigens at different times during this process.[126] Although, a few parenteral vaccine formulations for cancer immunotherapy based on dexosomes are under Phase I or II clinical trials,[129, 130] more detailed research is required to understand and effectively utilize this delivery systems in mass populations.

1.1.1.2.6. Niosomes

Niosomes are unilamellar vesicles made up primarily of non-ionic surfactant molecules. They are very similar to liposomes except that synthetic amphiphiles are used in place of phospholipids for preparation of vesicles, which makes niosomes resistant to oxidative degradation. Initial investigations into the immunogenicity of these delivery systems showed that niosomes were generally better stimulators of IgG2a than Freund's complete adjuvant, but poor stimulators of IgG1, and that the adjuvant activity of niosomes was dependent on the entrapment of antigen within vesicles, mixing free antigen with vesicles was not effective.[131] A subsequent investigation using 1-monopalmitoyl glycerol, cholesterol, and dicetyl phosphate in the molar ratio 5:4:1 demonstrated that both 560 nm and 225 nm vesicle preparations induced higher interferon-γ concentrations than inoculation with 155 nm vesicles, in response to restimulation of splenocytes with the ovalbumin (antigen).[132] Niosomes have generally been studied for parenteral administration, although they have also shown success through the

oral route with some formulation modifications.[133, 134] The specific mechanism of action for niosomes has not been investigated but uptake of niosomes by GALT via M-cells on oral administration has been suggested.

1.1.1.3. Immunostimulating Complex (ISCOM) and ISCOMATRIX™

Immunostimulatory complexes (ISCOMs) are colloidal antigen delivery systems composed of antigen, cholesterol, phospholipid and saponin mixture Quil A. An ISCOM particle without antigen is called ISCOMATRIX™ which has been reported to comprise an in-built adjuvant property.[17, 76, 135] They are about 40 nm spherical aggregates of ring-like micelles.[1] ISCOMs are prepared by solubilising protein antigen, cholesterol and phospholipids with a detergent (usually Triton X-100) which is subsequently exchanged by Quil A.[78] The lipid components of the formulation can be removed if the starting antigenic material is a virus suspension.[136] Quil A saponins are derived from Quillaja saponaria (soap bark tree) and have been used as adjuvants in veterinary vaccines. Quil A saponins form strong complexes with cholesterol and probably enhance antigen transport through membranes of APCs by forming pores in membranes.[137] Although initial studies with ISCOMs as vaccine delivery systems shown effective antigen delivery by both mucosal and parenteral routes resulting in stimulation of Th1 and Th2 CD4+ activity, MHC-I restricted CD8+ activity and secretory IgA, local reactions (pain and erythema) at the site of injection in human trials as well as difficulty in incorporation of some antigens, raised questions about the success of ISCOMs as delivery systems. Recently many of these problems have been overcome by use of either some additional excipients or physical incorporation of antigens with ISCOMATRIX™. ISCOMs and ISCOMATRIX™ vaccines have now been shown to induce strong antigen-specific cellular or humoral immune responses to a broad range of antigens of viral, bacterial, parasite origin or tumor in a number of animal species including non-human primates and humans. The ability of ISCOMs and ISCOMATRIX™ to induce cytotoxic lymphocytes (CTL) in humans makes them ideal for use in vaccines directed against chronic infectious diseases as well as for therapeutic

cancer vaccines. Additionally, ISCOMs and ISCOMATRIX™ also demonstrates significant potential as oral and intranasal adjuvants.[17]

1.1.1.4. Cochleates

Cochleates are bilayer sheets of stable protein phospholipid-calcium precipitates. The calcium ion stabilizes the formed spirals by ionic interaction (Figure 1.1).

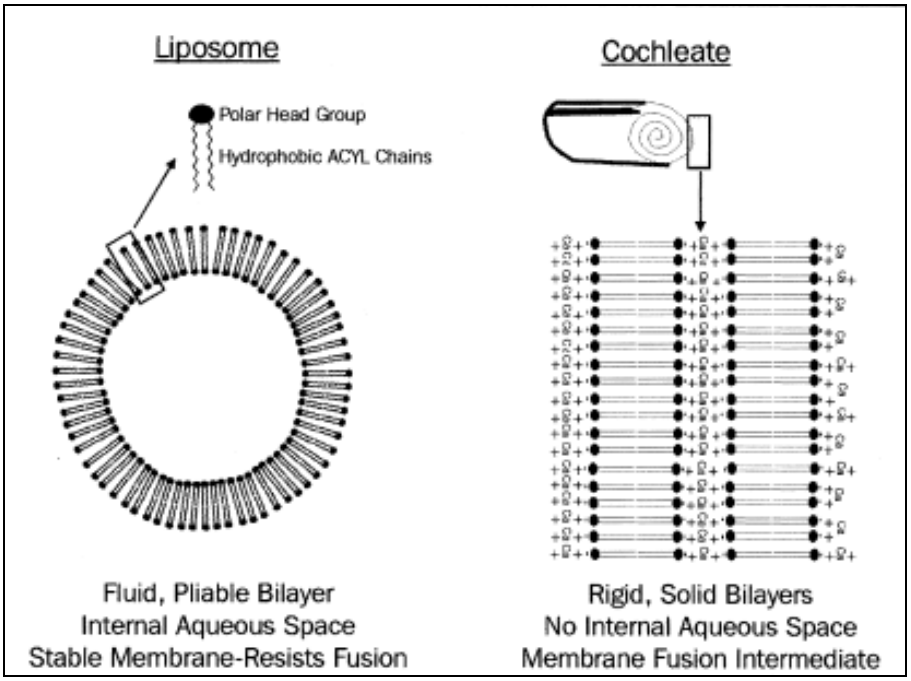


Figure 1.3. Simplified comparison of structure and properties of a liposome and cochleate.[138]

Cochleate vaccines may be beneficial for mucosal administration specifically through the oral route because the delivery vehicle is very stable. They are prepared through the calcium-induced fusion of liposomes composed of negatively charged phospholipids, in particular phosphotidylserines, for example, dioleyol phosphotidylserine.[138, 139] The inclusion of phosphotidylserine in liposomes was shown to enhance recognition and processing of liposomal

antigen by macrophages.[140] Gould-Fogerite and coworkers[138] hypothesized that as the cochleate outer structure is calcium rich, a partial fusion event occurs between the outer layer of cochleate and the cell membrane, which results in the delivery of a small amount of the encochleated antigen into the cytoplasm of the APC. Delivery of antigenic material to the cytoplasm allows access to the MHC Class-I presentation pathway which facilitates stimulation of CD8þ CTL. Also, some endocytic uptake of encochleated antigen leading to MHC Class-II presentation probably occurs.[138, 141] Hoffmann et al.,[142] however, reported contrary to the above results, that phosphotidylserine containing liposomes specifically inhibited immune responses to antigen in mice. Proteins, peptides and DNA formulated into cochleates are effective vaccines when given by mucosal or parenteral routes.[138, 143] Additionally, the efficacy and optimization of scale-up production of proteoliposome-derived cochleate delivery systems containing different antigens for intranasal immunization of Balb/c mice were evaluated in a series of studies. Proteoliposome derived cochleates are prepared to combine adjuvant properties of proteoliposomes with stability of cochleates that make the delivery system capable of inducing both systemic and mucosal antibody as well as Th1 type of immunity.[144-146]

1.1.1.5. Virus like particles and SMBV

The development of anti-viral vaccines has almost exclusively been based on live attenuated virus strains or inactivation of infectious virus. These vaccines induce strong immune responses but have some serious drawbacks including the possibility of accidental infection before inactivation, reversion of inactivating mutation or attenuated virus and potential to cause harm in immuno-compromised individuals.[147, 148] Virus like particles (VLPs) are virus-resembling particles composed of one or several viral proteins that, when over-expressed, spontaneously self-assemble into highly repetitive particles.[102, 149] VLP vaccines combine advantages of whole virus vaccine and recombinant subunit vaccine. VLPs are structurally similar to viruses but, because they lack viral nucleic acids, are completely non-infectious. VLPs can be made using recombinant techniques

in expression systems that do not rely on viral replication. Moreover, VLP based vaccines can induce high antibody titers and strong cytotoxic T-lymphocyte responses against a target molecule. The superb immunogenicity of VLPs arises from the fact that they incorporate key immunological features of viruses such as repetitive surfaces, a potent stimulator of B cell responses,[10] particulate structure, and sometimes induction of innate immunity through activation of pathogen-associated molecular pattern–recognition receptors.[150] VLPs can also be used to generate antibody responses toward antigens not derived from the VLPs parental virus. This is achieved by linking a foreign antigen to the surface of a VLP usually by chemical conjugation. This method has been used to couple a diverse range of antigens, including proteins, polypeptides, capsular polysaccharides, and small organic molecules. In this way, the underlying viral fingerprint of the VLP is imparted to the attached epitope, thereby rendering it as potent a B cell immunogen as the VLP.[151] A novel use of this principle is to attach disease-associated self-molecules to VLPs and to use the resultant conjugate vaccines to overcome B cell tolerance and induce neutralizing autoantibodies.[150, 151] This technological innovation is the foundation that underlies Immunodrugs. Immunodrugs are therapeutic VLP vaccines designed to induce autoantibodies against disease-related proteins. They are comprised of VLPs derived from the RNA bacteriophage Qβ to which self-antigens are covalently attached. One Immunodrug, AngQb, achieved clinical proof of concept for lowering blood pressure in hypertensive patients.[152] Currently, VLP-based vaccines, Recombivax® and Energix® for hepatitis B virus (HBV) have been licensed commercially.[151]

Synthetic Biomimetic Supramolecular Biovector (SMBV) is a nanoparticulate system consisting of a positively or negatively charged polysaccharide core surrounded by a lipid membrane (dipalmitoyl phosphatidylcholine and cholesterol). The diameter of the particles ranges between 50 and 100 nm. The similarity to viruses in structure and size (Figure 1.2) leads to a strong activation of the immune system against the presented antigens.

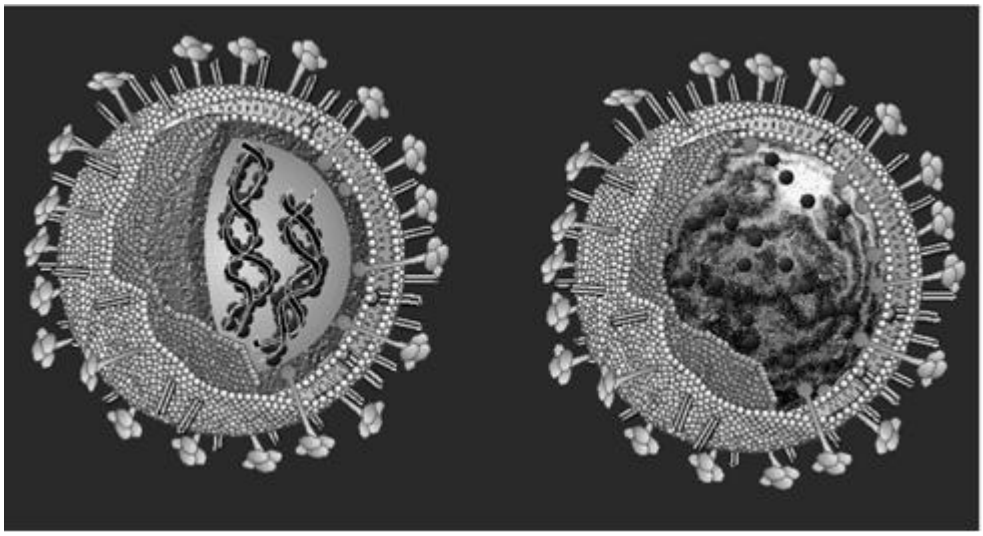


Figure 1.4. Schematic comparative presentation of a virus (left) and a SMBV™ loaded with antigens (right).[153]

The SMBV system is able to deliver a broad range of antigens because of its dual character structure, that is, hydrophilic core and lipophilic cover, and the ability to adapt the charge within the core.[153] The SMBV core is usually composed of maltodextrin modified with a quaternary ammonium moiety and these nanoparticulate structures are generally prepared by high pressure homogenization. The loading of antigens can also be undertaken after completing the preparation of ‘‘empty SMBV’’ and this could be beneficial with the antigens that are unstable at high temperature/presssure.[154] The phase-I clinical studies on human volunteers using a nasally administered SMBV-based trivalent influenza vaccine were well tolerated and induced mucosal and serum antibodies against all three influenza strains.[153, 155] Whilst it has been hypothesized that the mechanism by which SMBV is able to promote humoral as well as mucosal immunity is linked to the presentation of antigens to the nasal mucosa,[154] this has yet to be verified. Studies have shown that SMBV are strongly muco-resident in the nose of adult volunteers and might allow the direct binding of the antigen to the nasal mucosa.[78] Furthermore, the antigen to SMBV ratio of 1/40 is reported as optimal for both systemic and mucosal immune response, probably because

this ratio of antigen and SMBV allows efficient binding of antigen to APCs.[154, 156]

1.1.1.6. Aquasomes and Dendrimer based delivery systems

Aquasomes are three-layered structures (i.e., core, coating, and antigen/drug) that are self-assembled through noncovalent bonds, ionic bonds, and van der Wals forces.[157, 158] They consist of a ceramic core whose surface is noncovalently modified with carbohydrates to obtain a sugar ball, onto which is adsorbed the therapeutic agent. The core provides structural stability to a largely immutable solid.[159] Kossovsky et al[160] demonstrated the efficacy of an organically modified ceramic antigen delivery vehicle. These particles consisted of diamond substrate coated with a glassy carbohydrate (cellobiose) film and an immunologically active surface molecule in an aqueous dispersion. These aquasomes (5–300 nm) provided conformational stabilization as well as a high degree of surface exposure to protein antigen (muscle adhesive protein). For muscle adhesive protein, conventional adjuvants had proven only marginally successful in evoking an immune response. However, with the help of these aquasomes, a strong and specific immune response could be elicited by enhancing the availability and in vivo activity of antigen.[160] The use of ceramic core–based nanodecoy systems was proposed by Goyal et al[161] as an adjuvant and delivery vehicle for hepatitis B vaccine for effective immunization. Self-assembling hydroxyapatite core was coated with cellobiose, and finally hepatitis B surface antigen was adsorbed over the coated core. The antigen-loading efficiency of plain hydroxyapatite core (without cellobiose coating) and coated core were reported to be approximately 50% and 21% respectively. The preparation was found to be better than the conventional adjuvant alum on subcutaneous immunization in Balb/c mice. The nanodecoy systems were also found to be able to elicit a combined Th1 and Th2 immune responses.[161]

Dendrimers are well-defined (monodisperse) synthetic globular polymers with a range of interesting chemical and biological properties.

Chemical properties include the presence of multiple accessible surface functional groups that can be used for coupling biologically relevant molecules and methods that allow for precise heterofunctionalization of surface groups. Biologically, dendrimers are highly biocompatible and have predictable biodistribution and cell membrane interacting characteristics determined by their size and surface charge. Despite the molecular characteristics of dendrimers, they have at present only to a limited extent been used as carrier molecules to enhance immunogenicity of antigens for vaccine purposes, with an exception of the lysine-based multiple antigen peptides (MAP) carrier dendrons.[162] Although MAP dendrimers are developed specifically for presenting small peptide antigens to the immune system, the accommodation of nonpeptidic haptens (e.g., carbohydrates) and of several different types of peptides simultaneously (B and T cell epitopes) have also been demonstrated. The use of dendrimer based vaccine delivery is still in premature stages and further development and immunological investigations are needed to evaluated immunostimulant and carrier properties of these delivery systems.[163]

1.1.2. Non-Particulate (Liquid) Delivery Systems

Simple solutions have always been the first choice as delivery systems for vaccines due to simplicity and cost-effectiveness. However, most acellular safer sub-unit antigens are not sufficiently effective without adjuvants to induce immune responses. Many of these vaccines were found effective when formulated in particulate vaccine delivery systems, resulting in exhaustive research in the field of particulate delivery systems in the last 3-4 decades. On the other hand, non-particulate delivery systems with in-built adjuvant properties have also been developed and are in commercial use. One of the commercially successful non-particulate delivery systems with in-built adjuvant properties is MF59^{TM}, an oil-in-water emulsion (o/w) consisting of small, uniform, and stable microvesicles, consisting of a drop of oil surrounded by a monolayer of non-ionic detergents. The oil is squalene, which is obtained from shark liver. Squalene droplets are stabilized by addition of two non-ionic surfactants, a low hydrophilic–lipophilic balance (HLB)

surfactant, polysorbate 80 (Tween 80), which is widely used as an emulsifier in foods, cosmetics and pharmaceuticals, including parenteral formulations, and sorbitan triolate (Span 85). The exact mechanism of action for MF59™ emulsion is not known but administration of MF59™ induced a significant influx of macrophages at the site of injection, which was significantly suppressed in mice deficient for chemokines receptor 2 (CCR2).[164, 165] Thus, it is likely that one of the effects of MF59™ is to trigger the production of chemokines in cells resident at the injection site. Ongoing studies show that a local immuno-stimulating environment is generated and that human immune cells can be directly activated *in vitro* by MF59™. Irrespective of its mechanism(s) of action, the studies done on mice strongly suggest that MF59™ enhances functional and protective antibody responses[166-168] and/or induces strong T-cell responses[166, 169, 170] to several different types of antigen including bacterial toxoids (e.g., tetanus toxoid and diphtheria toxoid), outer membrane vesicles (e.g., *Neisseria meningitidis*), polysaccharide conjugates (e.g., meningococcal C conjugate vaccines), recombinant antigens (e.g., hepatitis B surface antigen, meningococcal B), and viral antigens (e.g., influenza antigens). Toxicology studies in animal models and clinical studies ranging from phase 1 to 4 have demonstrated the safety with different vaccines. In an extensive set of preclinical studies, clinical studies and postmarketing data, the MF59™ emulsion has been found to be a safe and potent vaccine adjuvant, resulting in the licensure of an MF59™-adjuvanted influenza vaccine in more than 20 countries.[171] Furthermore, multiple emulsion delivery systems along with an additional immunostimulant have been reported as effective in animal models through the oral route of administration.[172]

1.1.3. Live Attenuated Vectors And Edible Vaccines

Live attenuated vectors are effective vaccine delivery systems because they are replicating microorganisms that have inherent adjuvant activity. The ability of some microorganisms to colonize in host, and the potential for including genes from unrelated microorganisms encoding relevant antigens, represent an attractive possibility for use of these microorganisms as vaccine delivery systems. Various

bacterial (e.g. *Salmonella, Escherichia coli, Mycobacterium, Lactobacillus* and *Listeria monocytogenes*) and viral (e.g. *Poxviurses, Alphavirus, Adenovirus* and *Poliovirus)* species have been investigated as vectors for vaccine delivery.[173, 174] Live, recombinant vectored vaccines have the advantage that they can stimulate both humoral and cell-mediated immune responses, and have a great potential for immunization either alone or in combination with a subunit antigen.[101] There are a few live attenuated vector based veterinary and human vaccines available in commercial markets in few countries [175, 176] but further investigations are required for full utilization of this technology in humans.

A major hurdle to the successful development of non-replicating oral vaccines is the lack of efficiency of the uptake of antigen in the gastrointestinal tract. A variety of approaches have been taken to protect antigens from digestive enzymes. One of the promising approaches is use of plants as recombinant biofactories to express a number of proteins including pharmaceuticals and potential vaccines. The use of plants as vectors has been reported advantageous as vaccine antigen production in plants is safe and potentially very cheap and infinitely scalable; plants can often be used to produce biologically active proteins far more easily than can bacteria or yeast; and the use of food plants could allow edible vaccines to be locally and cheaply produced in the developing world.[177] Transgenic plants represent a potentially stable and cheap propagation source. However, development and selection of a suitable transgenic line can take many months and production at high yield is often not attainable or stable, often owing to the phenomenon of post-transcriptional or siRNA-dependent gene silencing.[178] Additionally, the potential to induce tolerance and the potential for the vaccines to inadvertently enter the food chain has been widely reported as possible disadvantages of this technology.[179] Overall, there are still major hurdles in the way of routine vaccine production via plants and this technology can only be expected to be commercially viable for vaccine delivery and development after further research.

1.1.4. Challenges and Limitations in the Development of Vaccine Delivery Systems

Despite fast development in the level of sophistication in the design of new antigen delivery systems, a number of challenges, such as efficient loading of antigen into the carrier, targeted and specific delivery in the body whilst evading biological defence mechanisms, and enhancement of vaccine efficacy and safety, must be met for these systems to be scientifically and clinically relevant. For licensing of new or newly formulated vaccines, non-clinical and clinical data regarding safety and efficacy are required, next to pharmaceutical quality data. Compliance with the regulatory guidelines and the risks associated with vaccine development make it a costly process, urging the protection of Intellectual Property. However, licensing of a promising novel vaccine formulation, with demonstrated higher efficacy and safety, does not guarantee success. Higher costs and patient conservativeness may limit market uptake.[180] Challenges and limitations in the development of vaccine delivery systems can be summarised in following points:

- The need for increasing subunit vaccine efficacy.
- The need for improving needle-free delivery.
- The need for enhancing vaccine safety.
- Target identification and mapping the need.
- Lack of animal models which closely resemble human physiology in terms of expression of various antigen receptors for targeting purposes.
- Regulatory hurdles for developing new vaccine delivery systems.
- Poorly standardized methods for characterization.
- Potential instability of antigens in delivery systems.
- The need for simple formulations; easy and cheap to manufacture as well as scale up in production.

1.2. IMMUNOLOGICAL ADJUVANTS

The term “adjuvant” is derived from the Latin “adjuvare” which means ‘help’ and was first used by Ramon in 1926 for a substance used in combination with a specific antigen that produces more immunity than the antigen used alone.[181] For convenience and to avoid confusion the use of the term “adjuvant” is avoided for vaccine delivery systems as not all of vaccine delivery systems show immunostimulant properties and simply act as carrier for antigen. Various non-specific and specific strategies/components have been investigated as immunological adjuvants for different antigens. The strategies or formulation components investigated for enhancing or modifying immune responses against antigens range from specific targeting ligands for particular receptors/cells to adsorption of antigens on chemical compounds. The immunological adjuvants for vaccines can be divided into the following main categories,

1.2.1. Mineral salts

Aluminium salts, principally aluminium hydroxide or aluminium phosphate, have been the most widely used adjuvants and are commonly referred to as alum. These adjuvants are components of several licensed human vaccines, including diphtheria-pertussis-tetanus (DPT), diphtheria-tetanus (DT), DT combined with Hepatitis B (HBV), Haemophilus influenza B or inactivated polio virus, Hepatitis A (HAV), Streptococcus pneumonia, meningococcal and human papilloma virus (HPV).[182] Formulation is achieved through adsorption of antigens onto the aluminum hydrogel. The mechanisms of action of the aluminum salts frequently cited include: depot formation facilitating continuous antigen release; particulate structure formation promoting antigen phagocytosis by antigen presenting cells such as dendritic cells, macrophages and B cells and; induction of inflammation resulting in recruitment and activation of macrophages, and increased MHC class II expression and antigen presentation.[183, 184] Advantages of aluminum adjuvants include their safety record, augmentation of antibody responses, and relatively simple formulation for large-scale production. The major limitations of aluminium adjuvants include their inability to elicit cell-mediated Th1 or CTL

responses that are required to control most intracellular pathogens such as those that cause salmonellois, tuberculosis, malaria, leishmaniasis, leprosy and AIDS.[181] Moreover, vaccines containing alum cannot be frozen because this leads to loss of potency. Accidental freezing is a widespread phenomenon occurring in up to 70% of vaccines in developing countries.[185] Additionally, alum has been occasionally associated with serious side effects such as erythema, granulomas and induction of IgE and IL-4 (allergy and immediate hypersensitivity).[186-188] Alternatively, calcium phosphate has been used to adsorb antigens which does not induce IgE production in animals or humans.[186] Mineral salts are not suitable for mucosal immunization because of their mode of action by formation of antigen depots and reported they are to be ineffective when used in conjuction with DNA-based vaccines.[188, 189]

1.2.2. Microorganism-derived adjuvants

Microorganism-derived substances have a high degree of immunogenicity and immune enhancing capabilities. Many species of bacteria have been used as a source of potential adjuvants such as *Mycobacterium spp.*, *Corynebacterium parvum, C. granulosum, Bordetella pertussis, E.coli* and *Neisseria meningitides*.[190] Bacteria toxins were the first investigated group of bacteria derived substances used as adjuvants. The important bacterial toxins investigated as immunological adjuvants include inactivated pertussigen, heat-labile toxin (LT) and cholera enterotoxin (CT).[188] These bacterial toxins showed enhancement of different branches of immune responses (mucosal and/or humoral and/or cellular) and have been investigated through both mucosal and parenteral routes of administration in various animal studies but the toxicity associated with these bacterial toxin adjuvants does not allow their use in humans. One important example of toxicity linked to bacterial toxins was the withdrawal of heat labile toxin (LT) adjuvanted, intranasal trivalent influenza vaccine which was approved for distribution and use in Switzerland following successful clinical studies, but was withdrawn following reports of Bell's palsy in some recipients. The pathogenesis of Bell's palsy as associated with the intranasal influenza vaccine is unclear although the presence of

E. coli enterotoxin in the vaccine was suggested as a possible risk inducing factor.[191, 192]

The toxicity issues related to bacterial toxins derived adjuvants lead to investigations for use of more specific and purified microorganism-derived components (e.g. muramyl dipeptide, monophosphoryl lipid A, CpG motifs) as adjuvants.[193, 194] Muramyl dipeptide (MDP) is a bacterial derived less toxic compounds capable of stimulating both humoral and cellular immune responses.[195] DNA containing CpG motifs and synthetic oligodeoxynucleotides containing unmethylated CpG motifs are one of the most potent cellular adjuvants.[196] Monophosphoryl lipid A (MPL) was the first immunological adjuvant capable of activating T-cell effector responses to be used in a licensed vaccine.[197] MPL in various formulations such as in emulsion formulations has been investigated in many clinical studies for induction of strong humoral and cellular immune responses against different antigens.[198, 199] The adjuvant activity of most microorganism-derived substances is mediated through activation of toll-like receptors (TLRs) which mediate the danger signals activating the host immune defence system.[200]

1.2.3. Miscellaneous adjuvants

There are various other substances which have been investigated as immunological adjuvants but with no commercial success for human use yet. The important categories of substances explored as immunological adjuvants include cytokines (granulocyte macrophage-colony stimulating factor and interleukin-12), polysaccharides (inulin, chitosan and dextran), saponins (Quil-A) and some synthetic polymers.[181] The exact mechanism of action behind these substances acting as immunological adjuvants is not known and more in-depth research is warranted.

1.3. TARGETING LIGANDS

Targeted delivery of antigen to particular cells or receptors is one of the approaches for a vaccine formulation as this strategy has the potential to provide a better efficacy to risk ratio for immune responses induced by incorporated antigen. The organised mucosa-associated lymphoid tissues (O-MALT) are antigen-sampling and inductive sites of the mucosal immune system. At these sites, antigens are transported across the mucosal epithelial barrier to prime underlying lymphocytes for a subsequent immunological response. Specialised cells termed membranous epithelial (M) cells are responsible for this transepithelial antigen transport, and putative M cells have been identified at multiple mucosal sites.[201] M cell specific surface carbohydrates have proven invaluable for M cell identification in experimental studies and, since microorganisms frequently use carbohydrates as cell surface receptors, they offer a mechanism to account for the M cell selective interaction exhibited by many microorganisms. The site and role of M cells in the immunological system makes them one of the potential sites for targeting of antigen formulations.[202]

Many lectins such as Ulex europaeus 1 (UEA1) and wheat germ agglutinin lectin (WGA) have been used for the targeting of antigen formulations to M-cells through the mucosal routes (oral, nasal, rectal) in animal models.[203, 204] The lectin-binding characteristics of the M cell apical membranes exhibit considerable species and site-related variations, and there is also evidence of heterogeneity among M-cells within individual domes which limits the success of lectins as M-cell targeting agents in humans. The M-cell specific plant lectins such as UEA-I and WGA have been reported to be only effective in a mouse model because L-fucose, the M-cell surface carbohydrate to which these lectins specifically bind, is reported to be absent on human M-cell surface.[205, 206]

Another group of sites that has been identified as ideal for vaccine targeting is toll-like receptor (TLR) expression implicated in diverse cell types such as airway and gut epithelial cells, B-cells, mast cells, NK cells, dendritic cells, regulatory T-cells, macrophages, monocytes, neutrophils, basophils and endothelial cells.[207, 208] TLRs are

transmembrane signaling proteins expressed by cells of the mammalian immune system, showing specific binding to different ligands of varied molecular nature.[209] The discovery and elucidation of TLR function has provided new knowledge on the host pathogen interface, and a more comprehensive view of immunological events and pathways.[210] In addition to microbial interaction and intervention, a number of agents that can be used as adjuvants have been associated with specific TLR involvement (Table 1.3) in the initiation and qualitative direction of immune responses. With the exposition of TLRs and their immunological role, development of a vaccine formulation incorporating a ligand/adjuvant for specific targeting of a particular TLR is emerging as a promising strategy for efficient and safe delivery of antigens. However the success of this strategy will be dependent on the invention/development of safer and specific immunological adjuvants for TLRs and further elucidation of immunological roles and mechanisms of TLRs.

Table 1.1. Selected adjuvants and natural ligands recognized by Toll like receptors (TLRs).[190, 210]

TLR type	**Adjuvant/Natural ligands**
TLR 1	Triacyl lipopeptides, *E.coli* type II heat labile entertoxins
TLR 2	Muramyl dipeptide (MDP) derivatives, viral glycoproteins, *E.coli* type II heat labile entertoxins
TLR 3	Polyinosinic-polycytidylic acid (synthetic dsRNA analog)
TLR 4	Monophosphoryl lipid A (MPL) and MDP derivatives
TLR 5	Flagellin
TLR 6	Diacyl lipopeptides (macrophage-activating lipopeptide-2)
TLR 7	ss RNA/guanosine analog, Imiquimod
TLR 8	ss RNA, small synthetic compounds
TLR 9	CpG oligonucleotides
TLR 10	Unknown
TLR 11	Profilin

1.4. ROUTE OF ADMINISTRATION

One important factor which is linked not only to efficiency of a vaccine but also to the cost of vaccination and acceptability of a vaccine by population is route of administration. Conventionally, most of the vaccines are administered as injections by parenteral routes mainly to avoid any potential loss of activity or stability of antigen on exposure to mucosal environment and also due to requirement of mostly lesser dose of antigen for being effective compared to mucosal administration. But in the past few decades with the development of novel vaccine delivery systems and newer immunological adjuvants, various other routes such as intranasal, oral, sub-lingual, transdermal and rectal have been investigated as promising routes for delivery of vaccines. Different delivery systems with or without an immunological adjuvant were found useful for particular (type of) antigens and for different routes of administration (Table 1.2 and 1.3).

Table 1.2. Delivery systems studied (selective examples) for delivery of antigens through mucosal routes.

Delivery System	**Antigen**	**Immunostimu-lant**	**Route of Administration**	**Pre-clinical / clinical**	**Result(s) / outcome(s)**	**Ref.**
PLG microspheres	*E. coli* surface antigen 6	LT_{R192G}	Oral	Human	Low serological immune response were elicited, further modifications in delivery strategy is required	[211]
Dry powder chitosan delivery system	Genetically inactivated diphtheria toxoid CRM_{197}	None	Intranasal	Human	Effective systemic immune response on single dose but mucosal immune response only on booster dose	[212]
DOTAP/chole-sterol liposomes	Human sperm surface antigen (CD52)	Diphtheria toxoid	Intranasal	Female mice	CD52-specific IgA and IgG antibodies in sera and vaginal secretions	[213]
Meningococcal Proteosomes	*Shigella flexneri* 2a lipopolysaccha-ride	In-built	Intranasal	Human Phase I/II	Induction of *S. flexneri* 2a LPS-specific IgA, IgG, and IgM antibody-secreting cells in a dose-responsive manner	[214]
ISCOMATRIX	A/New Caledonia 20/99 H1N1	In-built	Intranasal	Female Balb/c mice	Antibody response in both serum (IgG) and nasal secretions (SIgA)	[135]

	influenza virus			& merino	surpassed the levels obtained with unadjuvanted vaccine administered s.c.	
Virosomes	Tri-valent Influenza virus vaccine	LTK 63	Intranasal	Human Phase I	Induction of higher mucosal immune response by nasal administration compared to parenteral administration	[215]
Attenuated *Salmonella enterica* serovar Typhi	*Helicobacter pylori* urease	In-built adjuvant property	Oral	Human	Induction of mucosal IgA response against vector and strong serological IgG response against antigen in fraction of volunteers studied	[216]

Table 1.3. Delivery systems studied (selective examples) for delivery of through parenteral routes.

Delivery System	**Antigen**	**Immunostimu-lant**	**Route of Administration**	**Pre-clinical / clinical**	**Result(s) / outcome(s)**	**Ref.**
Monophospho-ryl lipid A containing liposomes	Recombinant protein (R32NS181) Malaria antigen	In-built and $Al(OH)_3$	Intramuscular	Human	Superior systemic immune responses compared to previously reported vaccines; no systemic toxicity.	[217]
Proteoliposo-mes	*N. meningitides* pore protein A	In-built	Information not available	Human Phase II/III	Significantly higher serum antibody levels compared to control groups	[218]
Virosomes	Hepatitis A	In-built	Intramuscular	Human	Epaxal® is registered and available in various commercial markets.	[219]
ISCOMs	Influenza vaccine	In-built	Intramuscular	Human Phase I/II	Induction of strong and rapid antibody responses, T helper responses and to a certain extent cytotoxic T cell responses.	[220]
Virus like	Human	In-built and	Intramuscular	Human	Vaccine was found efficacious	[221]

particles	papillomavirus (HPV-16/18)	$Al(OH)_3$		Phase III	in prevention of incident and persistent cervical infections with HPV-16/18.	
MF59™ emulsion	Influenza vaccine	In-built	Intramuscular	Human	Fluad® is a registered vaccine product and has been found safe and efficient in various clinical studies.	171, 222, 223

Mucosal immunization may offer advantages in the immunity generated and provide a noninvasive needle-free option for antigen delivery. If suitable delivery strategies can be developed, there is the potential for acceptable and safe modes of administration suited for self or mass administration of vaccines. Additionally, immunization via mucosal routes has been reported to induce both mucosal and systemic immune responses at least in some monogastric species including human.[224] Of the mucosal sites, the oral route of administration is considered to be the most acceptable and accessible. In addition, the presence of organized lympho-epithelial structures known as GALT (gut associated lymphoid tissue) and NALT (nasal associated lymphoid tissue) through oral and nasal routes respectively makes these routes attractive for vaccine delivery. Although, mucosal delivery of vaccines may offer some important advantages the difficulty of developing mucosal and particularly oral vaccines using non-living approaches should not be underestimated. Unfortunately, the advantages offered by the oral route of immunization are usually negated by the hostile gastrointestinal environment, with a high probability of degradation of antigens by acid and enzymes, dilution of the vaccine formulation by the gastrointestinal contents and elimination of antigens by peristalsis and ciliary movement, necessitating the use of higher doses of antigens and specialized formulations. As a result, oral vaccination has tended to produce immune responses that are either poor or none at all compared to parenteral vaccination.[225]

The intranasal route of administration has an advantage over the oral route in that lower doses of antigen are required because there is no significant dilution of the vaccine formulations by nasal fluids and no exposure to low pH or a broad range of secreted degradative enzymes.[30] Nasal delivery of nonreplicating vaccines such as immunogenic proteins, peptides and DNA for mucosal as well as systemic immunity is receiving increased research focus as a potentially useful immunological concept.[226] The successful development of economical and safe nasal non-replicating vaccines for immunization of human population is directly correlated with the selection and development of optimized formulations and delivery systems. Figure1.3 depicts a hypothetical simplified overview of pathways eliciting immunogenic responses via nasal

immunization [8, 227-229] and highlights the potential of particulate delivery systems in nasal immunization.

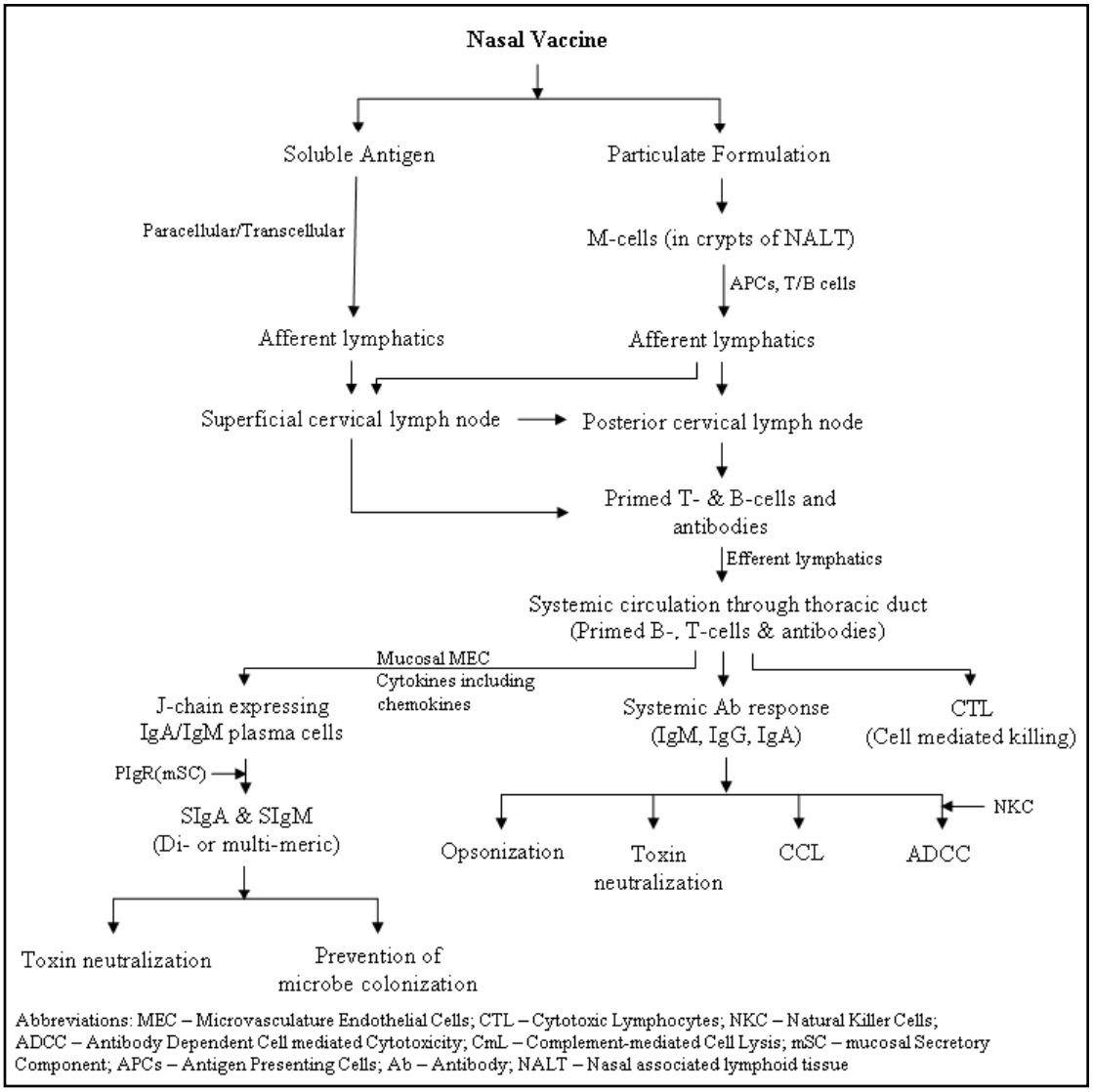


Figure 1.5. Hypothetical simplified overview of pathways eliciting immunogenic responses via nasal immunization.

NALT, the prime inductive site for nasal mucosal immunity, is arranged on both sides of the nasopharyngeal duct in rodents.[230, 231] The collection of oropharyngeal tissue

and NALT equivalent tissues is called Waldeyer's ring in humans. The inductive sites for nasal immunity in the NALT or the Waldeyer's ring are comprised of B- and T-cell follicles, specialized M cells (membranous cells) containing follicle associated epithelium with intervening antigen presenting cells (dendritic cells and macrophages) and regional draining lymph nodes, that is, cervical lymph nodes.[2, 227, 228] The M cell is a specialized antigen sampling epithelial cell consisting of a large basolateral pocket filled with T-cells, B-cells and macrophages. When an antigen is present on the nasal mucosa, especially an antigen in the form of a particle, it is actively transported, primarily by M cells, to reach antigen presenting cells (APCs) including dendritic cells, macrophages, B-cells and follicular dendritic cells for processing and presentation. The successful development of economical and safe nasal non-replicating vaccines for immunization of mass human population is directly correlated with the selection and development of optimized formulations and delivery systems for newer vaccines.

Nasal immunization is also a commercial reality with a nasal influenza vaccine. Flumist® (Medimmune, Inc.) has been available in the United States since 2003 and was proven to be safe and efficacious. However, it did not establish a significant market share of the total influenza vaccine market. The Flumist® vaccine was initially indicated for active immunization of healthy individuals aged 5–49 years which excluded the main target group for influenza vaccination (young children and the elderly). In addition, Flumist® was available only in a frozen formulation, requiring storage and transport at temperatures of -15ºC or below thereby inflating the high retail price and causing inconvenience for pharmacies. Recently, a new formulation of Flumist® vaccine, CAIV-T (cold adapted influenza vaccine-trivalent), which can be stored in a standard refrigerator and is indicated for the active immunization of eligible individuals aged 2–49 years, has gained FDA approval.[232] The Flumist® experience highlights the potential of intranasal route as a mucosal vaccine delivery approach and the need to focus on developing novel and/or better scientific approaches for commercial success. To achieve the ultimate success in vaccine delivery, one may need to employ multiple strategies to meet the challenges in formulation, manufacturing, regulatory, and of course, customer acceptance and satisfaction.

1.5. SIGNIFICANCE OF THE RESEARCH

The literature review of vaccine delivery systems highlights the need for further research in this field as very few delivery systems have ben successfully developed into commercial products. This phenomenon could be due to the research and development efforts being focused on the development of individual antigen rather than on delivery systems. Despite the efficacy of killed and attenuated vaccines, they do bear the potential risk of residual toxicity and might contain toxic components or reversion to virulence.[137] The technological advances in biotechnology have made possible the large scale production of highly purified bio-engineered DNA and proteins. However, in many instances, purified antigens are poorly immunogenic and induce only antibody responses on parenteral administration. A safe and efficient delivery system, preferably with built-in adjuvant activity, could significantly improve the efficiency and utility of these purified antigens.[78] One more important conclusion which can be drawn on the basis of the literature review is need for efficient and safe immunological adjuvants to be incorporated in a vaccine delivery system for induction of a strong and wide-range of immune responses. Most of the immunological adjuvants recently investigated have been derived from pathogens. This makes a vaccine delivery system containing one of these immunological adjuvants gaining regulatory approval difficult due to safety issues. The selection of the route of administration for vaccines delivery systems has also been reported as an important factor affecting type of immune response elicited by a vaccine. It is evident that there is an urgent need for a better understanding of the relationship between the design and performance of the vaccine delivery systems with consideration of the route of administration.

The development and evaluation of a particulate vaccine delivery system with or without incorporation of a non-pathogen derived potential immunological adjuvant with M cell targeting potential was undertaken in this research project to tackle some of the above issues related to vaccine delivery systems and immunological adjuvants. This research project may offer a novel way of achieving strong immune responses against the acellular protein antigens with the potential to offer significant advantages for human immunization and prevention of infectious diseases.

1.6. OVERALL OBJECTIVES

The principle objective of the research was to design, develop and evaluate a biodegradable nanoparticulate delivery system with a non-pathogen derived potential immunological adjuvant which has the M-cell targeting prospective. The research objectives were sub-divided into following points:

- Design, formulation and optimization of a chitosan (CS) and dextran sulphate (DS) based biodegradable nanoparticulate vaccine delivery system.

- Development of incorporation method(s) for a subunit protein antigen (pertussis toxoid) and/or a potential immunological adjuvant with M cell targeting prospective (IgA) into the optimized nanoparticulate system.

- *In-vitro* evaluation of the antigen and/or IgA - loaded nanoparticulate system in terms of particle characteristics, release profile, entrapment efficiency and stability.

- *In-vivo* evaluation of M-cell targeting ability of a IgA-loaded (antigen-free) nanoparticles via intranasal administration.

- *In-vivo* immunological evaluation of the optimized antigen loaded formulation(s) in a mouse model. The *in-vivo* immunological evaluation of formulations was conducted to assess,

    - Immunological potential of nanoparticulate delivery system through intranasal versus subcutaneous route of administration.

    - Efficiency of IgA as an immunological adjuvant through intranasal and subcutaneous route of adminstrations.

    - Differences in type of immune responses produced by the optimized formulation(s) through different route of administrations in presence or absence of IgA in formulation.

1.7. THESIS OVERVIEW

The research work of the project has been compiled in three main chapters, each incorporating an introduction, experimental, results and discussion section. The three main research chapters are followed by a general discussion and conclusions chapter. The three main chapters of thesis explain three varied parts of my research work as explained below,

- **Preparation and optimization of chitosan–dextran sulfate (CS-DS) nanoparticles**: This chapter describes the preparation method of CS-DS nanoparticles and how important formulation factors were optimized to obtain a stable and efficient CS-DS nanoparticulate formulation. An optimized blank CS-DS nanoparticle formulation was then used in later studies which were described in further chapters.

- **Preparation and i*n-vitro* characterization of the CS-DS nanoparticle formulation loaded with a model antigen and/or potential adjuvant/targeting agent:** This chapter explains how a model protein antigen and/or a novel potential immunological adjuvant (IgA) were loaded into CS-DS nanoparticles and what the characterstics of the systems are. The chapter also addresses the characterization methods and quantification of antigen and IgA.

- **Immunological characterization of the antigen and/or IgA loaded CS-DS nanoparticle formulations *in-vivo*:** The chapter focuses on the *in-vivo* evaluation of the loaded CS-DS nanoparticulate formulations through the mucosal (intranasal) and parenteral (subcutaneous) routes of administration. The chapter comprises comparison of immunological responses induced by different loaded CS-DS nanoparticle formulations with conventional antigen formulation. Also, a preliminary study to evaluate M-cell targeting ability of CS-DS nanoparticles loaded with IgA by nasal route of administration is reported in this chapter.

CHAPTER 2

PREPARATION AND OPTIMIZATION OF CHITOSAN-DEXTRAN SULFATE NANOPARTICLES

2.1. INTRODUCTION OF CHITOSAN-DEXTRAN SULFATE NANOPARTICLES

2.1.1. Chitosan

Chitosan is generally produced from crustaceans such as crabs and prawn shells but may also be derived from fungal chitins. It is a polysaccharide derived from deacetylation of chitin and has structural characteristics similar to glycosaminoglycan (Figure 2.1).[57]

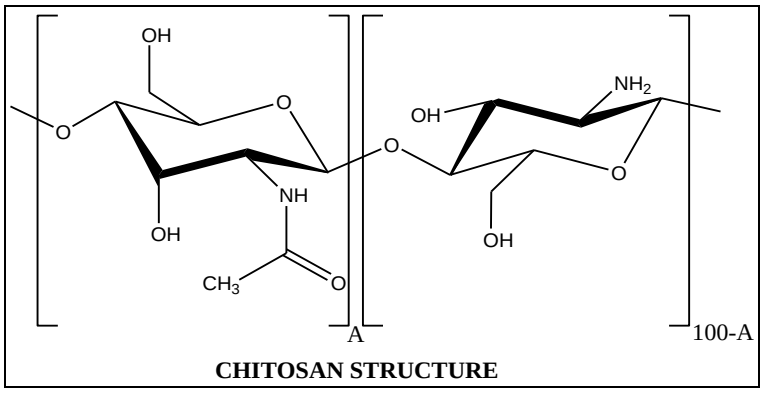


Figure 2.1. Chemical structure of the chitosan (A = % degree of acetylation).

Chitosan contains primary amine groups that can be protonated by selected acids or pH adjustment as the p*Ka* of chitosan amine is 6.3. This unique property renders chitosan some useful features or applications. For instance, the interaction of chitosan protonated amine groups with cell membranes results in a reversible structural rearrangement of protein associated with tight junctions which leads to their opening.[233] Also, protonation of amine groups of chitosan promote adhesion of chitosan formulations to negatively charged mucosal surfaces.[234] These properties aid to stimulate the absorption and/or uptake of therapeutically active materials by epithelial cells and M-cells from chitosan-based formulations on mucosal administration.[55] The cationic nature of chitosan in solution has also been

exploited by researchers in the formulation of DNA based antigens for mucosal delivery as ionic interaction between chitosan and DNA enhances the entrapment efficiency of antigen in particulate delivery systems.[61, 235-237]

The promising properties of chitosan as formulation ingredient has prompted the development of its various derivatives such as glycol-conjugated chitosans (chitosan conjugated with mannose, galactose, glucose, lactobionic acid or glucoseamine), lipid-conjugated chitosans (palmitic or oleic acid conjugated chitosan), mono-N-carboxymethyl chitosan and N-trimethyl chitosan (TMC).[55, 238] Chitosan has functional groups (amine and hydroxyl) which allow chemical modification of the molecule and its physical properties. Thiolated chitosans, obtained by modification of the primary amine groups with cysteine, thioglycolic acid and 2-iminothiolane, are a class of derivatives that showed improved mucoadhesive properties and have been used in mucosal delivery systems.[239-241] These derivatives of chitosan have shown *in situ* gelling properties due to the formation of inter- and intramolecular disulfide bonds at physiological pH. Another chitosan derivative synthesized by modification of its amine groups is mono-N-carboxymethyl chitosan (MCC). MCC is a negatively charged chitosan derivative with a property of significantly decreasing transepithelial electrical resistance (TEER) of Caco-2 cell monolayers at concentrations of 3-5% (w/v).[242] Quaternary chitosan derivatives are soluble over a wide pH range due to their permanent positive charge. Quaternary chitosan derivatives such as N-alkylated chitosan derivatives have been reported to possess antibacterial and antifungal properties.[243] N-trimethyl chitosan (TMC) is a partially quaternized derivative of chitosan, which has been extensively studied for its absorption enhancing and mucoadhesive effects for hydrophilic macromolecules, particularly through mucosal routes.[244, 245]

It is surprising that only a few of chitosan derivatives showed higher immunological adjuvant properties in *in-vivo* studies compared to non-derivatised chitosan formulations.[246, 247] TMC nanoparticles loaded with influenza subunit antigen shown higher immunogenicity compared to TMC solution formulation of antigen following intranasal vaccination in mice.[245] The TMC is a water soluble

chitosan derivative and provides mucoadhesive as well as absorption enhancing effects even at neutral pH. Sayin et al. prepared negatively charged MCC and positively charged TMC nanoparticles for nasal vaccination.[248] Both tetanus toxoid (TT) loaded TMC and MCC/chitosan particles were efficiently taken up by murine macrophages regardless of their surface charge but mice that received either TT–chitosan or TT–TMC nanoparticles intranasally developed higher serum antibodies than those that received intranasally TT–MCC particles, and comparable to the control mice vaccinated subcutaneously with free TT. TT formulated with soluble TMC or MCC induced similar antibody responses as compared to the nanoparticle formulations.[248]

It is clear from the studies that chitosan derivatives have strong mucoadjuvant activity and enhance local and systemic immunity upon mucosal immunization. Moreover, upon parenteral administration, particulate chitosan vaccines are also taken up by APCs and induced high systemic immune responses in vaccinated animals.[249] It is important to note that in the absence of antigen, chitosan and its derivatives hardly show immunostimulating effects.[250] Type and immunogenicity of antigens as well as the type and adjuvant activity of chitosan also play an important role in the type of immune responses and adjuvant activity of chitosan formulations. The differences in physicochemical properties such as charge density and molecular structure of chitosan derivatives have a great impact on their interaction with APCs and their adjuvant activity, but the mechanisms involved need further investigations.[55]

2.1.2. Techniques for preparation of chitosan nanoparticles

Chitosan nanoparticles formulations can be prepared by both chemical and physical methods. Chemical processes such as the reaction of the primary amine goups of chitosan with a di-aldehyde (usually glutaraldehyde) crosslinker, have some major drawbacks particularly in the case of proteins and peptides delivery. The use of organic solvents and crosslinking agent during the chemical process may adversely affect the stability of proteins and peptides.[61] Consequently, physical

methods by which chitosan and its derivatives are crosslinked to yield nanoparticles are preferred.[55] The presence of amine functional groups in chitosan molecule enables chitosan to carry a positive charge in acidic aqueous media, which in turn allows the interaction of negatively charged macromolecules and polyanions with chitosan to form nanoparticles by ionic gelation or complex coacervation.[251, 252] Ionic gelation and complex coacervation are very similar methods and sometimes used interchangeably as the only difference is use of an electrolyte (e.g. tripolyphosphate) in former and of an ionic polymer (e.g. alginate) in later, for gelation of chitosan.[253, 254] These nanoparticle manufacturing techniques have advantages of not requiring the use of organic solvents or homogenisation or heat, therefore minimizing possible degradation of active ingredient during the prepration process. Tri-polyphosphate (TPP) is a most widely investigated polyanion for formation of chitosan nanoparticles because of its non-toxic property and quick gelling ability. Many researchers have explored the capacity of chitosan–TPP nano-system for association of peptides, proteins, oligonucleotides, antigens and plasmids DNA for potential pharmaceutical usage.[255-257] Preparation methods by polyionic crosslink of cationic chitosan molecules with proteins, nucleic acids and genes were particularly useful for carrying and delivering macromolecules as therapeutic agents. Aside from its strong electrostatic interaction and binding with negatively charged proteins/genes, the chitosan–protein or chitosan–gene system has the ability to gel spontaneously on contact with multivalent polyanions due to the formation of inter- and intramolecular crosslinkage mediated by these polyanions. Additionally, incorporation of highly charged density polyanions onto chitosan nanoparticles can improve nanoparticle properties concerning protein association efficiency and modulation of active materials release. Other anionic crosslinkers reported to be useful in formation of chitosan nanoparticles include sodium sulfate, negatively charged cyclodextrins, poly-γ-glutamic acid and dextran sulfate.[55, 254, 258]

2.1.3. Dextran Sulfate

Dextran sulfate (DS) is a biodegradable and biocompatible polyanion with negatively charged sulfate groups (Figure 2). The polysaccharide itself is

expressed by bacteria, with most research focusing on dextran expressed from Leuconostoc mesenteroide. The structure of dextran consists of linear (1-6)-a-D-glucose, with branches extending mainly from (1-3) and occasionally from (1-4) or (1-2) positions accounting for a 5% degree of branching (Figure 2.2). Dextran is highly water soluble and easily functionalized through its reactive hydroxyl chemistries. Characterization of many types of dextran have indicated that branching, average molecular weight, and molecular weight distributions can vary widely depending on the conditions and strain of bacteria used for expression.[259, 260] Dextran was investigated as a blood plasma replacement in the early 1940s and has since become of interest as a biodegradable and biocompatible material. Biodegradation occurs through natural enzymatic splitting of saccharide bonds by dextran-1,6-glucosidase found in spleen, liver, lungs, kidneys, brain, and muscle tissue as well as by dextranases expressed by bacteria in the colon.[261, 262]

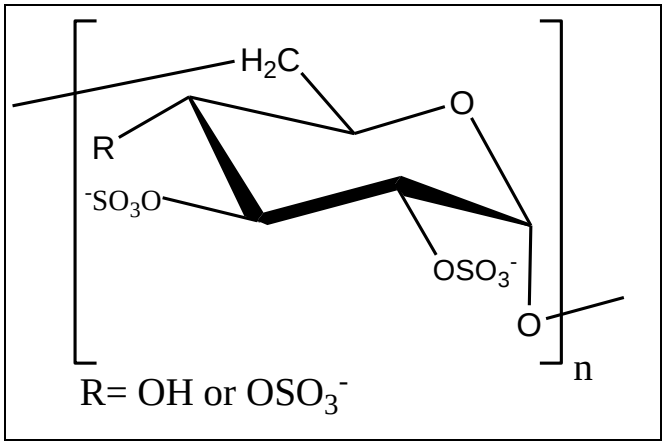


Figure 2.2. Chemical structure of the dextran sulfate (DS).

Dextran has been reported as an immunological adjuvant/carrier for antigens/vaccines in some studies[263-265]. Diwan et al[265] used the 'pre-formed' dextran microspheres for intramuscular administration of diphtheria toxoid to rats and reported that significantly higher antibody titers (24 times) were obtained against diphtheria toxoid compared to immunization with the conventional diphtheria toxoid absorbed on alum. They further reported that the immune response

was sustained for nine months, with gradual decline of antibody titers thereafter. In another study, the same group[266] reported that cross-linked 'pre-formed' dextran microspheres, containing tetanus toxoid induced serum antibody to tetanus toxoid for long periods, eliminating the need for additional booster doses. These studies indicate that dextran or its derivatives contain potential adjuvant properties for induction of strong humoral immune responses against a co-administered antigen.

Dextran sulfate is derived by esterification of dextran under mild conditions using sulfuric acid. Dextran sulfate has also been investigated for various biomedical and pharmaceutical applications. It has been used in the isolation of ribosomes and to release DNA from histone complexes. It can also promote hybridization of probes with DNA and complexes with fibrinogen. In-situ formation of hydrogels is appealing for biomaterial applications and therefore has been employed in the formation of dextran hydrogels via addition reactions. Hiemstra et al. have investigated gelation of vinyl sulfone-esterified dextrans, with degrees of substitution of 2-22, with bifunctional or four-arm PEG-SH.[267, 268] These materials have shown rapid gelation times and are also hydrolytically degradable via hydrolysis of the conjugated sulfone. The above dextran conjugated materials have been successfully investigated as controlled release delivery vehicles of indomethacin (a low molecular weight hydrophobic anti-inflammatory drug), bovine serum albumin, lysozyme, and immunoglobulin G.[268-270] The relatively low cost and availability of dextran as well as its hydroxyl functionality for chemical modification has increased the utilization of dextran in the field of polysaccharide polymer conjugates for biomaterials.

Dr. Yan Chen research group[57, 254] have previously reported preparation of chitosan nanoparticles by complex coacervation method using dextran sulfate as a cross-linking agent. The studies reported the application of chitosan-dextran sulfate nanonparticles as a potential protein and peptide delivery system. These *in-vitro* investigations found charge and weight ratio of chitosan to dextran sulfate as an important formulation factor affecting particle size, zeta potential, release profile and entrapment efficiency.

In summary, chitosan and dextran sulfate have the following properties which make them a potentially suitable ingredient for a nanoparticulate vaccine delivery system:

- Absorption enhancement and mucoadhesive nature of chitosan.
- Immunological adjuvant properties of chitosan and dextran.
- Biodegradability and non-toxic nature of chitosan and dextran sulfate.
- Large-scale availability of both polysaccharides at cheap cost.
- Aqueous solubility of both chitosan and dextran sulfate
- Formation of particles (micro- and nano-particles) by simple and mild physical methods.
- Availability of both chitosan and dextran sulfate in various molecular weights and possibility of preparation of wide range of derivatives.
- Being approved ingredient by regulatory bodies for one or more medical applications in human beings.

2.1.4. Objectives of this section

The principle objective of this section of the thesis was to prepare chitosan-dextran sulfate nanoparticles using a simple aqueous method and optimize the important formulation factors using particle size and zeta potential as evaluation parameters.

2.2. EXPERIMENTAL

2.2.1. Materials

Low molecular weight chitosan (MW ~ 150,000, 75-85% deacetylated, Catalogue no. 448869), dextran sulfate sodium salt (MW ~ 5000, Catalogue no.

31404), and acetic acid (purum, Catalogue no. 45740) were purchased from Sigma-Aldrich chemicals Ltd, Australia. All other chemicals used for experiments were of molecular biology grade and used as received unless otherwise specified. Purified water (Milli-Q water) was used in all preparations and procedures.

2.2.2. Methods

2.2.2.1. Preparation of nanoparticles

CS-DS nanoparticles were prepared using the method developed in our laboratory with some modifications.[57] In brief, a known weight of low molecular weight chitosan was dissolved in acetic acid and dextran sulfate was dissolved in purified sterile water to produce a CS solution and DS solution respectively. These solutions (CS and DS) were used for preparation of CS-DS nanoparticles by complex coacervation / ionic gelation method. Specifically, CS solution and DS solutions were mixed at room temperature under high speed magnetic stirring for 15 minutes to form nanoparticles as a result of ionic interactions between chitosan and dextran sulfate.

2.2.2.2. Particle size and zeta potential of nanoparticles

Particle size of nanoparticles in a formulation was determined using photon correlation spectroscopy using a Zetasizer 3000HS (Malvern Instruments, Malvern, Worcestershire, UK). Photon correlation spectroscopy uses the rate of change of light fluctuations to determine the size distribution of the particles scattering light. The measurements were performed at 25ºC with a detection angle of 90°, and the raw data were subsequently correlated to Z average mean size using a cumulative analysis by the Zetasizer 3000HS software package. Each sample was measured 10 times.

The zeta potential of particles was determined by laser Doppler anemometry using a Zetasizer 3000HS (Malvern Instruments, Malvern, Worcestershire, UK). The samples were injected into the flow cell chamber and

measurements were carried out at 25ºC. For each sample, the mean ± standard deviation (SD) of 3 repeat measurements was established.

2.2.2.3. pH and short-term storage stability study

The stability of developed nanoparticle formulations was assessed at different environmental pH using particle size and zeta potential as evaluation parameters. The pH of prepared CS-DS nanoparticulate formulations was adjusted to different pH values (3.7, 4.5, 5.5, 6.5, 7.5 and 10.0) by dropwise addition of 0.1M NaOH into prepared nanoparticle formulations. The pH adjusted nanoparticle formulations were then characterized for particle size and zeta potential using Zetasizer as explained in section 2.2.2.2. An optimum nanoparticle formulation was selected based on its pH stability and immunological rationales (explained in section 2.3.1 of this chapter) for all of further studies.

The short-term stability of optimized nanoparticulate formulation was evaluated for one week by measurement of particle size and zeta potential using the Zetasizer at specified time periods. The experimental data was statistically analyzed using Graphpad Prism software (v5 demo, GraphPad Software, Inc., CA, USA).

2.2.2.4. Characterisation of nanoparticles by electron microscopy

The morphology and size of optimized nanoparticulate formulation was characterized using focused ion beam field emission electron electron microscope (FESEM or FIBSEM) and transmission electron microscopy (TEM). For FIBSEM, a clean glass coverslip was taped over an aluminium SEM stub using carbon tape and a drop of sample (nanoparticle formulation) was spread on top of the glass coverslip. The sample was coated with platinum film using sputter coater device and micrographs/pictures were taken with a Zeiss Neon 40EsB FIBSEM (Carl Zeiss SMT AG, Germany). For TEM, a drop of sample was placed on top of the copper grids coated with carbon and allowed to air dry.

Electron micrographs were taken with a JEOL JEM2011 transmission electron microscope (Jeol Ltd, Japan).

2.3. RESULTS AND DISCUSSION

Empty CS-DS nanoparticles were prepared by complex coacervation / ionic gelation method which is a simple and aqueous based formulation technique. This nanoparticle preparation method is particularly useful for incorporation of proteins and peptides because of lack of use of organic solvents and high energy procedures which can cause degradation of most biological compounds. Chitosan with a pKa value of ~ 6.3 when dissolved in an acidic medium becomes polycationic due to protonation of primary amine functional groups in its structure.[257, 271] Dextran sulfate sodium salts provide polyanionic solutions of dextran sulfate (Figure 2.2) when dissolved in water as one of its pKa value is in negative and another one near 2.[272] These two oppositely charged polysaccharides can form nanoparticles on mixing by ionic cross-linking due to electrostatic interactions between oppositely charged ions. Tiyaboonchai et al[273] demonstrated the presence of spectral shifts in the sulfate and amine regions in the FT-IR spectra of CS-DS nanoparticles on comparison with FT-IR spectra of chitosan and dextran sulfate. The spectral shifts have been reported as confirmation of existence of an electrostatic interaction between the sulfate groups of dextran sulfate and the amine groups of chitosan.

The use of dextran sulfate as negatively charged polymer in formation of chitosan nanoparticles by complex coacervation / ionic gelation method has been reported to offer advantages. It has been reported that small sized chitosan-tripolyphosphate (CS-TPP) nanoparticles dissolve very quickly in low pH hydrochloric acid solution,[274] whereas CS-DS nanoparticles were stable in low pH.[275] It has been indicated that CS-DS nanoparticles have enhanced stability and increased mechanical strength compared with CS-TPP nanoparticles,[254, 276] probably because of the larger structure of dextran sulfate which provide stronger cross-linking with CS compared to TPP. Additionally, Tiyaboonchai et al[277] have studied that dextran sulfate is capable of

reducing the cationic charge-related cytotoxicity of polyethylenimine (PEI) nanoparticles *in vitro*.

2.3.1. Formulation characterization and optimization

The previous work done in our laboratory on CS-DS nanoparticles[254] and preliminary experiments helped in identifaction of important formulation factors to be optimized for obtaining a stable CS-DS nanoparticles formulation. For preliminary studies, particle size and zeta potential were used as characterizing parameters. Concentration of chitosan and dextran sulfate in solutions used to prepare nanoparticles affected the particle size of particles forming by ionic crosslinking / coacervation method. The particles in the range of nanometers (1-1000nm) were only obtained when low concentrations of chitosan and dextran sulfate (0.1% w/v - 0.25%w/v) were used to prepare nanoparticles. The 0.1%w/v concentration of chitosan and dextran sulfate solutions provided formulations having nanoparticles of less than 500nm size. These results are in agreement with previous similar studies[57, 257] and indicate that the size of coacervation nuclei formed during the preparation increases with increase in polymer concentration used. Therefore, for all further studies 0.1% w/v chitosan dissolved in 0.2% v/v acetic acid solution and 0.1% w/v dextran sulfate solution (DS) in purified water were used. The pH of CS and DS was recorded as 3.6 and 4.2 respectively. Another formulation factor identified as important for optimization of nanoparticle formulation was ratio of chitosan and dextran sulfate. Different ratios of volumes of chitosan and dextran sulfate solutions (each with a concentration of 0.1% w/v) were used to study the effect of chitosan and dextran sulfate ratio on the properties of formed CS-DS nanoparticles. Table 2.1 lists the weight and molar ratios of chitosan to dextran sulfate in various CS-DS nanoparticle formulations prepared.

Table 2.1. The ratio of chitosan to dextran sulfate in various CS-DS nanoparticle formulations prepared.

Weight ratio of chitosan to dextran sulfate	Molar Ratio of chitosan to dextran sulfate
1 : 3	1 : 90
1 : 2	1 : 60
1 : 1.5	1 : 45
1 : 1	1 : 30
1.5 : 1	1 : 20
2 : 1	1 : 15
3 : 1	1 : 10

Table 2.2 and Figure 2.3 depicts the effect of weight ratio of CS to DS on particle size and zeta potential of nanoparticles. The weight ratio of CS to DS decides the overall charge of chitosan-dextran sulfate nanoparticles formed with charge of nanoparticles being the charge of that dominant component. It indicates that despite the large difference in molecular weight of chitosan and dextran sulfate used for nanoparticle preparation, the charge to weight ratio is in similar range for chitosan and dextran sulfate. This is also supported by the formation of precipitation in case of formulation prepared with 1:1 weight ratio of CS to DS. The presence of a high magnitude of zeta potential on the nanoparticles increases the stability of the formulation, as charged particles are less likely to form agglomerates due to ionic

repulsive force.[66] However, it was noticed that the positively charged nanoparticles were significantly larger in size than negatively charged nanoparticles (Table 2.2) when size of nanoparticles with a particular CS:DS weight ratio was compared with size of nanoparticles with same DS:CS weight ratio (student t-test, $p < 0.01$). This might be because of the larger molecular size of chitosan compared to dextran sulfate and increased repulsion between positively charged amine groups of chitosan with increase in proportion of chitosan in formulation. This was previously reported that charge on macromolecules affect their contraction-extension/folding-unfolding which in turn affects their conformational structure and size.[257]

This study also showed that the order of mixing of chitosan and dextran sulfate solutions is important for preparation of a stable formulation i.e. the smaller volume solution should be added into larger volume solution to get stable formulation. Addition of larger volume component into smaller volume component during the preparation of nanoparticles resulted in visible precipitation instantaneously or within half an hour of preparation. This might have happened because of agglomeration of nanoparticles during charge inversion of nanoparticles in the process of preparation.

Schatz et al.[278] reported that the formation of polyelectrolyte complexes on mixing solutions of polyanions and polycations is spontaneous, entropy driven, and can lead to the formation of water-soluble complexes or precipitates, corresponding to two extreme cases. Schatz et al.[278] also compiled the important factors impacting the particle formation process; (a) the reactivity of two strong polyions lead to insoluble polyelectrolyte complexes and the interaction between strong and weak ions should be able to yield stable colloidal complexes, (b) the polymer concentrations in the reaction medium, (c) external parameters such as pH, ionic strength, order of mixing of reactants, and rate of mixing. Furthermore, it has been previously reported in literature[279, 280] that chitosan is a weak polybase and dextran sulfate is a strong polyacid. Drogoz et al.[281] investigated the various stages of the complexation process of chitosan and dextran sulfate and described that colloids were positively or negatively charged according to the nature of the polymer in

excess. The study conducted for this thesis also shows similar results and further confirms that nature of polymer in excess determines the charge of nanoparticles. Drogoz et al.[281] also described that the existence of various mechanisms of colloidal polyelectrolytes formation, according to the nature of the polymer in excess, could be attributed to the differences in chemical reactivity (strong versus low) of the ion in excess and the confirmation and flexibility of the macromolecular chains related to their electrostatic potential.

Table 2.2. The effect of ratio of CS to DS and order of mixing on particle size and zeta potential of CS-DS nanoparticle formulations.

Order of addition during preparation	Weight ratio of CS to DS	Visual observantion	pH of formulation	Particle size ± S.D (nm)	Polydispersity index ± S.D	Zeta potential ± S.D (mV)
CS (0.1%) into DS (0.1%)	1 : 3	Colloidal	3.9	165.7 ± 16.2	0.355 ± 0.064	-38.2 ± 5.2
CS (0.1%) into DS (0.1%)	1 : 2	Colloidal	3.8	161.8 ± 22.7	0.245 ± 0.149	-34.2 ± 2.1
CS (0.1%) into DS (0.1%)	1 : 1.5	Colloidal	3.8	158.6 ± 15.6	0.315 ± 0.148	-27.4 ± 1.6
DS (0.1%) into CS (0.1%)	1 : 1	Precipitation	n/a	n/a	n/a	n/a
DS (0.1%) into CS (0.1%)	1.5 : 1	Colloidal	3.8	258.0 ± 1.6	0.340 ± 0.014	39.1 ± 0.2
DS (0.1%) into CS (0.1%)	2 : 1	Colloidal	3.8	300.2 ± 17.2	0.253 ± 0.099	47.2 ± 0.2
DS (0.1%) into CS (0.1%)	3 : 1	Colloidal	3.7	314.7 ± 9.2	0.290 ± 0.212	53.2 ± 4.4

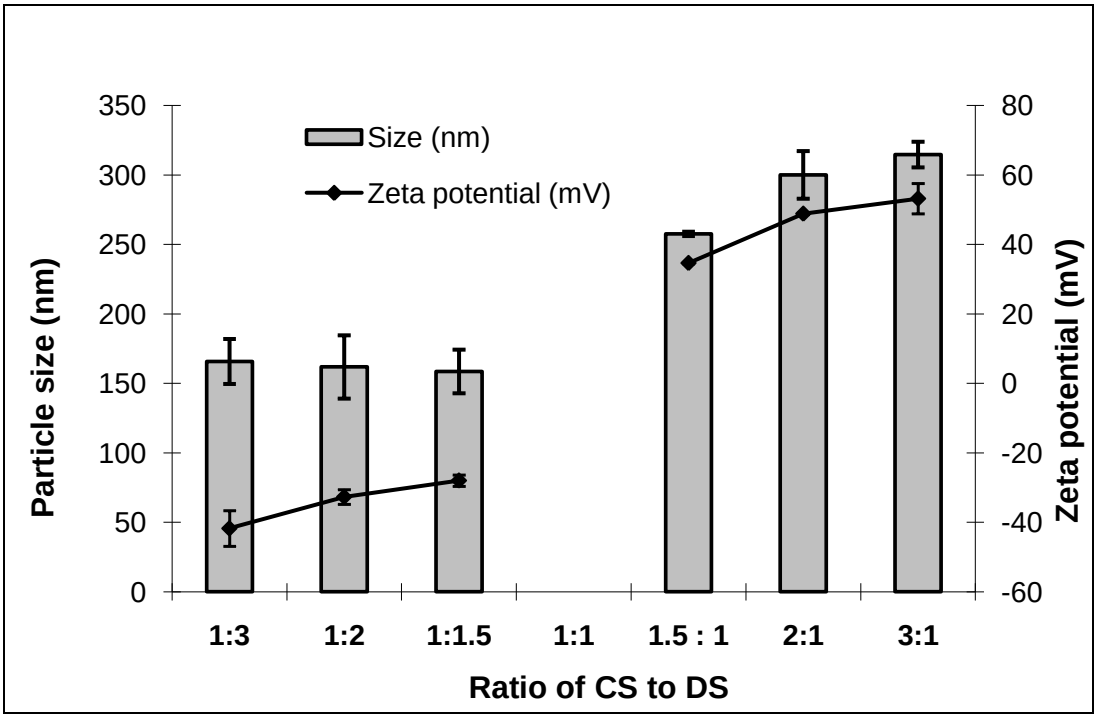


Figure 2.3. Effect of weight ratio of CS to DS on particle size and zeta potential of CS-DS nanoparticles.

To determine the isoelectric pH point of CS-DS nanoparticle formulations and to study the effect of pH change on particle size of CS-DS nanoparticles, the pH of nanoparticulate formulations was adjusted using 0.1M NaOH and the nanoparticles were characterized for size (Figure 2.4) and zeta potential (Figure 2.6). In broad sense, an increase in pH especially above pH 7 resulted in abrupt and steep increase in particle size of nanoparticles formulated with different weight ratios of CS to DS. It indicates the instability of CS-DS nanoparticles in basic pH environment. This instability of nanoparticles can be explained by neutralization of charge over nanoparticles with increase in pH (Figure 2.4) which increases the probability of agglomeration of nanoparticles as electrostatic repulsive force between nanoparticles was reduced. During the measurement of particle size of nanoparticle formulations using zetasizer higher variability was observed when the particle size exceeded 2000nm and accurate measurements could not be obtained (Figure 2.4).

This might be due to continuous formation of agglomerates and settling of precipitates in formulation. Also, different formulations showed precipitation or significant increase in particle size at different pH values. A correlation between initial zeta potential of nanoparticles and pH at which visible precipitation occurred, can be made. The nanoparticle formulations which showed initial higher zeta potential (positive or negative) produced visible precipitation at higher pH compared to those formulations which showed lower zeta potential (Table 2.2 and Figure 2.5). The isoelectric pH point for different CS-DS nanoparticle formulations prepared from different CS : DS weight ratios, was calculated assuming linear correlation between change in zeta potential of nanoparticles with change in pH of formulation (Table 2.3 and figure 2.7).

This study illustrated that size and zeta potential of CS-DS nanoparticles can be modulated by change in pH of residual environment. One has to point out that the increased particle size, as a consequence of pH change, is more a reflection of the aggregation of nanoparticles, rather than the change of the size of individual nanoparticles. Panyam et al. reported that by varying the surface charge, one could potentially be able to direct nanoparticles either to lysosomes or to cytoplasm. The research group suggested that nanoparticles which show transition in their surface charge from anionic at pH 7 to cationic in the acidic endosomal pH (pH 4-5) were found to escape the endosomal compartment whereas the nanoparticles which remain negatively charged at pH 4-5 were retained mostly in the endosomal compartment. Accordingly, easy modulation of particle size and zeta potential of nanoparticulate formulations could be important for induction of efficient *in-vivo* immune responses for vaccine delivery.

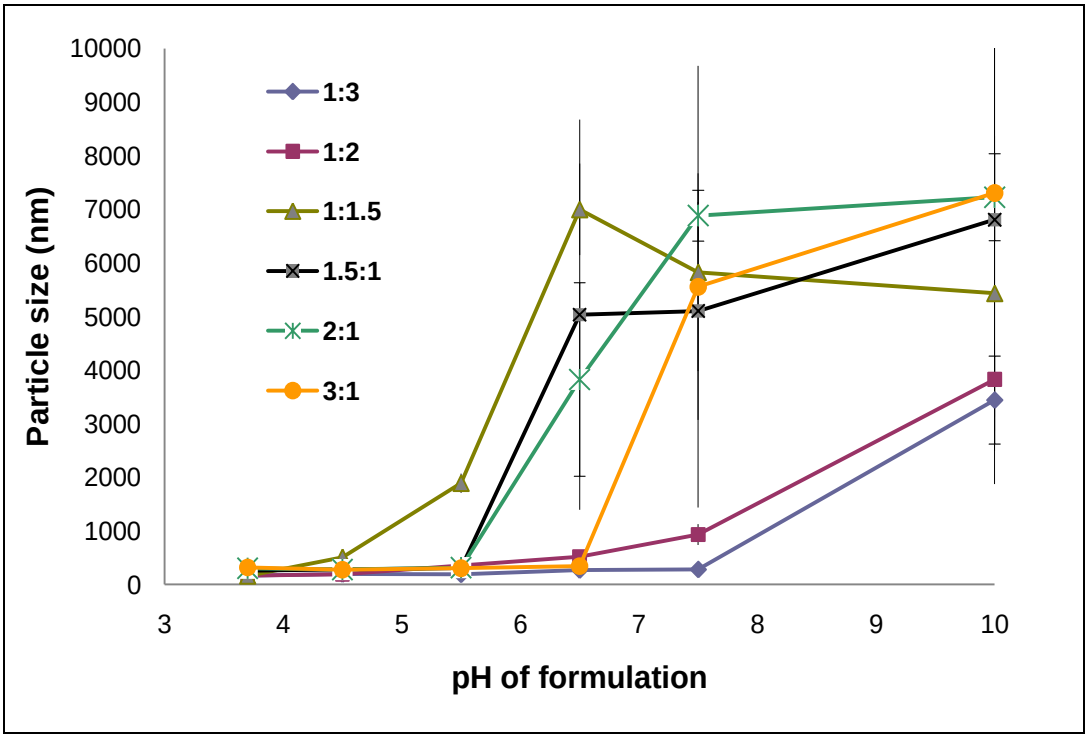


Figure 2.4. Effect of pH adjustment on particle size of CS-DS nanoparticles prepared from different CS to DS weight ratios at room temperature.

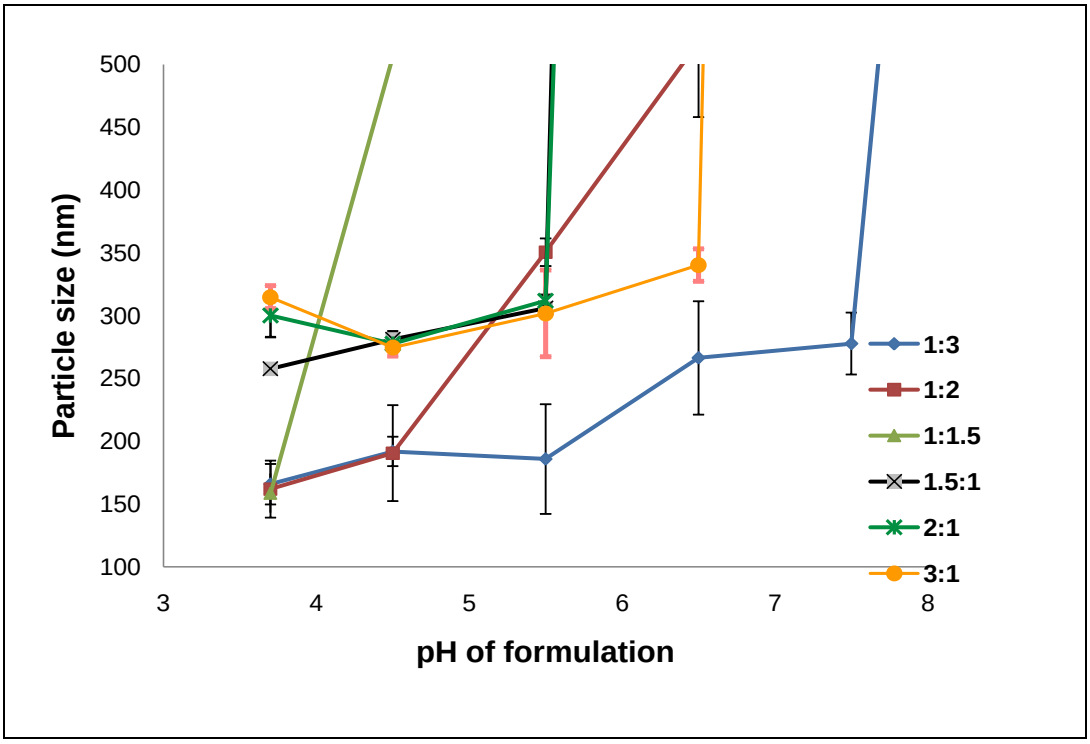


Figure 2.5. Effect of pH adjustment in range of pH 3.5 – 8.0 on particle size of CS-DS nanoparticles prepared from different weight ratios of CS:DS at room temperature (highlighting modulation of particle size with increase in pH before precipitation).

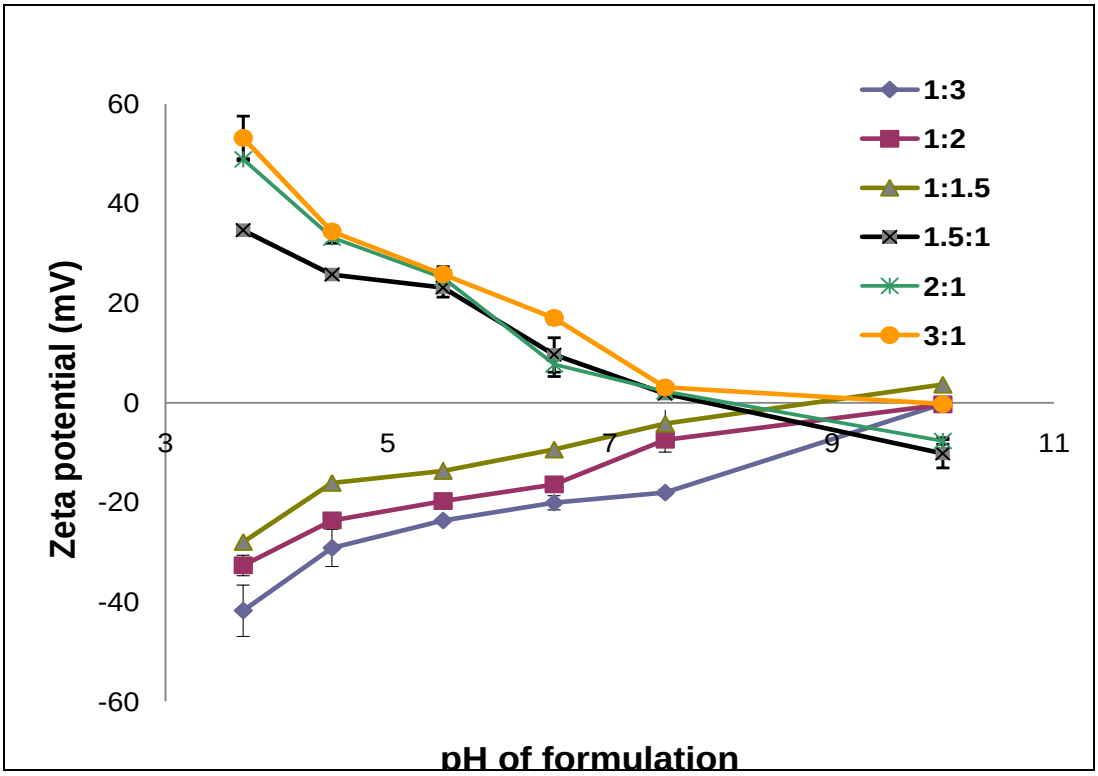


Figure 2.6. Effect of pH of final formulation on zeta potential of nanoparticles prepared from different weight ratios of CS:DS.

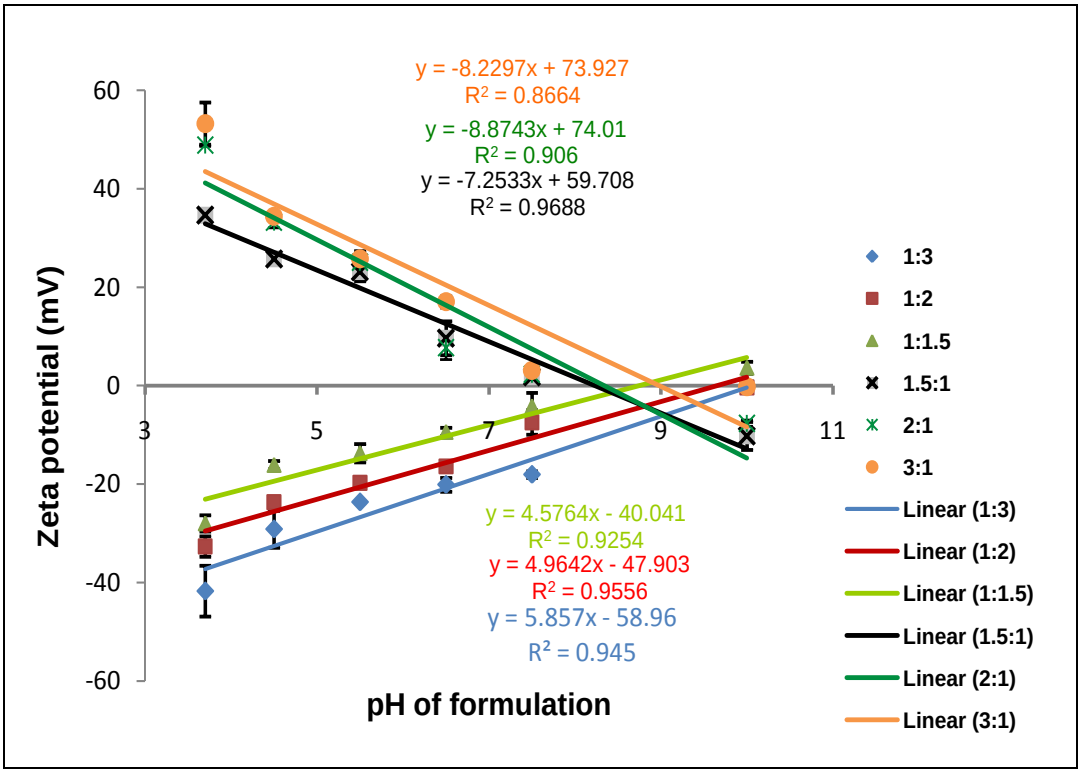


Figure 2.7. Determination of isoelectric pH point for CS-DS nanoparticle formulations prepared from different weight ratios of CS:DS, using linear model.

Table 2.3. Determination of isoelectric pH point for CS-DS nanoparticle formulations using linear model.

Formulation (CS:DS ratio)	Slope of linear trend line	Intercept of linear trend line	Coefficient of correlation (R)	Isoelectric point pH
1 : 3	5.858	-58.96	0.9723	10.06
1 : 2	4.964	-47.90	0.9775	9.64
1 : 1.5	4.576	-40.04	0.9620	8.75
1.5 : 1	-7.253	59.71	0.9843	8.23
2 : 1	-8.874	74.01	0.9518	8.34
3 : 1	-8.230	73.93	0.9308	8.98

Above results indicate that negatively charged CS-DS nanoparticles have smaller particle size and higher isoelectric pH point compared to their positively charged counter parts. These initial *in-vitro* studies suggested that negatively charged nanoparticle formulation prepared using 1: 3 ratio of CS and DS should be used as optimum and stable formulation for further *in-vitro* and *in-vivo* studies. However, considering the *in-vivo* situation, it is more likely that positively charged particles have higher possibility and efficiency to interact with negatively charged mucosal and epithelial cell surfaces. A high negative charge over the particle surface could reduce the interaction of particles with mucosal and epithelial cell (APCs and M-cell) surface as a result of ionic repulsive forces which can lead to possibility of decreased delivery of an incorporated antigen in delivery system to APCs.

Sogias et al.[282] elucidated the mechanisms of interactions between chitosan and mucins (mucus gels) and found that electrostatic attraction appears to be the major mechanism for CS mucoadhesion but is also accompanied by contributions from hydrogen bonding and hydrophobic effects. Sayin et al.[248] prepared both positively and negatively charged nanoparticles using chitosan or chitosan derivatives, CS and TMC for positively charged nanaoparticles and MCC for negatively charged nanoparticles. On intranasal administration of nanoparticle formulations, the group found that CS and TMC nanoparticles which have positively charged surfaces induced higher serum IgG titres when compared to those prepared with MCC which were negatively charged and smaller in size. Thiele et al. compared cationic, polyamine-coated polystyrene microparticles and anionic, protein-coated polystyrene microparticles with respect to their phagocytosis and phagosomal fate in dendritic cells and macrophages. The group concluded that hydrophilic, cationic microparticles were efficiently phagocytosed by dendritic cells and electrostatic attraction between the positively charged microparticle surface and the negatively charged cell surface is likely to mediate binding and subsequent internalization.

The above literature reports indicate that hydrophilic CS nanoparticle formulation with a positive surface charge has higher potential to be immunologically effective *in-vivo* than negatively charged chitosan nanoparticle formulations. Based on this rationale, a positively charged CS-DS nanoparticle formulation with higher isoelectric pH point of 8.9, prepared using 3:1weight ratio of CS and DS was selected for further studies. In addition to positive charge, higher isoelectric pH point provides wider pH range of stable formulation which could be important in characterization and *in-vivo* performance of delivery system.

2.3.2. Electron microscopy and stability study of selected CS-DS nanoparticle formulation

The morphology and size of selected nanoparticle formulation was studied using the focused ion field emission scanning electron microscopy (FIBSEM) and transmission electron microscopy (TEM). FIBSEM and TEM micrographs (Figure

2.8) confirmed the nanosize of CS-DS particles and show the particle size distribution of nanoparticles. Also, it was observed that all the nanoparticles were not of spherical shape and shape of nanoparticles irregularly varied along with surface morphology. The detailed study of the morphology and structure of CS-DS nanoparticles could not be performed by electron microscopy as the nanoparticles were unstable under high energy beam in microscopes and it was realised that nanoparticles structure (shape and size) was getting affected by internal conditions of microscope during the tenure of microscopy. This suggests that the CS-DS nanoparticles are not perfect solid bodies but more likely the CS-DS nanoparticles are either softer particles or partially / fully made up of gel-like structure when dispersed in water which gets affected under the exposure of high vacuum. The exposure of nanoparticles to high energy may have also affected the surface morphology. Although various research groups[249, 254, 283] have previously used simple SEM and TEM techniques similar to method used in this study, there are many recent publications[53, 284] reporting use of cryo-techniques for sample preparation before use of electron microscopy of chitosan nanoparticle formulations. It suggests that cryo-sample preparation techniques might be a better tool for stable microscopic study of chitosan nanoparticles. Unfortunately, formulations could not be studied by cryo-technique due to lack of facility and expertise in Western Australia.

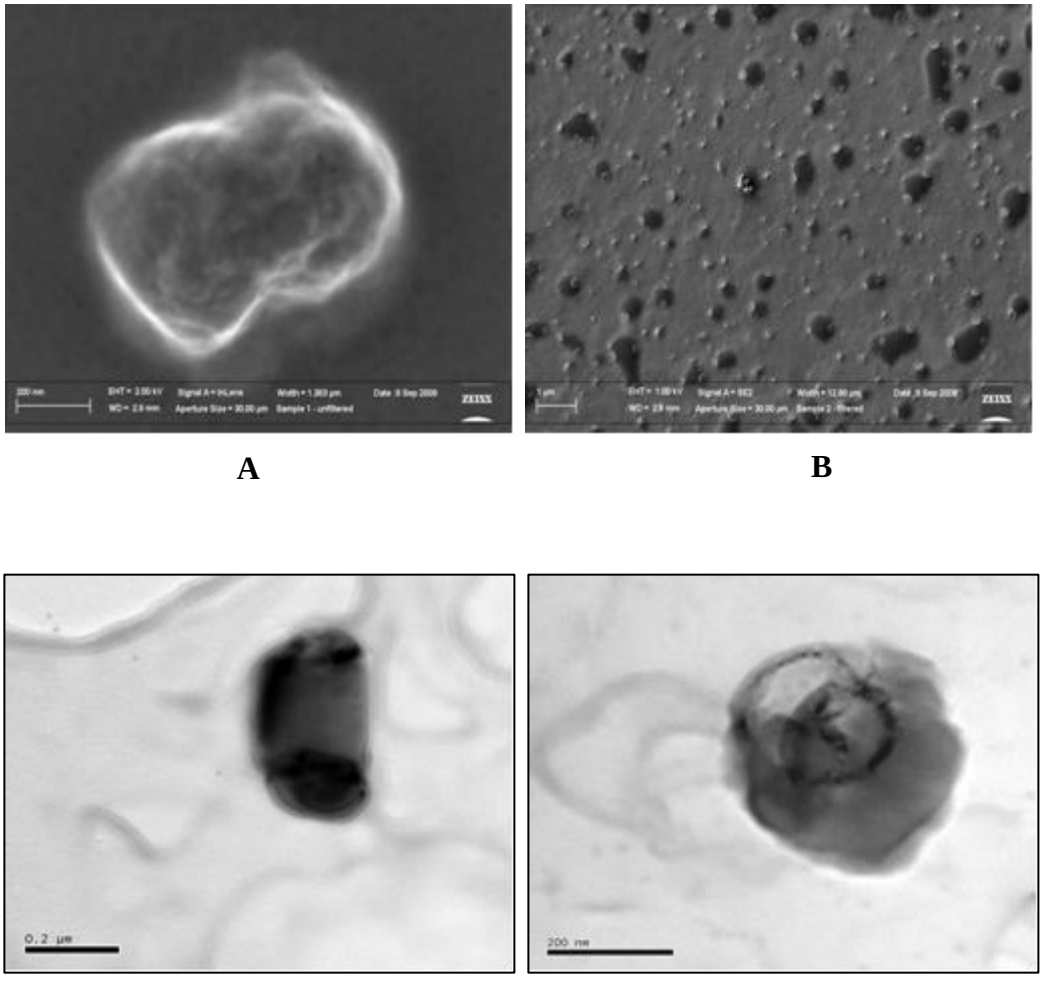


Figure 2.8. FIBSEM (A and B) and TEM (C and D) micrographs of CS-DS nanoparticle(s).

The short-term storage stability of selected CS-DS nanoparticle formulation was investigated at room temperature using particle size and zeta potential as evaluation parameters (Table 2.4). The particle size and zeta potential of selected CS-DS nanoparticle formulation didn't showed any significant change for 3 days (72 hours) but on seventh day a significant increase in particle size (One way ANOVA followed by post-Dunnet test, $p < 0.01$) and significant decrease in zeta potential (One way ANOVA followed by post-Dunnet test, $p < 0.05$) of nanoparticles were

recorded (Table 2.4). The increase in particle size with corresponding decrease in zeta potential indicates agglomeration of nanoparticles. As the formulation was neither sterile nor preserved, it was expected that the microorganism activities might have taken place which could also contribute to the instability of nanoparticles. It can be interpreted on the basis of data obtained from short term stability study that the vaccine formulation should be prepared fresh for all further studies and any unused formulation should be discarded after 48 hours of preparation. The long-term stability in aqueous environment and effect of temperature on the stability of formulation was not studied assuming that any long-term storage of vaccine formulation would be in freeze-dried form. The development and stability study of lyophilisation of CS-DS nanoparticle formulation were not undertaken as part of this thesis work and could be investigated in the future.

Table 2.4. Short-term stability study of CS-DS nanoparticulate formulation.

Time (days)	**Particle size ± S.D (nm)**	**Polydispersity index ± S.D**	**Zeta potential ± S.D (mV)**
0	314.7 ± 9.2*	0.29 ± 0.21	53.2 ± 4.4**
1	320.8 ± 4.1	0.30 ± 0.16	51.2 ± 5.4
3	334.2 ± 8.5	0.28 ± 0.21	45.8 ± 5.8
7	358.0 ± 13.5*	0.51 ± 0.22	36.9 ± 8.8**

*Means are significantly different ($p < 0.05$), **Means are significantly different ($p < 0.01$).

To conclude, development and optimization of an empty CS-DS nanoparticle formulation was undertaken which can be used as potential vaccine delivery system for further *in-vitro* and *in-vivo* studies.The ratio of chitosan to dextran sulfate, order of mixing and pH of nanoparticle suspension were identified as important formulation factors governing size and zeta potential of CS-DS nanoparticles. These formulation factors could be utilized for manipulation of particle size and surface charge of CS-DS nanoparticles. An optimized blank CS-DS nanoparticle formulation with particle size 314.7 ± 9.2 nm, zeta potential +53.2 ± 4.4 mV and prepared with CS to DS weight ratio of 3:1 was found stable for atleast 24 hours in aqueous environment without any significant change in particle size and zeta potential. The particle size range of opitimized empty CS-DS nanoparticles was confirmed using electron microscopy techniques but shape and surface properties of nanoparticles could not be completely evaluated as the simple electron microscopy techniques were found unsuitable.

CHAPTER 3

PREPARATION AND *IN-VITRO* CHARACTERIZATION OF THE CS-DS NANOPARTICLE FORMULATION LOADED WITH A MODEL ANTIGEN AND/OR POTENTIAL ADJUVANT / TARGETING LIGAND

3.1. INTRODUCTION

The current trend in new vaccine development is directed towards the use of antigenic subunits or highly purified recombinant molecules with better immunogenicity/risk ratio for the development of effective vaccines, and away from the use of killed or attenuated pathogens.[285-287] The live vaccine systems (killed or attenuated pathogens) have been reported to be associated with adverse reactions in a significant percentage of vaccines. These can range from simple headache to encephalitis, intussusception and even death.[288] Alternatively, highly purified recombinant molecules or synthetic peptides or sub-units of pathogens are recognized for offering the best safety profile. However, these safer antigens/vaccines lack strong immunogenicity and may require co-administration of immunological adjuvants and multiple administration for complete protection.[287] Various adjuvants or strategies have been investigated as formulation components of vaccines to enhance the induction of immune responses against safer antigens. It is now generally accepted that for induction of a strong immunological response via both antigen-presentation pathways (MHC-I and MHC-II) against the antigens may require combination of various components/strategies such as use of particulate vaccine delivery systems, incorporation of safe immunological adjuvants in vaccine formulation and development of targeted vaccine formulations.

3.1.1. Selection of pertussis toxin (PTX) as a model protein antigen

Pertussis (whooping cough) is a prevalent cause of infant death worldwide and continues to be a public health concern especially in developing countries. Estimates from the World Health Organisation (WHO) suggest that, in 2003, about 17.6 million cases of pertussis occurred worldwide and that about 279,000 patients died from this disease.[289] *Bordetella pertussis*, the most common causative agent of pertussis, is a Gram-negative coccobacillus with exclusive affinity for the mucosal layers of the human respiratory tract. *B. pertussis* has evolved various mechanisms, known as virulence factors, for attachment, invasion and survival within eukaryotic cells such as epithelial and several phagocytic cells[290-293]. The important virulence factors which are considered to play a key role in the establishment of B. pertussis

infection are filamentous hemagglutinin (FHA), pertussis toxin (PTX), adenylate cyclase toxin (AC-Hly) and pertactin (P69). In addition to these key factors other factors such as fimbriae, dermonecrotoxin, tracheal cytotoxin, tracheal colonization factor, serum resistance factor and lipopolysaccharide also play roles leading to clinical manifestations of pertussis.[294, 295] PTX acts as both an adhesion and a toxin therefore it is considered to be a major virulence factor of *B. pertussis*. Margaret Pittman introduced the term "Pertussis toxin" which is also known as lymphocytosis-promoting factor, islet-activating factor and pertussigen. PTX (MW 105,060) is exotoxin composed of noncovalently associated subunits designated S-1, S-2, S-3, S-4 and S-5 with M_rs of 26220, 21920, 21860, 12060 and 10940, respectively.[296, 297] The toxin contains one copy of each subunit except S-4, which is present in two copies. PTX plays an important role in the pathogenesis of whooping cough that might be used by *B. pertussis* to evade and impair the host immune and cells surface functions *in vivo* [294, 298]. Inactivated pertussis toxin commonly known as pertussis toxoid (PTXd) is an important and essential component of commercial acellular DTaP (Diphtheria-Tetanus-acellular Pertussis) vaccine.

Recent reports from the WHO and independent research conclude that protection following pertussis vaccination wanes after 6–12 years [289]. As a result, symptomatic as well as asymptomatic pertussis is increasingly reported in older children, adolescents and adults even in communities with high immunization coverage [289]. Many studies have highlighted the multiple causes for the resurgence of the disease, such as use of better diagnostic tools, different effectiveness of the vaccines used, differences in vaccine coverage and loss of vaccine efficacy due to the emergence of new *B. pertussis* strains overproducing PTX.[299-302] It has also been previously reported that conventional adjuvants such as aluminium compounds failed to augment vaccines performances against whooping cough in some cases.[181, 303] The background importance of PTXd in the pertussis vaccine along with the resurgence of disease in highly immunized countries lead to the selection of PTXd as a model protein antigen for this thesis studies.

3.1.2. Selection of IgA as an immunological adjuvant with M cell targeting potential

The introduction of immunological adjuvants in chapter 1 highlights the importance of investigations leading to development of novel immunological adjuvants for incorporation in vaccine formulations. Secretory immunoglobulin-A (SIgA) is essential in protecting mucosal surfaces by ensuring immune exclusion. SIgA exhibits the striking feature to adhere to the apical membrane of M-cells[304, 305], promoting uptake of proportion of SIgA as reflected by the detection of gold-coated SIgA in the pocket of M-cells[306]. Further study of the interaction of M-cells with SIgA molecules demonstrated that IgA, with or without bound secretory component, but not IgG or IgM, bound selectively to murine and human M-cells.[307] Adherence of IgA to M-cells required Cα1 and Cα2, suggesting that no known IgA receptor was involved in the interaction process.[308] Recent studies indicated that SIgA exert the immunomodulatory and weak mucosal immunostimulant effects after being transported through M-cells of the Peyer's patch.[308, 309] In a rather isolated study, Zhou et al[310] prepared and evaluated IgA coated liposomes for rectal administration of ferritin antigen. They also compared M-cell targeting efficiency of IgA coated liposomes in Peyer patch tissues with uncoated liposomes. They concluded that IgA can significantly enhance the local secretory immune response to antigen in liposomes, apparently by increasing liposome uptake by at least 10 times via M cells.

The studies on transportation of SIgA through M-cells, as well its immunological role and use of IgA as a potential M-cell targeting agent in some preliminary studies signify the potential of IgA as a novel immunological adjuvant. The endogenous nature of IgA, readily availability of antibody from industry, non-pathogen origin and lack of inter-species variation in interaction between IgA and M-cells are some of the advantages IgA can offer as a vaccine formulation component. The conclusions of the studies highlighted above regarding IgA and lack of studies exploring IgA's suitability as an immunological adjuvant lead to the selection of IgA as a potential immunological adjuvant and M-cell targeting agent in this study.

3.1.3. Objectives of this section

The broad objective of this part of research project was the development of an effective method for incorporation of PTXd and/or mouse IgA into optimized CS-DS nanoparticle formulation and characterization of these loaded formulations for *in-vitro* parameters.

3.2. EXPERIMENTAL

3.2.1. Materials

Mouse IgA-Kappa from murine melanoma (Catalog no. M1421), 3,3',5,5'-tetramethylbenzidine (TMB-2 liquid substrate system, Catalog no. T8665), tris bufferd saline powder (Catalog no. T6664), phosphate buffered saline (Catalog no. P4417), glutaraldehyde grade I (8% in water, catalog no. G7526) tween-20 (Catalog no. P9416), bovine serum albumin (Catalog no. A3294), L-lysine (Catalog no. W384704), glycerol (Catalog no. 49767), and carbonate-bicarbonate buffer capsules (Catalog no. C3041) were purchased from Sigma-Aldrich Ltd., Australia. Ham's F12 nutrient mixture (Catalog no. 11765-054), fetal calf serum, certified (Catalog no. 16000-036), trypsin-EDTA (Catalog no. 25200-056) and Opti-MEM-I reduced medium (Catalog no. 31985-062) were purchased from Invitrogen Australasia Pty Ltd, Australia. Lyophilized pertussis toxin (List Biological Laboratories Ltd., USA, Catalog no.181) was purchased from Sapphire Biosciences, Australia. All chemicals used for experiments were of molecular biology grade and used as received unless otherwise specified.

Female Balb/c mice aged between 6-8 weeks were purchased from Animal Resource Center, Perth, Australia. All the animal purchase and studies were duly reviewed and approved by the Animal Ethics Committee of Curtin University of Technology.

3.2.2. Methods

3.2.2.1. Estimation of IgA

Mouse IgA ELISA Quantitation kit (Catalog no. E90-103, Bethyl Laboratories, USA) was used for estimation of IgA for determination of loading and release of IgA from nanoparticles. Flat-bottom microtiter plates (Catalog no. 442402, Nunc F96 MicroWell™ Plates, Thermo Scientific, Nunc A/S, Denmark) were coated with 10 µg/mL goat anti-mouse IgA affinity-purified antibody solution in carbonate-bicarbonate pH 9.6 coating buffer and incubated for 60 minutes at 25 ± 2ºC. Plates were washed 4 times with wash buffer (Appendix 7.1) and each well was blocked with 300 µL of blocking buffer (Appendix 7.1) for 30 minutes at 25 ± 2ºC. Plates were washed 4 times with wash buffer, 100 µL of test sample or standard was added per well, and incubated for 60 minutes at 25 ± 2ºC. Plates were washed 5 times with wash buffer and 100 µl of HRP-labeled goat anti-mouse IgA antibody solution in dilution buffer (0.02 µg/mL) was added to each well and incubated for 60 minutes at 25 ± 2ºC. Plates were washed 5 times, developed for 30 min at 25 ± 2ºC with HRP substrate (3,3',5,5'-tetramethylbenzidine), stopped with 1M HCl, and read at 450 nm with a microplate reader (Victor^{3}V™ Wallac 1420 multilabel counter, PerkinElmer life and analytical sciences, Turku, Finland). Formula and preparation method of different buffers used in ELISA are included in Appendix 7.1. A standard curve was plotted and best fit model was derived using Graphpad Prism statistical software (v5 demo, Graphpad Inc., USA). The accuracy and precision of ELISA assay were determined from four 400 ng/mL IgA samples. Precision was calculated as percent relative standard deviation (RSD) and accuracy was calculated as percent ratio of measured experimental IgA concentration to theoretical expected IgA concentration (400 ng/mL). Analytical sensitivity (detection limit) for ELISA method[311] of IgA quantification was calculated using the formula [average absorbance of the blank + 3 x (Standard Deviation for blank)]. Blank represents antigen and antibody free wells in the ELISA run.

3.2.2.2. Detoxification of PTX

PTX was detoxified before its entrapment into nanoparticles using modification of method previously described by Munoz et al[312]. In brief, a solution of purified PTX was made in 20 mM sodium phosphate containing 0.5 M NaCl (pH 7.6). A known volume of 0.2% w/v glutaraldehyde solution (made in the same buffer) was added to bring the final concentration of glutaraldehyde to 0.05% w/v. The mixture was incubated at room temperature for 2 h, and then a known volume of 0.2 M L-lysine solution (made in same buffer) was added to bring the final concentration of L-lysine to 0.02 M. The mixture was incubated for 2 h at room temperature and then dialyzed for 2 days against 250 mL of 20mM sodium phosphate buffer containing 0.5 M sodium chloride. To incorporate PTXd in CS-DS nanoparticle formulation, the buffer was replaced with sterile water and volume was reduced using 10,000 molecular weight cut-off (MWCO) membrane centrifugal filtration unit (Microsep, Pall Life Science, Australia).

Detoxification of PTX was evaluated by Chinese Hamester Ovary (CHO) cell assay.[313] In brief, confluent flasks of CHO cells were trypsinized and diluted in Ham's F12 nutrient mixture supplemented with 5% v/v fetal calf serum (FCS). A cell suspension containing 5 X 10^5 of CHO cells was then added to each well of a flat-bottom 9-well tissue culture plate. PTX and PTXd in Opti-MEM-I reduced medium (final concentrations in the volume of 2 mL was 0.07 µg/mL and 2 µg/mL) were added, and clustering of cells was evaluated at 20 hours. The phosphate buffer saline was used as negative control for comparison. The test was conducted in triplicate and photographs were captured at the end of test (20 hours) using Nikon inverted microscope. General details of cell-culture techniques and cell counting method are compiled in Appendix 7.2.

3.2.2.3. Quantification of PTXd using protein estimation kit

A fluorescent protein-probe based estimation method (Quant-iT™ protein assay kit, Catalog no. Q33210, Invitrogen, USA) was used for initial

quantification of PTXd in solution obtained from detoxification procedure of PTX. A standard curve was prepared using bovine serum albumin as the model protein and all the assays were performed in triplicate. In brief, 200µl of working Quant-iT reagent solution was added in triplicate in individual wells of 96-well microplate followed by addition of 10µl of sample in each well. Flourescence was measured using a microplate reader (Victor^{3}V™ Wallac 1420 multilabel counter, PerkinElmer life and analytical sciences, Finland) at excitation wavelength at 470 nm and emission wavelength at 590 nm. The accuracy and precision of assay were determined from four 30 µg/mL PTXd samples. Precision was calculated as percent relative standard deviation (RSD) and accuracy was calculated as percent ratio of measured experimental PTXd concentration to theoretical expected PTXd concentration (30 µg/mL). Analytical sensitivity (detection limit) for Quant-iT method of PTXd quantification was calculated using the formula [average flourescence of the blank + 3 x (Standard Deviation for blank)]. Blank represents sample free wells in the plate.

The protein estimation kits only provide a non-specific method for protein estimation as the quantification is based on the reaction between common functional groups present in protein structure and the reagent. Therefore, these kits cannot be used when two or more proteins (e.g. PTXd and IgA) are present in any solution. This limitation of protein estimation kit prohibited the use this method for estimation of PTXd in entrapment efficiency and release studies. To solve this problem to the development of an ELISA method for PTXd was tried as described below. For comparison, PTX was also used along with PTXd in development of ELISA method.

3.2.2.4. Estimation of PTXd and PTX by ELISA method

The estimation of PTXd and PTX was conducted in three steps which are briefly described here,

3.2.2.4.1. Production of hyper-immunized anti-PTXd serum in Balb/c mice

Twenty female BALB/c mice (Animal Resource Center, Perth, Australia) aged 8-10 weeks were immunized by the subcutaneous (s.c.) route with volume of 125 μl per mouse. Mice received four immunizations respectively at day 0 with 2 μg, at day 10 with 4 μg and at day 21 and day 28 with 6 μg of PTX adsorbed on alum (Imject® alum, Catalog no. 77161, Pierce Biotec. Ltd, USA). Blood was taken from each animal one week after the last immunizaiton dose using cardiac puncture technique under deep anaesthesia (Intraperitoneal, ketamine/xylazine combination) and all the animals were then sacrificed by cervical dislocation. The animal study protocol was reviewed and approved by the Animal Ethics Committee, Curtin University of Technology, Perth, Australia.

3.2.2.4.2. Purification of IgG antibodies from hyper-immunized serum and conjugation of purified IgG with horseradish peroxidase (HRP)

Protein-G IgG purification kit (Catalog no. 89926, Pierce Biotec. Ltd, USA) was used for purification of IgG antibodies from serum collected from hyper-immunized mice. Protein G column was equilibrated with about five gel-bed volumes of IgG binding buffer followed by addition of diluted serum sample to the immobilized protein G column. The column was again washed with about five gel-bed volume of IgG binding buffer and purified antibodies were then eluted and collected using gentle Ag/Ab elution buffer (Catalog no. 21027, Pierce biotec. Ltd, USA). Elution of antibody was monitored using UV absorbance at 280nm (UV-Vis spectrophotometer, Hewlett-Packard instruments, USA). Eluted antibody solution was dialyzed against 0.1M carbonate-bicarbonate buffer and stored at -20°C in aliquots. The flow rate through column was maintained around 1 mL/minute during all steps.

For conjugation of HRP with IgG, SureLINK® HRP conjugation kit (Catalog no. 840003, KPL Inc., USA) was used. In short, 150 µL of 1 mg/mL antibody solution was mixed with activated HRP for 1 hour at room temperature followed by addition of 10 µL of reducing agent and incubation for 15 minutes at room temperature. Equal volume of storage buffer was then added in reaction vial and incubation at room temperature was provided for 15 minutes. HRP-conjugated antibodies were stored at -20°C in aliquots.

3.2.2.4.3. Sandwich ELISA method

Sandwich ELISA method similar to ELISA method used for estimation of mouse IgA (kappa) was used to estimate PTXd (and PTX) for determination of entrapment efficiency and release profile with the exception of using purified anti-PTXd IgG for coating and anti-PTXd IgG conjugated with HRP as secondary antibody. Formula and preparation method of different buffers used in ELISA are included in Appendix 7.1.

The sandwich ELISA method developed only provided an accurate and reproducible estimation method for PTX and not for PTXd (explained in section 3.3.3). This led to the use of PTX in place of PTXd for entrapment efficiency and release studies.

3.2.2.5. Preparation of IgA and PTX loaded CS-DS nanoparticles

CS-DS nanoparticles containing mouse IgA and/or PTXd were prepared by adding 105 µl of PTXd and/or mouse IgA solution (containing 140µg of IgA or PTXd or IgA and PTXd each) into 105 µl chitosan solution (0.2% w/v in 0.4% v/v acetic acid solution) followed by dropwise addition of 70µl of dextran sulfate solution (0.1% w/v in milli-Q water) with high speed magnetic stirring at room temperature.

For entrapment efficiency and release profile studies PTX was used in place of PTXd due to lack of accurate and reproducible method of estimation for PTXd.

3.2.2.6. Particle size and zeta potential of nanoparticles

Particles size of nanoparticles in a formulation was determined using photon correlation spectroscopy using a Zetasizer 3000HS (Malvern Instruments, Malvern, Worcestershire, UK). The measurements were performed at 25ºC with a detection angle of 90°, and the raw data were subsequently correlated to Z average mean size using a cumulative analysis by the Zetasizer 3000HS software package. Each sample was measured 10 times.

The zeta potential of particles was determined by laser Doppler anemometry using a Zetasizer 3000HS (Malvern Instruments, Malvern, Worcestershire, UK). The samples were injected into the flow cell chamber and measurements were carried out at 25ºC. For each sample, the mean ± SD of 3 repeat measurements was established.

3.2.2.7. Entrapment efficiency of PTX and/or IgA in nanoparticles

The quantity of PTX and/or IgA entrapped in the nanoparticles was calculated by the difference between the total quantity of protein added during the nanoparticle formation and the quantity of non-entrapped protein remaining in the aqueous filtrate. The latter was determined following the separation of protein-loaded nanoparticles from the aqueous medium by centrifugal-filtration using filtration units (Vivaspin® 500, Catalog no. KH-36228-12, Cole-Parmer, USA) fitted with membrane of one million Dalton cut-off at 2000 x g at 15 °C for 10 minutes. The particles were washed 3 times and filtration was repeated in between each washing. The PTX and/or IgA entrapment efficiency were calculated from the following equation (3.1):

$$\%\ Entrapment\ efficiency = \frac{Quantity\ added - Nonentrapped\ quantity}{Quantity\ added} \times 100 \qquad (3.1)$$

3.2.2.8. *In-vitro* release study of PTX and/or IgA from nanoparticles

The purpose of this study was to estimate the quantity of PTX which potentially could leak out of nanoparticles during the storage period. In addition, the release study may also help in understanding the corelation between pharmaceutical aspects of nanoparticulate vaccine delivery and induction of immune responses *in-vivo*. The CS-DS nanoparticles containing 10 µg of entrapped PTX and/or IgA (obtained from entrapment efficiency study as filtrand nanoparticles) were incubated with 2 mL of acetate buffer pH 5.1 medium. An acetate buffer acidic medium was used in release study to avoid agglomeration of CS-DS nanoparticles which could occur at basic pH as explained in chapter 2. The release studies were conducted at maintained temperature of 37°C. At pre-determined time intervals, the 100 µl samples were withdrawn and centrifuged at 16000 x g over 10 µl of glycerol bed to collect the supernatant for analysis. Glycerol bed has been previously used in studies[314] during centrifugation for effective separation of nanoparticles from released ingredients. The 100 µl of fresh release medium was used to replace the sample volume collected from release medium. Results are shown as the distributive percentage of PTX or IgA released with respect to the total associated protein.

3.2.2.9. SDS-polyacrylamide gel electrophoretic (PAGE) analysis of PTXd or IgA – loaded CS-DS nanoparticles

The stability of PTXd and IgA in nanoparticles was investigated using SDS-PAGE analysis. The electrophoresis was performed with gels composed of 10% acrylamide, cast and run in Tris–glycine buffer. The sample bands were stained using silver nitrate staining method.[315] All the samples were diluted with reducing sample buffer and diluted samples were heat treated for 5 minutes. 20 µl of each diluted and heat treated sample (each containing 2 µg of PTXd or IgA) was loaded for electrophoretic analysis. The Gel electrophoresis was performed using a Mini-PROTEAN 2D electrophoresis system (Bio-Rad Laboratories, Inc., USA) at voltage of 75 V for initial 15 minutes followed by 200 V for next 45 minutes. After electrophoresis, the pictures of gel were taken

using a digital camera (Sony Inc., Japan). The composition and preparation of SDS-PAGE gel mixtures are included in Appendix 7.3.

3.2.2.10. Statistical analysis

The raw data was processed to calculate mean with standard deviation using Microsoft excel 2007 (Microsoft Inc., USA). The experimental data was statistically analyzed using Graphpad Prism statistical software **(**v5 demo, **GraphPad Software Inc., CA, USA).**

3.3. RESULTS AND DISCUSSION

3.3.1. Estimation of IgA

Mouse IgA was quantified by sandwich ELISA method using a mouse IgA estimation kit (Catalog no. E90-103, Bethyl laboratories, USA). Mouse IgA solutions of known concentration were used to prepare calibration/standard curve. A non-linear correlation between concentration of IgA solutions and absorbance was observed in concentration range of 15.6 – 1000 ng/mL (Figure 3.1). The sigmoidal model - variable slope (equation 3.2) was selected as the best curve fit model for protein quantification on the basis of least sum of squares obtained for y-values and confidence intervals for each parameter value (Figure 3.2).

$$Y = \left[\frac{A-D}{1+\left(\frac{X}{C}\right)^{B}}\right] + D \qquad (3.2)$$

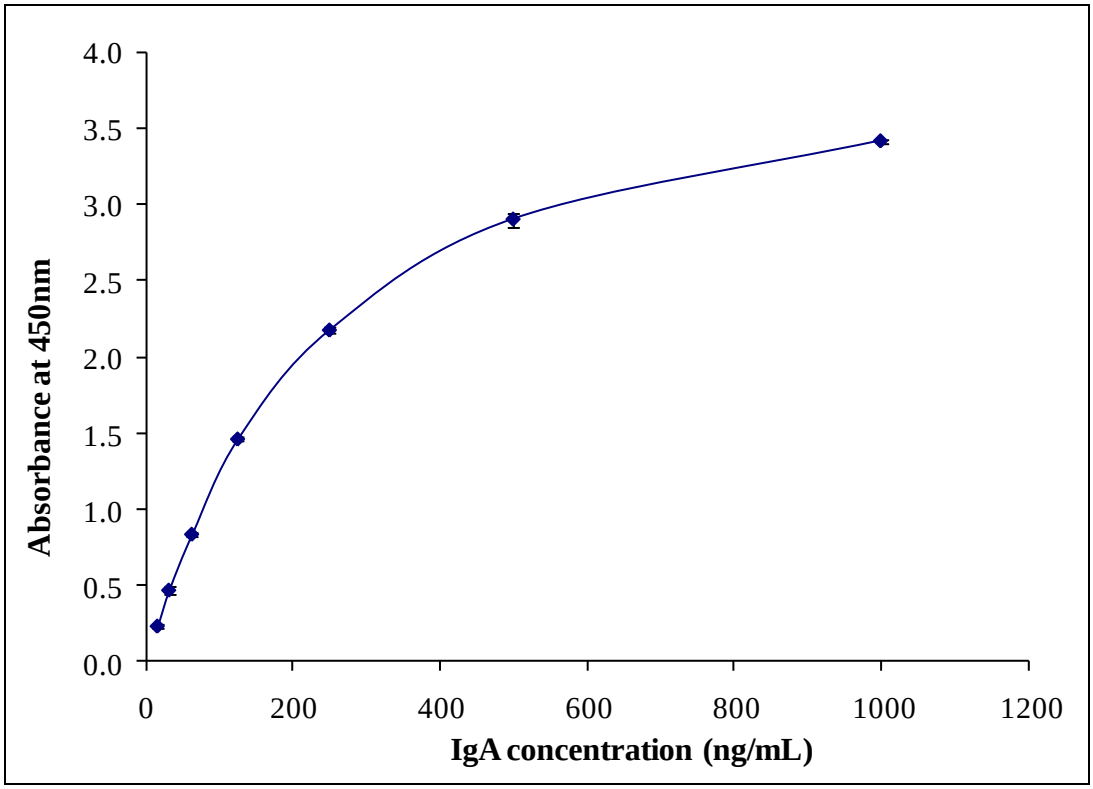


Figure 3.1. Standard (calibration) curve for IgA estimation using ELISA method.

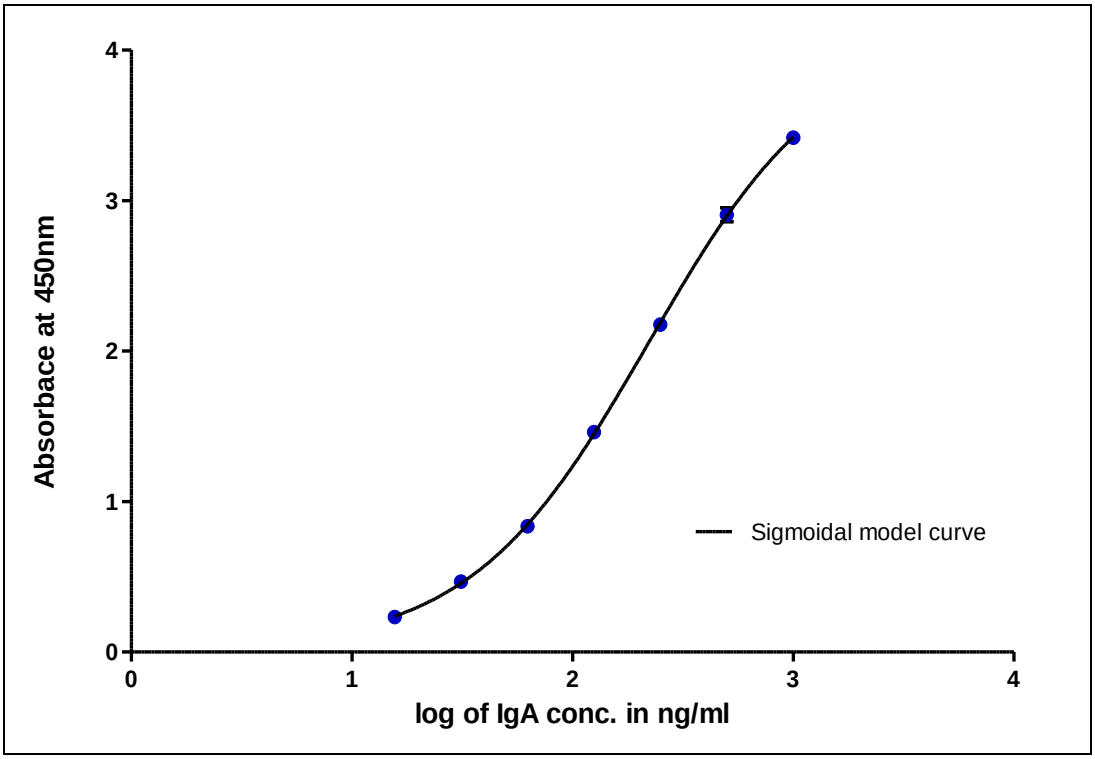


Figure 3.2. Log scale calibration curve for IgA estimation using ELISA method.

Statistical and analytical parameters for the estimation of IgA by ELISA method were determined (Table 3.1) and used for further calculations in in-vitro characterization studies.

Table 3.1. Statistical and analytical parameters for IgA estimation by ELISA method.

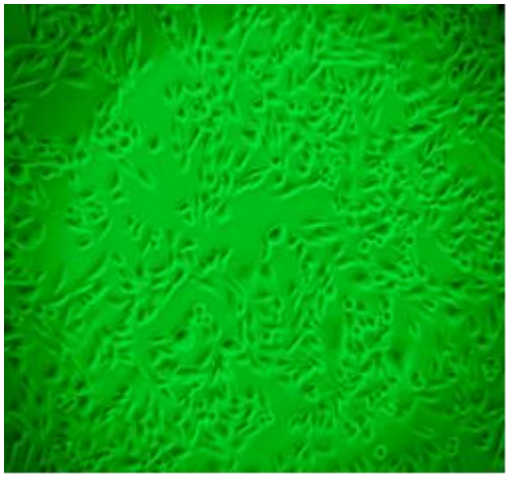
Parameter	**Results**
Accuracy (400 ng/mL)	98.2 ± 12.3 % (n = 4)
Precision (400 ng/mL)	4.3% (n = 4)
Sensitivity (ng/mL)	4.3
A	0.0491
B	1.070
C	220.1
D	4.117
Correlation of coefficient (R)	0.9997

3.3.2. Detoxification of PTX

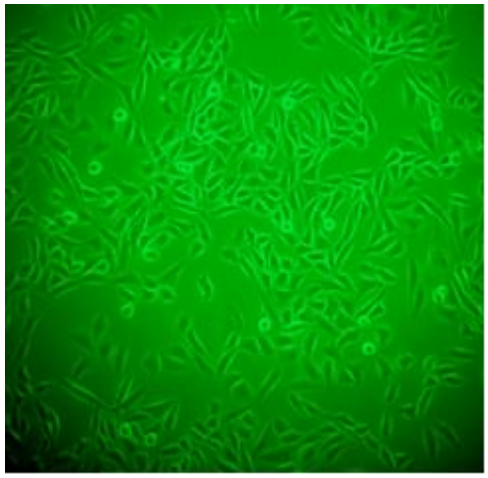
Bordetella pertussis infections, associated with whooping cough, have been significantly reduced by the introduction of pertussis vaccines and immunization programs. Although whole-cell pertussis vaccines have been used for past many decades and are still being used particularly in the developing world, the

more recent development and introduction of acellular pertussis vaccines has both increased compliance with vaccination programs and reduced the severity of adverse reactions to the vaccine. These acellular pertussis vaccines are composed of the PTXd with or without inclusion of pertactin (PRN), filamentous hemagglutinin (FHA), and fimbriae (FIM2 and FIM3/6). Of the components used in the formulation of the acellular pertussis vaccine, only the PTX is the toxic component if administered at the doses required for effective vaccination,[316] hence it is included in the detoxified form (PTXd) in the final formulation. Most of the commercial acellular pertussis vaccines contain chemically detoxified PTX with an exception of few using genetically detoxified PTX. In this investigation, glutaraldehyde was used for detoxification of PTX using modified method reported by Munoz et al.[312] The glutaraldehyde treatment of pertussis toxin is reported to cause cross-linking of pertussis toxin structures and reduction of toxicity of toxin.[317]

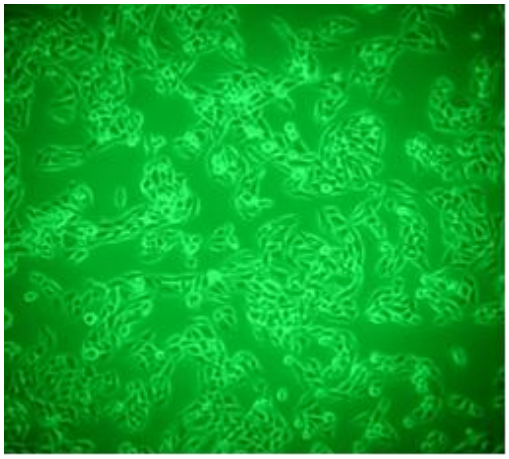
CHO cell agglutination assay is usually used testing for safety of acellular pertussis-containing vaccines.[313, 318, 319] Agglutination of CHO cells is a reflection of PTX activity as it requires both the binding and enzymatic functions of the toxin.[320, 321] The clustering of CHO cells was clearly observed (Figure 3.3 B1, B2) at both high (2 µg/mL) and low (0.07 µg/mL) doses of PTX which indicates the high sensitivity of assay as reported previously[320]. The absence of clustering of cells in results of CHO cell assay for PTXd (Figure 3.3 C1, C2) obtained by detoxification of PTX with glutaraldehye suggested PTX is detoxified and is suitable for use in *in-vivo* studies.



A1 - Buffer only



A2 - Buffer only



B1 - Pertussis toxin (2µg)

B2 - Pertussis toxin (70ng)

C1 - PTXd (2µg)

C2 - PTXd (70 ng)

Figure 3.3 CHO cell assay for evaluation of detoxification of PTX.

3.3.3. Estimation of PTXd (or PTX)

Two different methods were utilized for estimation and analysis of PTX and PTXd as explained previously in this chapter. Currently, there is no *in-vitro* specific standard quantitation method for PTXd which has been universally recommended and accepted in industrial or academic research. Non-specific protein estimation methods such as Lowry and Bradford methods have been widely used for estimation of proteins including PTXd. However, these estimation methods are general methods of protein quantification with no specificity for PTXd and have limited applicability in multi-protein studies such as ours. In addition, these methods usually need higher concentrations of protein (higher detection limit and lower sensitivity) for accurate quantification, which makes them unsuitable for studies involving very low dose of proteins (e.g. PTXd). Therefore, two different methods were used for estimation of PTXd (or PTX) in this study. The first method used a fluorescent reagent for non-specific protein quantification to estimate initial PTXd concentrations after detoxification before addition into any formulation. The second estimation method was pursued with an aim of specific sensitive quantification of PTXd using ELISA technique particularly for entrapment efficiency, release profile and other formulation experiments.

For initial quantification of PTXd after detoxification process, a protein quantification kit (Quant-iT™ Protein Assay kit) was used. The solutions of bovine serum albumin (BSA) of known concentrations were used as standard protein samples. A non-linear correlation between concentration of BSA solutions and fluorescence was observed in concentration range of 0 – 100 µg/mL. Curve-fit statistical models were studied to select the best fit model using Graphpad Prism 5 statistical software. Polynomial (second order) model (equation 3.3) was selected as the best curve fit model for protein quantification on the basis of least sum of squares obtained for y-residual values and confidence intervals for each parameter value (Figure 3.4).

$$Y = \beta_0 + \beta_1 X + \beta_2 X^2 \qquad (3.3)$$

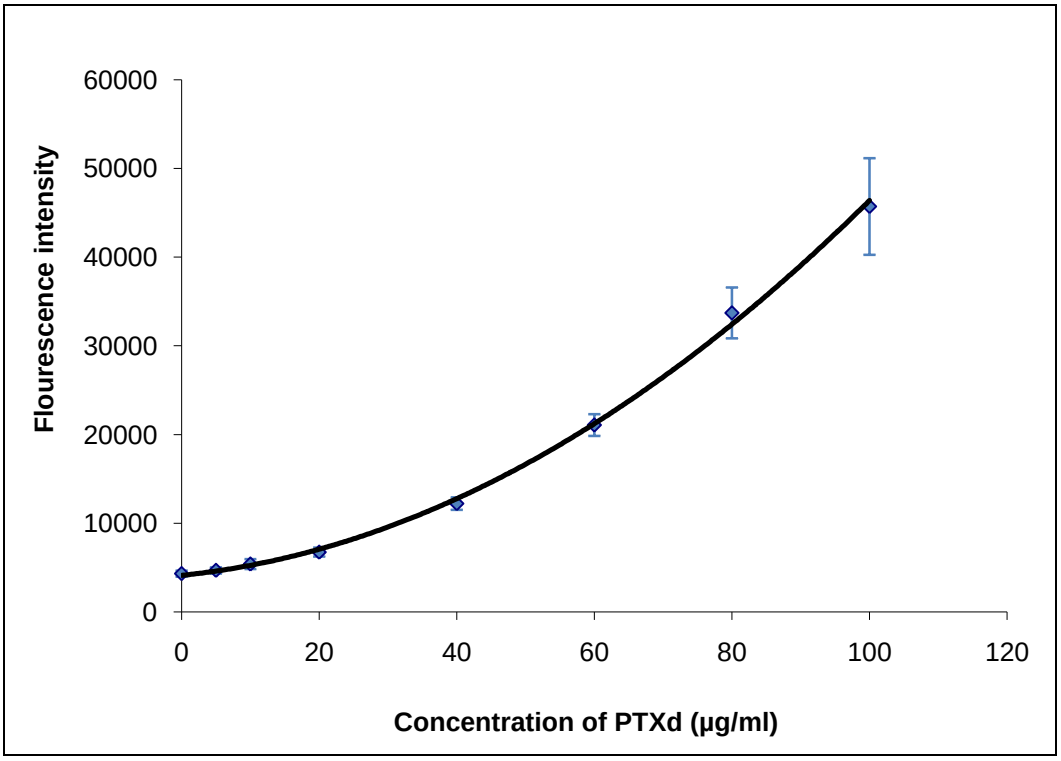


Figure 3.4. Standard (calibration) curve and model fitting for PTXd using Quant-iT protein quantification kit method.

Statistical and analytical parameters for the Quant-iT estimation method were determined (Table 3.2) and to avoid experimental and environmental errors, standard concentrations of BSA were run in each plate along with PTXd samples.

Table 3.2 Statistical and analytical parameters for Quant-iT protein estimation method

Parameter	**Results**
Accuracy (30 µg/mL)	109.7 ± 9.5 % (n = 4)
Precision (30 µg/mL)	8.12% (n = 4)
Sensitivity (µg/mL)	10.79
β_0	4117.70
β_1	78.50
β_2	3.44
Correlation of coefficient (R)	0.9992

Corbel et al.[317] have reviewed the issue of accurate determination of antigen content at the intermediate and formulation stages and reported that measurement of protein content has the disadvantage of not necessarily reflecting the quality or quantity of antigenically active material present. Moreover, non-specific nature of Quant-iT™ protein assay method and lower sensitivity (higher detection limit) of this assay method (Table 3.2) limited its applicability in *in-vitro* characterization (particularly entrapment efficiency and release studies) of PTXd loaded CS-DS nanoparticles. To tackle these problems, development of a sandwich ELISA method for quantitation of PTXd by induction and purification of anti-PTXd-IgG antibodies from mouse serum collected from hyper-immunised animals was undertaken. However, the developed ELISA method did not provided accurate, sensitive and reproducible quantification of PTXd (raw data included in appendix

7.4) which led to the use of PTX in entrapment efficiency and release studies. The developed sandwich ELISA was used to quantify PTX using known concentration solutions of PTX as standards (Figure 3.5). The developed sandwich ELISA method showed acceptable accuracy, sensitivity and reproducibility of results for PTX (Table 3.3) which allowed the use of this method in entrapment efficiency and release studies where lower detection limit is important for obtaining correct results. Antigen free and antibody free wells were used as negative controls and mean absorbance value of negative controls were subtracted from each positive sample absorbance before plotting the graph.

The curve fitting study revealed that correlation between concentration of PTX and absorbance is non-linear for the concentration range of 15.6 - 2000 ng/mL (Figure 3.5). Second order polynomial model (equation 3.4) was selected as best curve fit model on the basis of least sum of squares of y-value differences and confidence intervals for each parameter value (Figure 3.5).

$$Y = \beta_0 + \beta_1 X + \beta_2 X^2 \qquad (3.4)$$

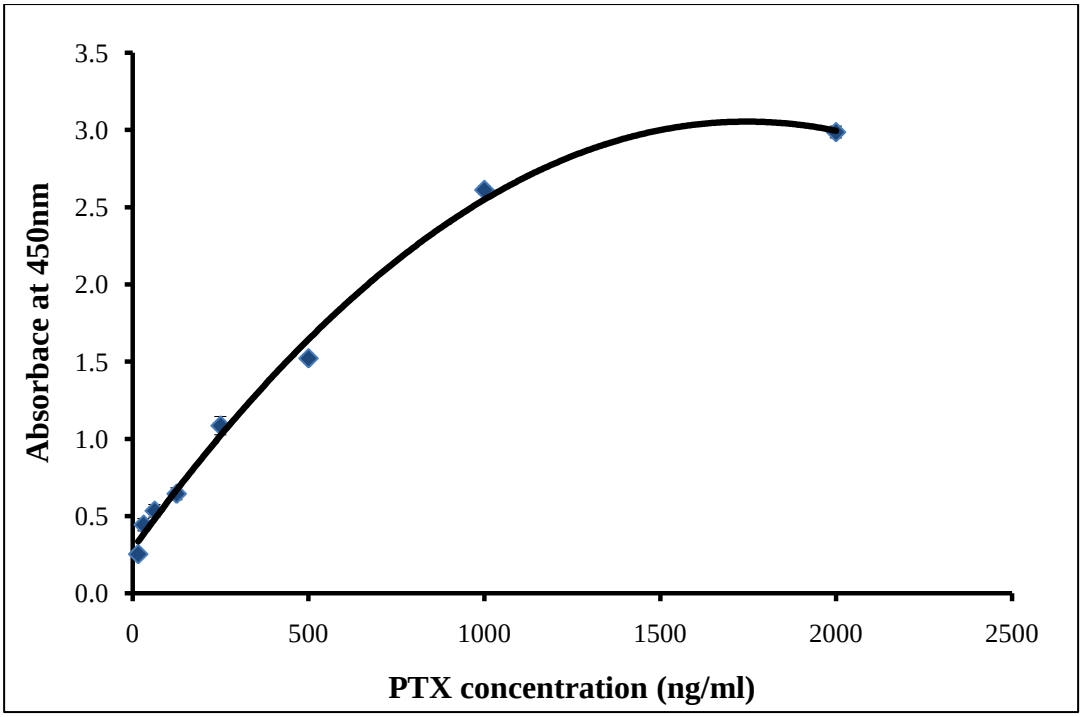


Figure 3.5. Standard (calibration) curve for PTX estimation using sandwich ELISA method.

Statistical and analytical parameters for the estimation of PTX by sandwich ELISA method were determined (Table 3.3) and to avoid experimental and environmental errors, standard concentrations of PTX were run in each plate along with samples. The accuracy and precision of assay were determined from four 400 ng/mL PTX samples. Precision was calculated as percent relative standard deviation (RSD) and accuracy was calculated as percent ratio of measured experimental PTX concentration to theoretical expected PTX concentration (400 ng/mL). Analytical sensitivity (detection limit) for sandwich ELISA method[311] of PTX quantification was calculated using the formula [average absorbance of the blank + 3 x (Standard Deviation for blank)]. Blank represents antigen and antibody free wells in the ELISA run.

Table 3.3. Statistical and analytical parameters for PTX estimation by sandwich ELISA method

Parameter	**Results**
Accuracy (400 ng/mL)	96.1 ± 9.2 % (n = 4)
Precision (400 ng/mL)	6.74% (n = 4)
Sensitivity (ng/mL)	34.97
β_0	0.2862
β_1	0.0032
β_2	$- 9 \times 10^{-7}$
Correlation of coefficient (R)	0.9975

Isben[322] has studied in detail the effect of chemical detoxification of PTX on epitope recognition by murine monoclonal antibodies using ELISA as evaluation technique. The author concluded that the detoxification procedures employed altered the level and pattern of epitope recognition in various degrees. The researcher also reported that modifications induced by chemical detoxifications clearly reduced or destroyed antibody recognition of some epitopes while increased binding of others. The research indicated that PTX detoxification using formaldehyde causes higher reduction in antibody recognition of epitopes compared to PTX detoxified using hydrogen peroxide or genetically detoxified. Moreover, it has been also reported that detoxification of PTX using aldehydes could modify both the B- and the A-subunits of PTX which potentially reduce its interaction with antibodies in ELISA assay.[323-325] These reports and the study conducted for this thesis indicate unsuitability of ELISA as a reliable method of estimation for PTXd.

3.3.4. *In-vitro* characterization of antigen and/or IgA loaded CS-DS nanoparticles

As explained above, the developed sandwich ELISA method was reasonably reproducible and accurate for estimation of PTX whilst the same method was found highly non-reproducible and non-reliable for PTXd estimation (raw data included in appendix 7.4). In the absence of any standard method for *in-vitro* estimation of PTXd especially for *in-vitro* characterization of antigen (PTXd) and adjuvant (IgA) loaded CS-DS nanoparticles, PTX was used in place of PTXd for *in-vitro* characterization (entrapment efficiency and release profile) of loaded nanoparticle formulation. It is important to mention here that detoxification of PTX may lead to increase in molecular size due to cross-linking which in turn may or may not affect entrapment and release process. Therefore, further exploration of quantifying methods for PTXd such as immuno-blotting to determine exact entrapment efficiency and release profile is warranted. Immunoblotting has been reported[326] as a selective technique for the detection of different *B. pertussis* protein antigens but utility of this technique for accurate and sensitive *in-vitro* quantification of PTXd needs further research studies.

Two different approaches were studied for incorporation of PTX or IgA into CS-DS nanoparticles and entrapment efficiency was used as an evaluation parameter. In first approach, PTX or IgA was added into chitosan solution before addition of dextran sulfate into chitosan solution whereas in second approach PTX or IgA was added into dextran sulfate solution before mixing of dextran sulfate with chitosan solution. Significantly higher entrapment ($p < 0.001$, student t-test) was achieved when PTX or IgA was added into chitosan solution during preparation of nanoparticles compared to addition of PTX or IgA into dextran sulfate solution (Table 3.4).

Table 3.4. Entrapment efficiency of IgA or PTX into CS-DS nanoparticles prepared by two formulation approaches

Formulation approach ↓	**IgA entrapment efficiency (%)** ± SD	**PTX entrapment efficiency (%)** ± SD
IgA into chitosan solution	94.19*** ± 0.06	-
IgA into dextran sulfate solution	8.54*** ± 0.31	-
PTX into chitosan solution	-	95.98*** ± 1.94
PTX into dextran sulfate solution	-	73.44*** ± 2.11

*** Means are significantly different ($p < 0.001$), comparison of values in same column.

The higher entrapment efficiency of proteins on addition into chitosan solution might be because of larger molecular structure of chitosan (~ 150K) compared to dextran sulfate (~ 5K) allow intermingling of PTX or IgA with chitosan before formation of nanoparticles. Huang et al.[327] have also reported entrapment efficiency of higher than 80% in CS-DS nanoparticles for a protein (vascular endothelial growth factor) and hypothesized the mechanism of entrapment in two stages; first intermingling of protein with high molecular weight polysaccharide and then formation of nanoparticles on addition of low molecular weight polyelectrolyte by interation of two polyions. Gan et al.[257] have systematically studied fabrication conditions for efficient loading of chitosan nanoparticles using BSA as model protein. The authors reported that since protein molecules are large macromolecules with complex 3-D structure, able to fold and

unfold at different solution conditions, their interactions with long cationic chitosan chain and the consequential encapsulation can be complicated, depending on 3-D conformation, electrostatic, and solution conditions. Further, the presence of a crosslinking agent such as aldehydes will chemically interact with free amine groups on both protein and chitosan molecules, resulting in more compact protein–chitosan nanoparticles. Additional adsorption of protein molecules on the surface of the formed particles may occur in sequence, leading to additional protein loading on the particles.[257]

In the light of experimental results and previous reports, antigen (PTX) and/or adjuvant were added into chitosan solution during preparation of nanoparticles for all further experiments. The entrapment efficiency of CS-DS nanoparticles was also observed higher than 90% when both IgA and PTX were incorporated into CS-DS nanoparticles i.e. 93.97 ± 0.508 and 95.88 ± 0.53 respectively for IgA and PTX. This indicates that two proteins and their quantity do not hinder the incorporation of each other into CS-DS nanoparticles and CS-DS nanoparticle formulation can be a potential delivery system for multiple proteins together.

Table 3.5 tabulates the particle size and zeta potential results of IgA- or PTXd- or IgA and PTXd-loaded CS-DS nanoparticles. It is important to mention that PTX was used in place of PTXd only for entrapment efficiency and release studies (where quantification of antigen was needed) and for particle size, zeta potential and stability (SDS-PAGE) studies PTXd was incorporated in CS-DS nanoparticles as in further *in-vivo* studies (next chapter of thesis) only PTXd can be administered as safe antigen to animals (as explained in section 3.3.2).

The incorporation of PTXd or/and IgA in CS-DS nanoparticles did not result in significant increase of particle size but, the zeta potential of CS-DS nanoparticles become less positive on incorporation of protein(s). This may have occurred because the carboxylic acid groups on the surface of a large protein molecule may interact with amine groups at certain sites at the spread chitosan chain, but still maintaining a compact 3-D protein structure without spreading.[328]

On the other hand, high positive zeta potential of loaded CS-DS nanoparticles indicate that still be a high proportion of free amine group on the chitosan chain remains unoccupied.

Table 3.5. *In-vitro* characteristics of loaded CS-DS nanoparticle formulations

Formulation	**Particle size ± S.D (nm)**	**Polydispersity index ± S.D**	**Zeta potential ± S.D (mV)**
CS-DS nanoparticles	314.7 ± 9.2	0.29 ± 0.21	+53.2 ± 4.4
IgA-loaded nanoparticles	290.8 ± 10.3	0.52 ± 0.07	+39.6 ± 4.1
PTXd-loaded nanoparticles	324.0 ± 3.1	0.45 ± 0.17	+41.6 ± 3.2
IgA and PTXd loaded nanoparticles	330.3 ± 1.1	0.39 ± 0.14	+40.1 ± 1.2

Lack of significant increase in particle size of nanoparticles with limited reduction in zeta potential on IgA and/or PTXd incorporation and entrapment efficiency higher than 90% achieved for these proteins when added into chitosan solution indicate involvement of strong attractive force between these proteins and nanoparticle components. This may be explained using a "Guest-host model" resulting in a "core/shell" structure established for polyelectrolyte complexes.[329] In brief, the model states that the polymer partners complex according to the ''zip'' mechanism and form particles consisting in long host molecule sequentially complexed with shorter molecules of oppositely charged guest polyelectrolyte resulting in a hydrophobic core formed from the aggregation of complexed segments whereas excess component in the outer shell ensuring the colloidal stabilization against further coagulation.[278, 281, 330] This model explains most of the

in-vitro characterstics observed in the studies conducted as part of this thesis for CS-DS nanoparticles.

The applicability of this model on CS-DS nanoparticles is further vindicated by the release profile of protein(s)observed in this study. All the loaded CS-DS nanoparticles showed less than 15% of initial rapid release followed by almost no release of protein(s) for long time period (Figure 3.6 and 3.7). This indicates quick desorption of loosely bound surface IgA and/or PTX from shell part of nanoparticles, followed by slow or no release from core of nanoparticles with dynamic adsorption-desorption continuously occurring during the later phase. Similar release profiles have been reported previously for chitosan nanoparticles incorporating different protein(s).[283, 331, 332] The lack of high percentage release in short time period is important in making sure that most of the vaccine dose remains in nanoparticle at the time of administration.

A small increase of less than 10% in protein(s) release was observed when both IgA and PTX were incorporated into CS-DS nanoparticles compared to nanoparticles loaded with only IgA or PTX. The increased release might have occurred due to a small increase in the proportion of protein bound to surface of nanoparticles in the presence of other competitive protein.

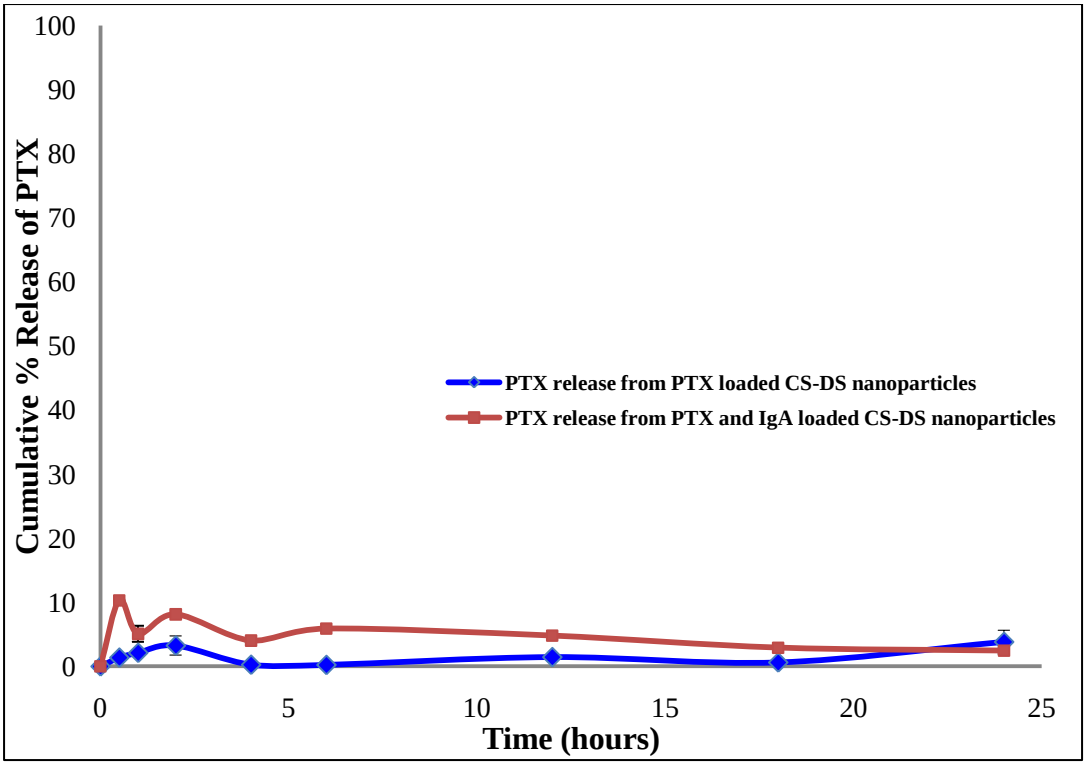


Figure 3.6. Release profile of PTX from CS-DS nanoparticle formulation in acetate buffer pH 5.1. All the data plotted is in the form of mean ± SD (n = 3).

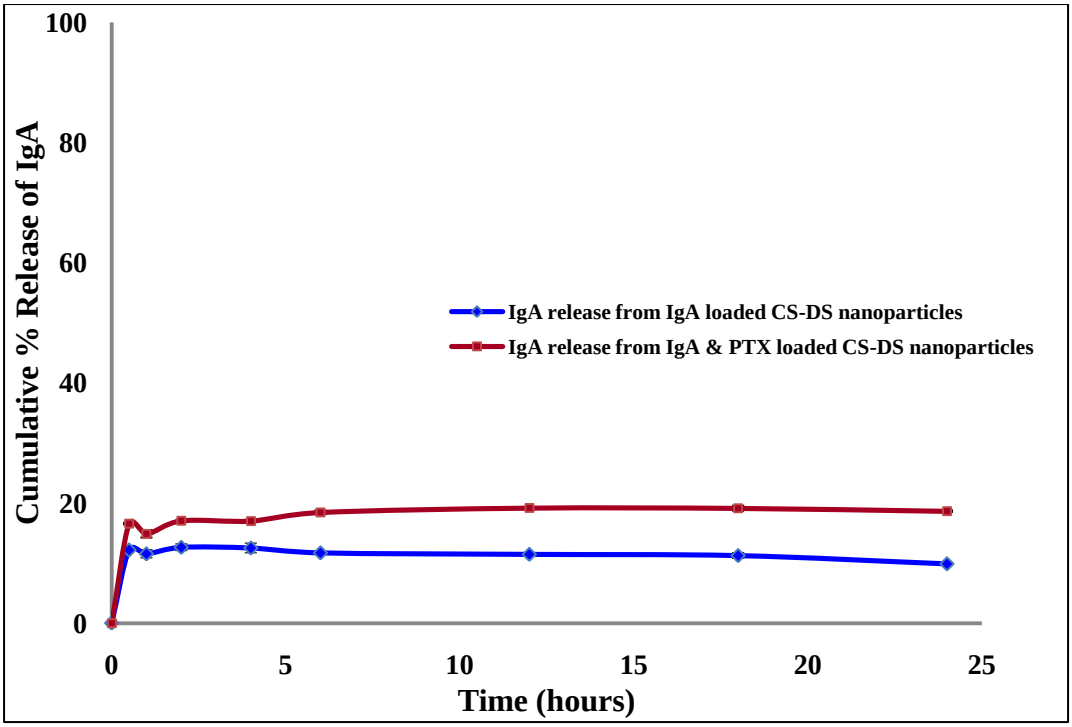


Figure 3.7. Release profile of IgA from CS-DS nanoparticle formulations in acetate buffer pH 5.1. All the data plotted is in the form of mean ± SD (n = 3).

To evaluate the integrity of protein(s) on entrapment into CS-DS nanoparticles, the SDS-PAGE bands of the nanoparticle containing IgA or PTXd were compared with that of native IgA or PTXd using the protein concentration of 100µg/ml (2 µg in 20 µl) in each sample. The results of study (Figure 3.8) showed identical bands for the entrapped IgA (lane C) and native IgA (lane B), each with two bands representing heavy and light chains of structure. Similarly, for native (lane D) and entrapped PTXd (lane E) identical bands were observed. There were no additional bands which indicate that the molecular weight of IgA and PTXd was not significantly affected by the entrapment procedure. The relatively lighter (faded) bands for nanoparticle incorporated PTXd (lane E) and IgA (lane C) compared to native PTXd (lane D) and IgA (lane B) indicate shielding of significant proportion of PTXd and IgA inside CS-DS nanoparticles. The absence of distinct PTX structural unit bands (S1, S2, S3, S4 and S5) for PTXd in SDS-PAGE results

indicates structural modification of molecule by detoxification. It has been previously reported[317] that SDS-PAGE results are of less value in characterization of PTXd obtained by chemical detoxification. However, the results of SDS-PAGE give sufficient information about molecular weight similarity of IgA and PTXd before and after incorporation into CS-DS nanoparticles. This is important for comparison of different loaded nanoparticle formulations against antigen solution formulations in *in-vivo* studies.

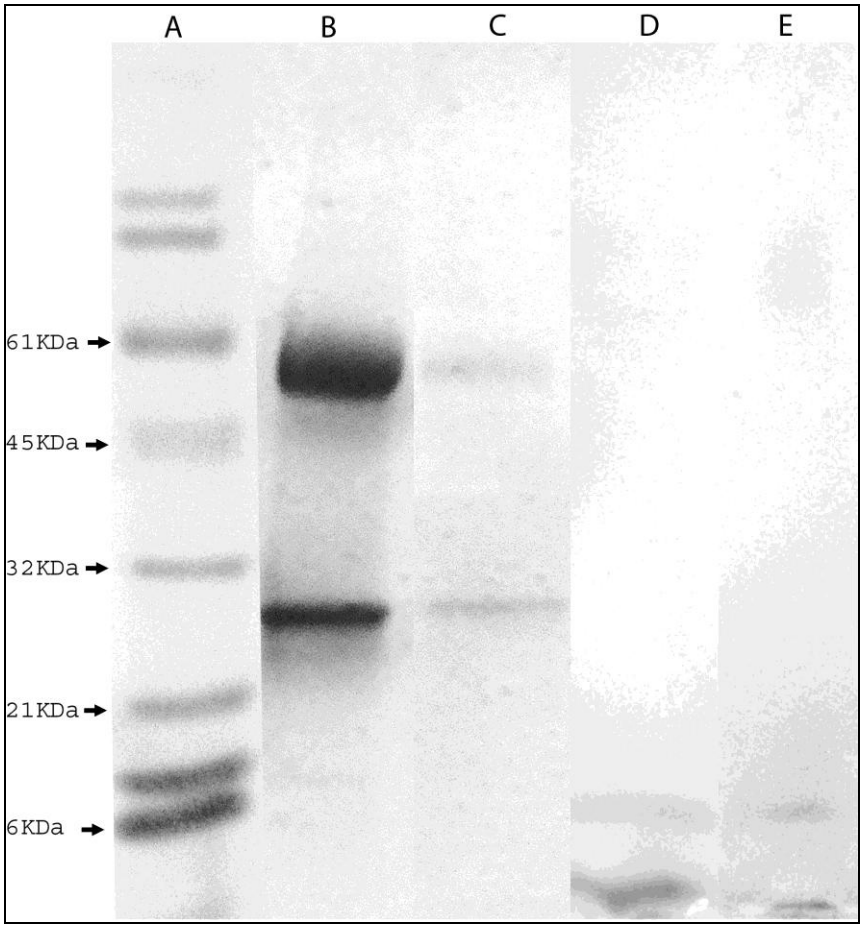


Figure 3.8. Gel electrophoretic analysis of, (A) Molecular weight marker; (B) mouse IgA; (C) CS-DS nanoparticles loaded with mouse IgA; (D) PTXd; (E) CS-DS nanoparticles loaded with PTXd.

To conclude, PTXd and/or IgA were efficiently incorporated into CS-DS nanoparticles using a simple aqueous method and loaded nanoparticles were

characterized for *in-vitro* evaluation parameters. The method of estimation for IgA and PTXd (or PTX) were developed and used in the determination of entrapment efficiency and release profile for loaded CS-DS nanoparticles. The *in-vitro* characterization of IgA and/or PTXd loaded CS-DS nanoparticles suggests the potential of CS-DS nanoparticles as vaccine delivery system with the following important advantages:

- ❖ High entrapment efficiency for protein antigen and adjuvant using simple preparation and incorporation method.

- ❖ Nanoparticulate size and positive zeta potential, both of which can potentially lead to induction of higher immunological responses *in-vivo*.

- ❖ Lack of significant release of incorporated protein(s) from nanoparticles during storage ensures that most of the protein(s) will be in nanoparticle incorporated form at the time of administration.

These results warrant the further evaluation of PTXd and/or IgA loaded CS-DS nanoparticles *in-vivo.*

CHAPTER 4

IMMUNOLOGICAL CHARACTERIZATION OF THE ANTIGEN AND/OR IgA LOADED CS-DS NANOPARTICLE FORMULATIONS *IN-VIVO*

4.1. INTRODUCTION

The immune system can be divided into innate and adaptive (acquired) immune systems although both of these immune systems work together as a complex integrated system.[333] A cascade of events occurs when cells participating in innate immune defences recognize foreign structural motifs on pathogens to eliminate or contain the threat. Innate immunity comprises of a variety of hematopoietic and cellular factors including the complement system, phagocytic cells, natural killer cells, naturally occuring antibodies, γδ T cells, and anti-microbial peptides. The innate immune system recognize components of foreign invaders using relatively few molecules, these microbial associated components were described by Janeway and Medzhitov as pathogen-associated molecular patterns (PAMPs).[334] Depending on the vigour of the innate immune response, the adaptive immune response may or may not be actively engaged.[188] In contrast to innate immunity, adaptive or acquired immunity involves recognization of antigen-specific epitopes, in case of protein antigens, via specialized cell surface receptors (B cell or T cell receptor) resulting in an antigen-specific immune response.[188]

There are two arms of acquired immunity, which have different set of participants but with one common aim of eliminating the foreign antigen. Of these two arms, one is mediated mainly by B cells leading to production of antibodies and referred to as antibody-mediated (humoral) immunity. The other is mediated by T cells (Th1 or Tc cells), which do not synthesize antibodies but instead synthesize and release various cytokines that affect other cells.[335] Cell mediated immunity is particularly effective against intracellular pathogens, some cancer cells and foreign tissue transplants. Antibody-mediated immunity works mainly against antigens present in body fluids and extracellular pathogens. Antibody mediated immune responses against an antigen can be further classified into two main types based on the site of induction; first is systemic immune response which represent induction of antibodies in blood and lymph fluids, and second is mucosal immune response which offer antibody-mediated protection at the mucosal surfaces mostly through secretory immunoglobulins (SIgA).

Induction of the appropriate immune response is essential determinant of vaccine efficacy/performance. One of the critically important innate immune cells involved with induction of immune responses are the dendritic cells. Dendritic cells are found in all body tissues and play a central role in stimulation and regulation of acquired immunity (cell mediated and humoral immunity).[336] In the blood and tissues, dendritic cells are in an 'immature'' state, capable of phagocytosis, and express low levels of costimulatory molecules as well as molecules associated with antigen capture and cellular migration (pattern recognition receptors – PRRs) such as TLRs, DCIR, CCR7, DC-SIGN, DEC-205 etc.[337] Upon activation via TLRs and/or other environmental cues, such as IL-8, dendritic cells undergo maturation. Following maturation, dendrtic cells lose much of their phagocytic capacity while increasing surface expression of migratory and costimulatory molecules, such as MHC I/II, CD80, CD86, and CD40. This process is accompanied by migration to the draining lymph node(s). Within the lymph node, dendritic cells continue maturation and serve as potent APCs to naive CD4+ and CD8+ T cells.[188]

Dendritic cells process antigen either via the major histocompatibility complex (MHC) class-I or class-II pathways. This depends upon whether the antigens are endogenous or exogenous. Exogenous antigens taken up by APCs are contained within a phagosome or early endosome. The phagosome fuses with a lysosome generating a phagolysosome in which proteolytic enzymes are activated following changes in the pH that leads to degradation of the antigen into small peptide fragments in order to facilitate their presentation to T cells and B cells. Antigens within phagolysosomes of APCs representing exogenous antigens are displayed as MHC-II and then presented on the cellular surface for stimulation of CD4+ T cells.[338]

Antigens of many viral and intracellular bacterial pathogens are presented by the endogenous pathway and processed in the cytosol. Processing of these antigens involve degradation in the proteasome and transport to the endoplasmic reticulum via TAP-I and TAP-2 encoded peptide transporters via the golgi apparatus as MHC class-I peptide complex to the surface of the APC for presentation to CD8+ T cells.[188, 338] Upon recognition of the MHC peptide complex and costimulation from APC, naïve T-helper

(Th) cells differenciate into effector Th cells.[339] These CD4+ T-helper (Th) cells can be further classified as Th1, Th17, and Th2.[340-342] A Th2-type immune response is characterized by the production of IL-4, IL-5, IL-10, IL-13 and TGF-β and the secretion of IgG1 and IgE antibody isotypes. Th1-type responses are characterized by the production of cytokines such as IFN-γ, GM-CSF, TNF-α, IL-2, IL-12, IL-18 and TNF-β and IgG2a antibodies and are considered to be indirect indicators of cell-mediated immunity (CMI) involving activation of macrophages and manifestation of delayed type hypersensitivity.[343] Immune responses of the Th1-type are directed more towards the clearance of intracellular pathogens such as *Mycobacterium tuberculosis* and *Shigella* species, and most viruses, whereas a Th2-type response is generally associated with the induction of antibodies that effectively neutralize toxins, viruses, and/or bacterial adhesion.[344, 345] Th17 responses are considered inflammatory in nature and are characterized by production of IL-17.[340] The role of Th17 cells in vaccinology or infectious disease has yet to be fully elucidated although an association with autoimmunity has been suggested.[338]

The induction of different arms of immune response depends on stimulation of particular antigenic processing pathway, which in turn is influenced by various factors such as formulation components of vaccine, nature of antigen and route of vaccine administration. Conventionally, nearly all the vaccines were administered through parenteral routes and such is the case with majority of the currently used commercial vaccines. However, interest in investigation of newer routes for vaccine delivery have resulted in various studies reporting possibility of vaccine delivery through different mucosal routes such as oral, nasal, pulmonary, sublingual and rectal routes. Mucosal administration of vaccines offers a number of advantages, including easier administration, reduced potential adverse effects and the potential reduction of the need for frequent boosters. In addition, mucosal immunization has been reported to induce both mucosal and systemic immune responses at least in some monogastric species. Table 4.1 compares the pros and cons of parenteral versus mucosal routes of administration for vaccine delivery.[346, 347]

Table 4.1 Comparison of the parenteral and mucosal routes of administration for vaccine delivery

Comparison parameters	**Parenteral delivery**	**Mucosal delivery**
Immunogenicity of antigen	Higher	Moderate or lower
Dose of antigen required	Lower	Moderate or higher
Acquired immunity	Systemic antibodies; Cell mediated immunity may be induced using delivery system or adjuvants.	Systemic and/or mucosal antibodies; Cell mediated immunity may be induced using delivery system or adjuvants.
Compliance and ease of administration	Lower compliance and injections are used. Medical expertise needed for administration	Higher compliance and easy administration with self administration possible
Examples of routes studied/used	Subcutaneous, intramuscular, intradermal	Oral, nasal, rectal, sublingual, pulmonary.

A comparative *in-vivo* immunological evaluation of different antigen loaded CS-DS nanoparticle formulations delivered via intranasal versus subcutaneous routes was undertaken due to the potential differences in type and level of induction of immune responses by a vaccine formulation administered through different routes of administration. The nasal mucosa is an attractive option for mucosal vaccine delivery because of the absence of acidity, lack of abundant secreted enzymes and small mucosal

surface area that result in a low dose requirement of antigen compared to oral vaccine delivery. Additionally, the most common causative agent of pertussis, *Bordetella pertussis*, has an exclusive affinity for the mucosal layers of the human respiratory tract. This makes intranasal vaccination attractive as induction of mucosal immune response in respiratory mucosa could be valuable in local protection against the infection. Furthermore, the presence of an organized lymphoid tissue (M cells incorporating NALT) in nasal mucosa could provide an efficient uptake pathway for nanoparticulate PTXd-loaded CS-DS formulations. The presence of M cells in nasal mucosa could also help to understand the effect of incorporation of a M cell targeting ligand (IgA) in PTXd loaded CS-DS nanoparticles on induction of *in-vivo* immune responses. The selection of IgA as a potential immunological adjuvant and M-cell targeting agent in this thesis work has already been included in chapter 3 section 3.1.2.

4.1.1. Objectives of this section

The goal of this part of project was to perform a comparative study of the immunological responses of antigen-loaded CS-DS nanoparticle formulations with or without IgA delivered via intranasal versus subcutaneous routes in a mouse model. The goal was accomplished by execution of the following studies:

- Evaluation of nasal M-cell targeting efficiency of IgA loaded CS-DS nanoparticulate formulation.
- Determining the immunogenic response of IgA as an immunological adjuvant for pertussis toxoid delivery via mucosal and parenteral routes.
- Determining the immunogenic response of CS-DS nanoparticluate formulation as vaccine delivery system through mucosal and parenteral routes.

4.2. EXPERIMENTAL

4.2.1. Materials

Lyophilized pertussis toxin (List Biological Laboratories Ltd., USA, Catalog no.181) was purchased from Sapphire Biosciences, Australia. Mouse IgA-Kappa from murine melanoma (Catalog no. M1421), 3,3',5,5'-tetramethylbenzidine (TMB-2 liquid substrate system, Catalog no. T8665), tris bufferd saline powder (Catalog no. T6664), phosphate buffered saline (Catalog no. P4417), glutaraldehyde grade I (8% in water, catalog no. G7526) tween-20 (Catalog no. P9416), bovine serum albumin (Catalog no. A3294), L-lysine (Catalog no. W384704), UEA-I conjugated with TRITC (Catalog no. L4889), goat anti-mouse IgA-FITC conjugated (Catalog no. F9384) and carbonate-bicarbonate buffer capsules (Catalog no. C3041) were purchased from Sigma-Aldrich Ltd., Australia.

Goat anti-mouse IgG1-HRP conjugated (Catalog no. A90-105P, Bethyl labs., USA), goat anti-mouse IgG2a-HRP conjugated (Catalog no. A90-107P, Bethyl labs., USA), goat anti-mouse IgG2b-HRP conjugated (Catalog no. A90-109P, Bethyl labs., USA) and goat anti-mouse IgA-HRP conjugated (Catalog no. A90-103P, Bethyl labs., USA) antibodies were purchased from Sapphire Biosciences, NSW, Australia. BD OptEIA™ mouse interferon-γ (IFN-γ) ELISA set estimation kit (Catalog no. 555138, BD biosciences), sterile scalpel blades no. 20 (Catalog no. 16827, BD biosciences) and ultra-fine needle insulin syringe (Catalog no. 16296, BD biosciences) were purchased from BD biosciences, Australia. The flat-bottom 96-well F96 maxisorp nunc immuno plates (Catalog no. 442404, Thermo Fisher scientific, Roskilde, Denmark) were obtained from in-vitro technologies, Australia. All other chemicals used for experiments were of molecular biology grade and used as received unless otherwise specified.

Female Balb/c mice aged between 6-8 weeks were purchased from Animal Resource Center, Perth, Australia. All the animal purchase and studies were duly reviewed and approved by Animal Ethics Committee of Curtin University prior to the start of the animal study.

4.2.2. Methods

4.2.2.1. Preparation of CS-DS nanoparticulate formulations for *in-vivo* studies

Blank CS-DS nanoparticles were prepared as a negative control formulation for *in-vivo* studies by dropwise addition of 100µl dextran sulfate solution (0.1% w/v in milli-Q water) into 300µl of chitosan solution (0.1% w/v in 0.2% v/v acetic acid solution) at room temperature with high speed magnetic stirring to form nanoparticles.

CS-DS nanoparticles incorporating mouse IgA and/or PTXd were prepared by adding 105µl of PTXd and/or mouse IgA solution (containing 140µg of IgA or PTXd or IgA and PTXd each) into 105µl chitosan solution (0.2% w/v in 0.4% v/v acetic acid solution) followed by dropwise addition of 70µl of dextran sulfate solution (0.1% w/v in milli-Q water) with high speed magnetic stirring at room temperature.

Half of each formulation was used for subcutaneous immunological animal studies and another half for nasal immunological animal studies. All the formulations were prepared on the same day of their administration into animals.

4.2.2.2. *In-vivo* uptake of IgA loaded nanoparticles in nasal epithelia

Two female Balb/c mice (aged 6-8 weeks) were anaesthetized with isoflurane as inhalational anaesthetic in conjuncture with air using ventilator just before administration of formulation through nasal route. CS-DS nanoparticulate formulation loaded with IgA was administered into mice nares (10 µL per nostril) by a 20 µL pipette tip. The animals were kept at a controlled temperature condition for half an hour after administration of formulation. The animals were sacrificed 30 min after intranasal administration of the formulations under deep anaesthesia induced by administration of ketamine (80mg/Kg) in combination with xylazine (16mg/Kg) by intraperitoneal route.

The exposed nasal mucosa was gently washed by tris buffer saline. The nasal cavity of the mice was flushed with 4% (w/v) paraformaldehyde solution in PBS pH 7.4 and the septum along with nasal associated lymphoid tissue (NALT) situated at the posterior part of the nose were excised using dissecting microscope (Olympus SZ61, Olympus corporation, Philippines). The excised nasal tissues were immersed in the 4% (w/v) paraformaldehyde solution in PBS pH 7.4 for 45 minutes. The tissues were then gently washed with tris buffer saline. The tissues were stained with FITC labelled anti-mouse IgA, UEA-1 conjugated with TRITC and Hoescht 33343 for 60 min and visualized by a confocal laser scanning microscope (Bio-Rad, Alphen a/d Rijn, The Netherlands). The confocal pictures were obtained by scanning the nasal epithelia in the x, y plane with a z-step. Another two female Balb/c mice (6-8 weeks) were used as negative controls and administered with buffer only. The nasal tissues excised from negative control mice were stained with either UEA-1 conjugated with TRITC or Hoescht 33343 and confocal pictures were obtained as described above.

4.2.2.3. *In-vivo* immunological comparative evaluation of formulations

Four groups of 6 female Balb/c mice aged 6-8 weeks each were used for *in-vivo* comparative evaluation of different formulations through subcutaneous route of administration for immunological responses. Alum (Imject Alum) adsorbed PTXd was used as a positive control in this study. In brief, PTXd was adsorbed over alum by dropwise addition of PTXd into alum suspension with continuous vortexing for 30minutes. Another five groups of 6 female Balb/c mice aged 6-8 weeks each were used for in-vivo comparative evaluation of different formulations through intranasal route of administration for immunological responses. For the easier administration of formulations to animals through intranasal route, each animal was anaesthetized with isoflurane as inhalational anaesthetic in conjuncture with air using ventilator just before administration of 10µl formulation in each nostril.The details of the study are included in Table 4.2.

Table 4.2. Experimental design for comparative *in-vivo* evaluation of CS-DS nanoparticulate formulations against conventional antigen formulation.

Animal group	Route of adm.	IgA dose (µg)	PTXd dose (µg)	Formulation	Dosing frequency	Sacrifice post first adm.	End-point collection
1	IN	0	10	PTXd solution	Day 1, 14 and 21 of study	30 days	Blood, spleen, lungs
2	IN	0	10	CS-DS nanoparticles loaded with PTXd	Day 1, 14 and 21 of study	30 days	Blood, spleen, lungs
3	IN	10	10	CS-DS nanoparticles loaded with IgA and PTXd	Day 1, 14 and 21 of study	30 days	Blood, spleen, lungs
4	IN	0	0	Buffer only (control group 1)	Day 1, 14 and 21 of study	30 days	Blood, spleen, lungs
5	IN	10	0	Blank CS-DS nanoparticles with IgA (control group 2)	Day 1, 14 and 21 of study	30 days	Blood, spleen, lungs
6	SC	0	10	PTXd adsorbed on imject alum	Day 1, 14 and 21 of study	30 days	Blood, spleen, lungs
7	SC	0	10	CS-DS nanoparticles loaded with PTXd	Day 1, 14 and 21 of study	30 days	Blood, spleen, lungs

8	SC	10	10	CS-DS nanoparticles loaded with IgA and PTXd	Day 1, 14 and 21 of study	30 days	Blood, spleen, lungs
9	SC	0	0	Blank CS-DS nanoparticles (Control group 3)	Day 1, 14 and 21 of study	30 days	Blood, spleen, lungs

SC = Subcutaneous administration; IN = Intranasal administration; adm. = administration

4.2.2.4. Collection and processing of blood and tissues from mice at the termination of immunological studies

Blood from each animal was collected in sterile centrifuge tubes on 30th day of first immunizaiton dose using cardiac puncture technique under deep anaesthesia induced by administration of ketamine (80mg/Kg) in combination with xylazine (16mg/Kg) by intraperitoneal route. All the animals were sacrificed by euthanasia by cervical dislocation under deep anaesthesia. Lungs and spleen from each mouse were also excised after dissection and kept into 1mL of PBS containing 0.1% w/v BSA and 2mL of complete DMEM (CDMEM) nutrient medium respectively.

The collected blood from animals was kept for half an hour on ice and then centrifuged at 4000 rpm (1500 x g) for 10 min to collect supernatant as serum. All the serum samples were kept frozen at -80ºC until tested by ELISA for anti-PTXd specific IgG, IgG1, IgG2a and IgG2b antibody isotypes and/or subclasses. Lungs collected from each mouse were homogenized in a glass tube with the help of a plunger and the homogenate was centrifuged at 13000 rpm (12000 x g) for 10 min to collect the supernatant which was kept frozen at -80ºC until being tested by ELISA to determine antigen specific IgA.

4.2.2.5. Preparation and culture of splenocytes

The individual spleens collected from each mouse were homogenised using a syringe plunger and passed through cell strainer (sieve) to produce spleen cell suspension. These spleen cell suspension were pooled to get two pooled cell suspension per animal group i.e, three individual mouse spleen cell suspensions from same animal group were pooled. The pooled cell suspensions were centrifuged at 1000 rpm for 10 minutes and cell pellet was redispersed in ACK buffer followed by incubation for 5 minutes at room temperature. Cell suspension was again centrifuged at 1000 rpm for 10 minutes and cell pellet was redispersed in complete Dulbecco's Modified Eagle Medium (CDMEM) nutrient medium. Cells were washed three times and

finally redispersed in 1mL of CDMEM nutrient medium. The concentration of cells was measured using hemocytometer cell counting and appropriate dilution was made to get cell concentration of 10^7 cells per mL. Using sterile 24-well flat bottomed tissue culture plates, 1mL of splenocyte suspension (1 X 10^7 cells/mL) from each pooled cell suspension was plated in triplicate along with 20µl of the mitogen solution [Con A - 5µg (positive control) or PTX - 2µg or PBS only (negative control)]. The plates were incubated in a humidified 5% CO_2 incubator for 72 hours at 37°C. The plates were centrifuged and the clear supernatants stored at -80ºC until being tested by ELISA to estimate IFN-γ concentration. General details of cell-culture techniques and cell counting method are compiled in Appendix 7.2.

4.2.2.6. Estimation of anti-PTXd serum antibodies using ELISA

Flat-bottom microtiter plates (Catalog no. 442402, Nunc immuno 96 microwell plates) were coated with 2µg/mL PTX solution in carbonate-bicarbonate pH 9.6 coating buffer and incubated overnight in fridge at 4 ± 2ºC. Wells were aspirated and plates were washed four times with wash buffer and each well was blocked with 300 µl of blocking buffer for 30 minutes at 25 ± 2ºC. Plates were aspirated followed by washing four times with wash buffer, 100 µl of diluted serum sample in dilution buffer was added per well, and incubated for 60 minutes at 25 ± 2ºC. Plates were aspirated followed by washing five times with wash buffer and 100 µl of diluted HRP-labeled goat anti-mouse IgG or HRP-labeled goat anti-mouse IgG1 or HRP-labeled goat anti-mouse IgG2a or HRP-labeled goat anti-mouse IgG2b antibodies in dilution buffer (0.4 µg/mL) was added to each well and incubated for 60 minutes at 25 ± 2ºC. Plates were aspirated followed by washing five times, developed for 30 min at 25 ± 2ºC with HRP substrate (3,3',5,5'-tetramethylbenzidine), stopped with 1M HCl, and read at 450 nm with a microplate reader (Victor^{3}V™ Wallac 1420 multilabel counter, PerkinElmer life and analytical sciences, Turku, Finland). Serial dilutions of each serum sample were made and run in ELISA for estimation of anti-PTXd titers for each animal of every group. At the end of

each washing set and before addition of next reagent, plates were inverted on absorbent paper to remove any residual buffer. Formula and preparation method of different buffers used in ELISA are included in Appendix 7.1.

4.2.2.7. Estimation of anti-PTXd IgA in lung homogenates

Flat-bottom microtiter plates (Catalog no. 442402, Nunc immuno 96 microwell plates) were coated with 2 µg/mL PTX solution in carbonate-bicarbonate pH 9.6 coating buffer and incubated overnight in fridge at 4 ± 2ºC. Wells were aspirated and plates were washed four times with wash buffer and each well was blocked with 300 µl of blocking buffer for 30 minutes at 25 ± 2ºC. Plates were aspirated followed by washing four times with wash buffer, 100 µl of diluted lung homogenate supernatant sample in dilution buffer was added per well, and incubated for 60 minutes at 25 ± 2ºC. Plates were aspirated followed by washing five times with wash buffer and 100 µl of diluted HRP-labeled goat anti-mouse IgA antibodies in dilution buffer (0.4 µg/mL) was added to each well and incubated for 60 minutes at 25 ± 2ºC. Plates were aspirated followed by washing five times, developed for 30 min at 25 ± 2ºC with HRP substrate (3,3',5,5'-tetramethylbenzidine), stopped with 1M HCl, and read at 450 nm with a microplate reader (Victor^{3}V™ Wallac 1420 multilabel counter, PerkinElmer life and analytical sciences, Turku, Finland). Serial dilutions of each lung homogenate supernatant sample were made and run in ELISA for estimation of anti-PTXd titers for each animal of every group. At the end of each washing set and before addition of next reagent, plates were inverted on absorbent paper to remove any residual buffer. Formula and preparation method of different buffers used in ELISA are included in Appendix 7.1.

4.2.2.8. Estimation of splenocyte IFN-γ using ELISA

Microwells of flat-bottom microtiter plates (Catalog no. 442402, Nunc immuno 96 microwell plates) were coated with 100µl per well of capture antibody diluted in coating buffer (carbonate-bicarbonate pH 9.6 buffer). Plates

were sealed and incubated overnight at 4°C. Wells were aspirated and washed four times with wash buffer. Plates were blocked with 300µl per well of blocking agent and incubated at 25 ± 2 °C for 1hours. Plated were aspirated followed by washing four times with wash buffer, 100 µl of splenocyte culture supernatant sample or recombinant mouse IFN-γ standard was added per well and incubated for 2hours at 25 ± 2ºC. Plates were aspirated followed by washing five times with wash buffer and 100 µl of working detector (biotinylated anti-mouse IFN-γ monoclonal antibody + streptavidin-horseradish peroxidase conjugate) was added to each well and incubated for 60 minutes at 25 ± 2ºC. Plates were aspirated followed by washing five times, developed for 30 min at 25 ± 2ºC with HRP substrate (3,3',5,5'-tetramethylbenzidine), stopped with 1M HCl, and read at 450 nm with a microplate reader (Victor^{3}V™ Wallac 1420 multilabel counter, PerkinElmer life and analytical sciences, Turku, Finland). Recombinant mouse IFN-γ standard solutions in the concentration range of 1000 pg/mL to 62.5pg/mL were run along with samples in the plate. At the end of each washing set and before addition of next reagent, plates were inverted on absorbent paper to remove any residual buffer. Formula and preparation method of different buffers used in ELISA are included in Appendix 7.1.

4.2.2.9. Statistical analysis

The normalized data was analyzed and processed to calculate mean with standard error of mean for each animal group using Microsoft excel 2007 (Microsoft Inc., USA). All animal group results were statistically compared by one-way ANOVA followed by post Tuckey test and comparative graphs were plotted using Graphpad Prism statistical software (v5 demo, Graphpad Inc., CA, USA). Details of calculations done for titer dtermination are explained in appendix 7.6.

4.3. RESULTS AND DISCUSSION

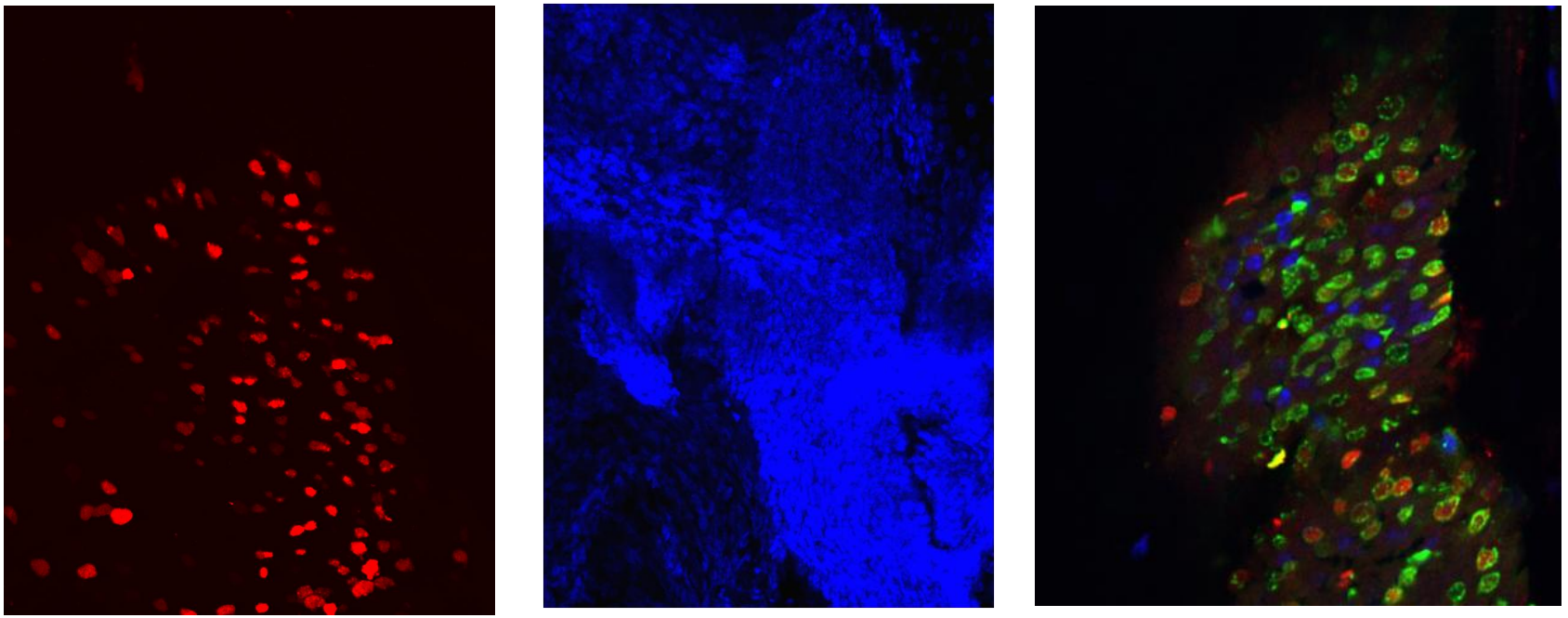
4.3.1. Selection of animal model for *in-vivo* studies

Mouse is the most frequently used animal in the literature for investigating immune responses against *Bordetella pertussis* because it can closely, albeit not in entirety, resembles the human immune response. Although humans are the only natural host for this bacterium, mice can be infected with *B. pertussis* with the exception of the absence of cough after infection, and have been used and accepted as a model for studies on immunity against this pathogen.[348, 349] As the animal experiments were designed to evaluate immune responses induced by nanoparticulate formulations, accompanied by the fact that the proposed work can not be carried out *in vitro*, mouse model was selected for the proposed studies. Female Balb/c mice were selected for all the *in-vivo* experiments because of their known less aggressive nature compared to the males of same breed which allows possibility of keeping a group of mice together in one cage without causing significant stress to any of the animals.

4.3.2. CLSM visualization of IgA-loaded CS-DS nanoparticles in nasal epithelia

To evaluate the site of uptake for IgA-loaded CS-DS nanoparticles on intranasal administration, a confocal laser scanning microscopy (CLSM) study using Balb/c mice as animal model was performed. First control animal nasal tissue was only treated with UEA-1 attached to TRITC flourescent marker for specific labeling of the M-cells in NALT as shown in Figure 4.1a. UEA-1 is a plant lectin reported to specifically bind with L-fucose present on the surface of M-cells in mice.[201, 350] Second control animal nasal tissue was only treated with Hoechst 33342 fluorescent marker for nonspecific labeling of every epithelial cell as Hoechst 33342 labels nuclei of each cell (Figure 4.1b). Visualization of IgA loaded nanoparticles in nasal epithelia after administration of loaded formulation administered through the nasal route to Balb/c mice was performed using anti-mouse IgA-FITC conjugated based on the specific antigen-antibody reaction of IgA-loaded nanoparticles with

anti-mouse IgA-FITC conjugated. FITC, TRITC and Hoechst all have distinct excitation and emission wavelengths which allow their concomitant use on a single tissue sample without any significant interference. The nasal tissues of animals administered with IgA loaded CS-DS nanoparticles were treated with all three markers (Hoechst 33342, UEA-I attached to TRITC and anti-mouse IgA FITC conjugated). The presence of FITC (green) attached to anti-mouse IgA around UEA-I conjugated TRITC (red) was observed (Figure 4.1c). This co-presence of green and red colour in confocal microscopy pictures indicates the preferential uptake of IgA loaded CS-DS nanoparticles in nasal associated lymphoid tissue (NALT) probably by M-cells. The z-scan series pictures of each of nasal tissue are compiled in Appendix 7.5.



(a) **(b)** **(c)**

Figure 4.1. CLSM visualization of nasal epithelia, (a) specific labelling of M-cells in NALT with UEA-TRITC; (b) non-specific labelling of epithelial cells in nasal mucosa with Hoechst 33342; (c) targeting of NALT by IgA-loaded nanoparticles.

As early as in 1992, Porta et al[351] reported that latex beads coated with sIgA instilled into mouse intestinal loops are taken up by M-cells present in Peyer's patch follicle-associated epithelial tissue. The group on the basis of their confocal study using fluorescent beads also stated that bead adsorption and uptake was greater at the edge compared with the apex of follicle domes. In an another study, Zhou et al[310] used IgA-coated liposomes for rectal delivery of a soluble model antigen and reported that the presence of IgA on the liposome surface increased the uptake of liposomes into Peyer's patch mucosa, and the local mucosal rectalo-colonic immune response to model antigen about 5-fold over uncoated liposomes. Inspite of these earlier studies through oral and rectal routes, any study reporting use of IgA loaded particulate delivery system for intranasal administration could not be found in literatue. The results of CLSM study carried out for this thesis support previous studies[310, 351] and further broaden the potential of IgA as M-cell targeting agent through mucosal routes. It is important to mention here that various previous studies[30, 314, 352, 353] have reported uptake of particulate delivery systems by M-cells situated in NALT on nasal administration of particulates. The confocal microscopy study in this thesis did not differentiate the contribution of nanoparticulate nature of delivery system and presence of IgA in nanoparticles on preferential uptake of IgA-loaded CS-DS nanoparticles by M-cells on nasal administration. Further studies are warranted to separately estimate the contribution of particulate nature of nanoparticles and IgA in preferential targeting of NALT by IgA loaded CS-DS nanoparticles.

4.3.3. *In-vivo* immunological comparative evaluation of formulations through subcutaneous route of administration

The developed PTXd loaded CS-DS nanoparticle formulations with or without mouse IgA were compared against a conventional alum adjuvanted PTXd formulation for induction of systemic, mucosal and cell-mediated immune responses through subcutaneous route of administration. Subcutaneous route is one of the parenteral routes normally used for vaccine administration and alum is the

regulatory approved as well as most commonly used adjuvant in vaccine formulations for parenteral administration.

For the quantitative comparison of humoral immune responses (systemic and mucosal), the raw absorbances data was processed to calculate titers. All the titer values for every animal and every immune response parameter were calculated using same method as described in detail in appendix 7.6. In brief, the mean of blanks (antigen and antibody free wells) was subtracted from normalized absorbances of samples and graphs were plotted with absorbance on Y axis versus dilution factor for sample on the X axis. A linear regression fit was performed for the absorbance values between 0.1 and 1.0 and linear trendline was extrapolated to X-axis to find the corresponding dilution factor as antibody titer. In other words, the cut-off point was 0.1 optical density (Appendix 7.6).

Table 4.4 and Figure 4.2 shows the comparative antigen-specific serum IgG levels induced by different formulations administered by subcutaneous route (raw data compiled in appendix 7.6). The PTXd loaded CS-DS nanoparticle formulations induced significantly higher serum IgG antibody levels compared to alum-adjuvanted PTXd formulation. The negative control group of animals administered with blank CS-DS nanoparticles showed negligible induction of serum IgG response. The level of significance in the difference between serum anti-PTXd IgG titer levels induced by IgA+PTXd loaded CS-DS nanparticles and alum adjuvanted PTXd formulation was higher ($p < 0.001$) compared to the difference between serum IgG titer levels induced by PTXd loaded CS-DS nanoparticles and alum-adjuvanted PTXd formulation ($p < 0.05$). Additionally, the level of significance for the difference between serum anti-PTXd IgG titers induced by PTXd loaded CS-DS nanoparticles formulation and alum adjuvanted PTXd formulation was found as $p = 0.057$. These results indicate that enhanced serum IgG response induced by IgA and PTXd loaded CS-DS nanoparticle formulation compared to alum adjuvanted PTXd formulation may be due to the synergistic adjuvant effect of CS-DS nanoparticles and IgA. However, lack of significant difference at $p < 0.05$ between serum anti-PTXd IgG titers induced by PTXd-loaded

CS-DS nanopaticle formulation and IgA+PTXd-loaded CS-DS nanoparticle formulation indicate that out of two contributing sources of adjuvant activity CS-DS nanoparticles may play a major role.

Table 4.3. Antigen specific serum IgG antibodies titer induced in animals by different formulations administered by subcutaneous route

Animal	PTXd + alum	PTXd in CS-DS nanoparticles	PTXd and IgA in CS-DS nanoparticles	Blank CS-DS nanoparticles
1	275.92	2276.67	1862.33	Negligible
2	239.35	259.78	1665.25	Negligible
3	138.00	452.54	1554.24	Negligible
4	87.80	861.88	2141.17	Negligible
5	439.80	1641.20	2091.50	Negligible
6	794.43	1540.25	2020.50	Negligible
MEAN	**329.22**	**1172.05**	**1889.16**	**N/A**
SE	**115.66**	**317.38**	**97.42**	**N/A**

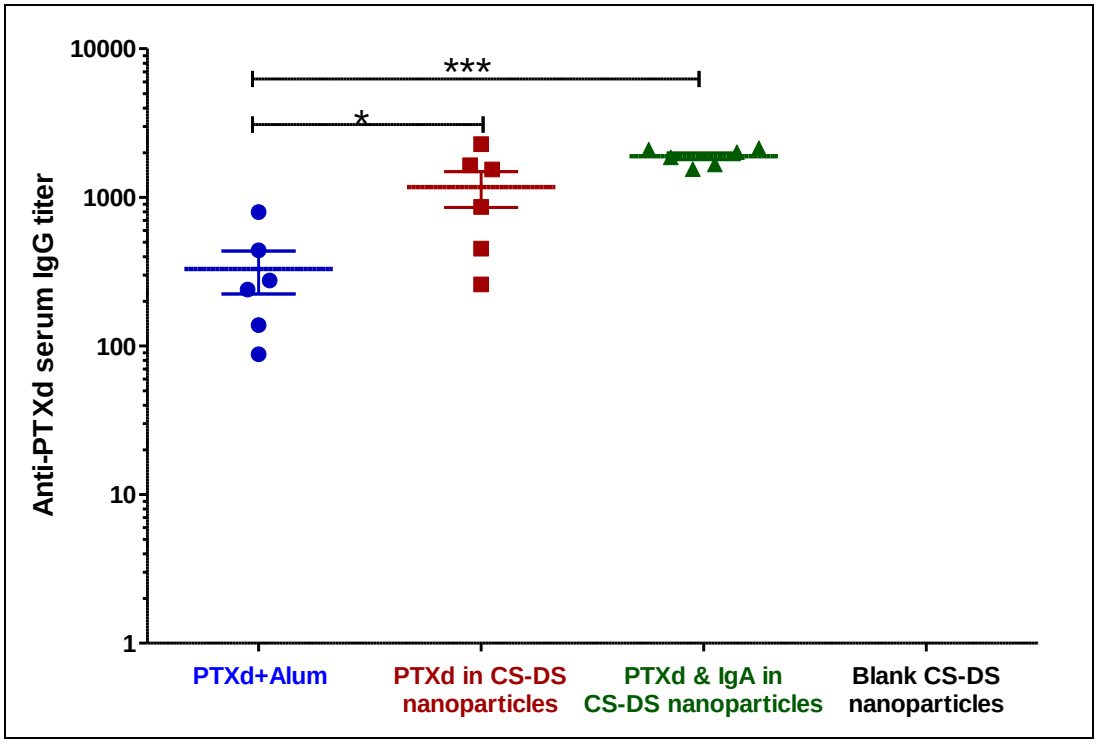


Figure 4.2. Comparative antigen specific serum IgG antibodies level induced by different formulations administered by subcutaneous route (* $p < 0.05$; *** $p < 0.001$; error bar represents standard error)

Production of immunoglobulin isotypes is associated with different T-helper responses. The serum IgG subclass profiles are utilized as an indicative of Th1 or Th2 responses against an antigen.[248, 354, 355] Th1 cells secrete IFN-γ and interleukin-2 (IL-2), promoting the production of IgG2a. Th2 cells secrete IL-4, IL-5 and IL-10, promoting the production of IgG1.[356] Table 4.5 and Figure 4.3 shows antigen specific serum IgG1 levels induced by different formulations administered through subcutaneous route (raw data compiled in appendix 7.6). The results for serum IgG1 are in accord with the results for serum IgG and indicate that CS-DS nanoparticle formulation with or without IgA induced significantly higher ($p < 0.05$ or $p < 0.01$) IgG1 antibody immune response compared to alum adjuvanted conventional formulation. Similar to serum IgG results this can be interpreted from IgG1 results that CS-DS nanoparticle formulation/delivery system has major effect

in induction of enhanced serum IgG1 antibody levels with a minor synergestic effect in presence of IgA in the formulation.

Table 4.4. Antigen specific serum IgG1 antibodies titer induced in animals by different formulations administered by subcutaneous route

Animal	PTXd + alum	PTXd in CS-DS nanoparticles	PTXd and IgA in CS-DS nanoparticles	Blank CS-DS nanoparticles
1	291.28	2179.25	2184.25	Negligible
2	990.22	1888.40	982.87	Negligible
3	154.00	287.16	988.60	Negligible
4	514.81	2000.50	1220.87	Negligible
5	522.00	1087.50	2034.40	Negligible
6	240.39	1022.83	2270.50	Negligible
MEAN	**452.12**	**1410.94**	**1613.58**	**N/A**
SE	**135.34**	**299.11**	**250.13**	**N/A**

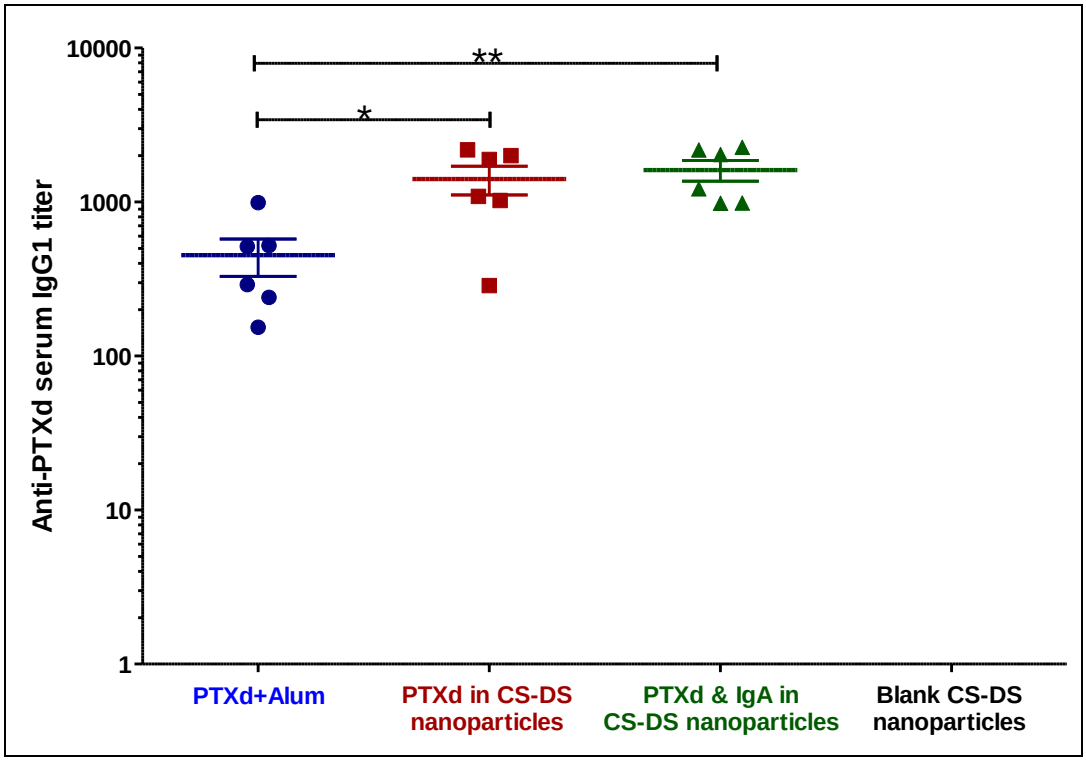


Figure 4.3. Comparative antigen specific serum IgG1 antibodies level induced by different formulations administered by subcutaneous route (* $p < 0.05$; ** $p < 0.01$; error bar represents standard error)**.**

The results obtained for antigen specific serum IgG2a antibody response are shown in Figure 4.4 and Table 4.6 (raw data included in appendix 7.6). There was no significant difference in the level of IgG2a antibody response induced by different PTXd formulations administered through subcutaneous route and overall, the serum IgG2a antibody response was lower compared to serum IgG1 antibody response for all PTXd formulations. Similar results were also obtained for IgG2b antibody response as shown in Figure 4.5 and Table 4.7. In addition, all the animals in the animal group administered with PTXd formulations did not show IgG2a and IgG2b immune response i.e, some of the animal(s) per animal group showed negligible or zero IgG2 immune response (Figure 4.4 and 4.5).

The results of IgG1 and IgG2a antibody response indicate induction of a predominant Th2 immune response by all PTXd formulations as the ratio of IgG2a to IgG1 antibodies in serum was found to be smaller than 1 for all PTXd formulations. On the other hand, the number of animals per group that showed IgG2a immune response was found higher in case of PTXd-loaded CS-DS nanoparticles compared to alum adjuvanted PTXd formulation as well as IgA and PTXd-loaded CS-DS nanoparticles formulation (Figure 4.4). This result suggests that the chitosan nanoparticulate formulation may have some adjuvant properties relating to Th1 cells.

To further investigate the cell-mediated immune response induced by different PTXd formulations, IFN-γ in splenocyte culture supernatants of each animal group was estimated (data included in appendix 7.6). The IFN-γ ELISA results showed that in the presence of Con-A all the splenocyte cultures obtained from different animal groups (including negative control group) irrespective of the administration of any formulation secreted high concentration of IFN-γ. Contrary to Con-A treatment, all the splenocytes cultures incubated with PBS secreted very low concentrations of IFN-γ. Surprisingly though, all the splenocyte culture supernatants irrespective of the differences in immunization of different groups showed absorbances higher than 1 in IFN-γ ELISA when the splenocytes were incubated with PTX (appendix 7.6). The results obtained from splenocyte cultures when incubated with PTX could be because of the use of PTX and not PTXd as stimulant in splenocyte culture incubation as PTX has been demonstrated as a strong mitogen for T-lymphocytes.[357-359] Hence, for complete evaluation of the Th1 (cell mediated) immunological adjuvant properties of CS-DS formulations, further immunological studies with higher doses of PTXd, larger size animal groups and modified methods for cell-mediated immune response estimation are warranted. These studies could not be carried out because of time and financial constraints.

Table 4.5. Antigen specific serum IgG2a antibodies titer induced in animals by different formulations administered by subcutaneous route

Animal	PTXd + alum	PTXd in CS-DS nanoparticles	PTXd and IgA in CS-DS nanoparticles	Blank CS-DS nanoparticles
1	Negligible	224.87	Negligible	Negligible
2	217.23	249.18	Negligible	Negligible
3	Negligible	Negligible	117.50	Negligible
4	122.08	238.67	205.28	Negligible
5	77.62	57.84	201.09	Negligible
6	59.56	279.88	231.31	Negligible
MEAN	**119.12**	**210.09**	**188.80**	**N/A**
SE	**35.24**	**39.12**	**24.69**	**N/A**

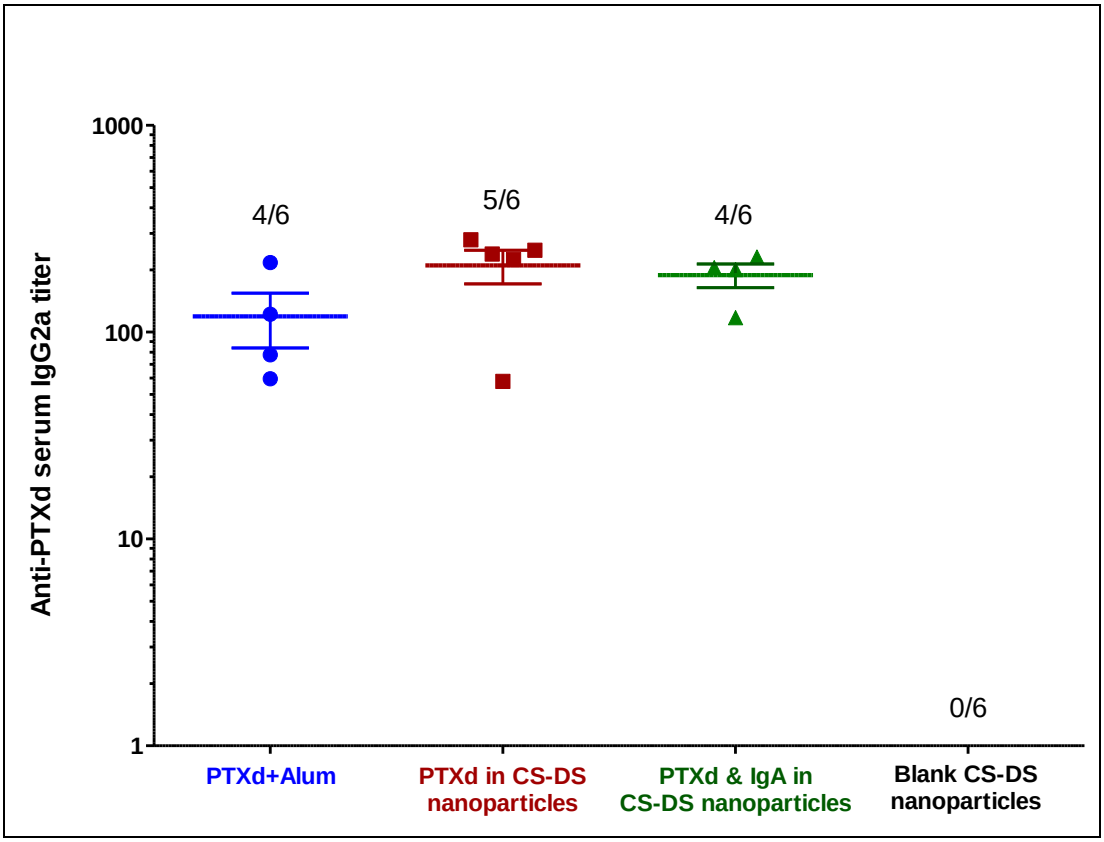


Figure 4.4. Comparative antigen specific serum IgG2a antibodies level induced by different formulations administered by subcutaneous route (error bar represents standard error; fractional numbers in graph represent the number of animals in a group showed measurable antibody titer as numerator and total number of animals in a group as denominator).

Table 4.6. Antigen specific serum IgG2b antibodies titer induced in animals by different formulations administered by subcutaneous route

Animal	PTXd + alum	PTXd in CS-DS nanoparticles	PTXd and IgA in CS-DS nanoparticles	Blank CS-DS nanoparticles
1	Negligible	394.18	Negligible	Negligible
2	204.81	241.26	Negligible	Negligible
3	Negligible	Negligible	52.08	Negligible
4	55.19	59.63	230.83	Negligible
5	60.99	68.84	216.43	Negligible
6	Negligible	50.95	233.00	Negligible
MEAN	**106.99**	**162.97**	**183.08**	**N/A**
SE	**48.93**	**67.70**	**43.82**	**N/A**

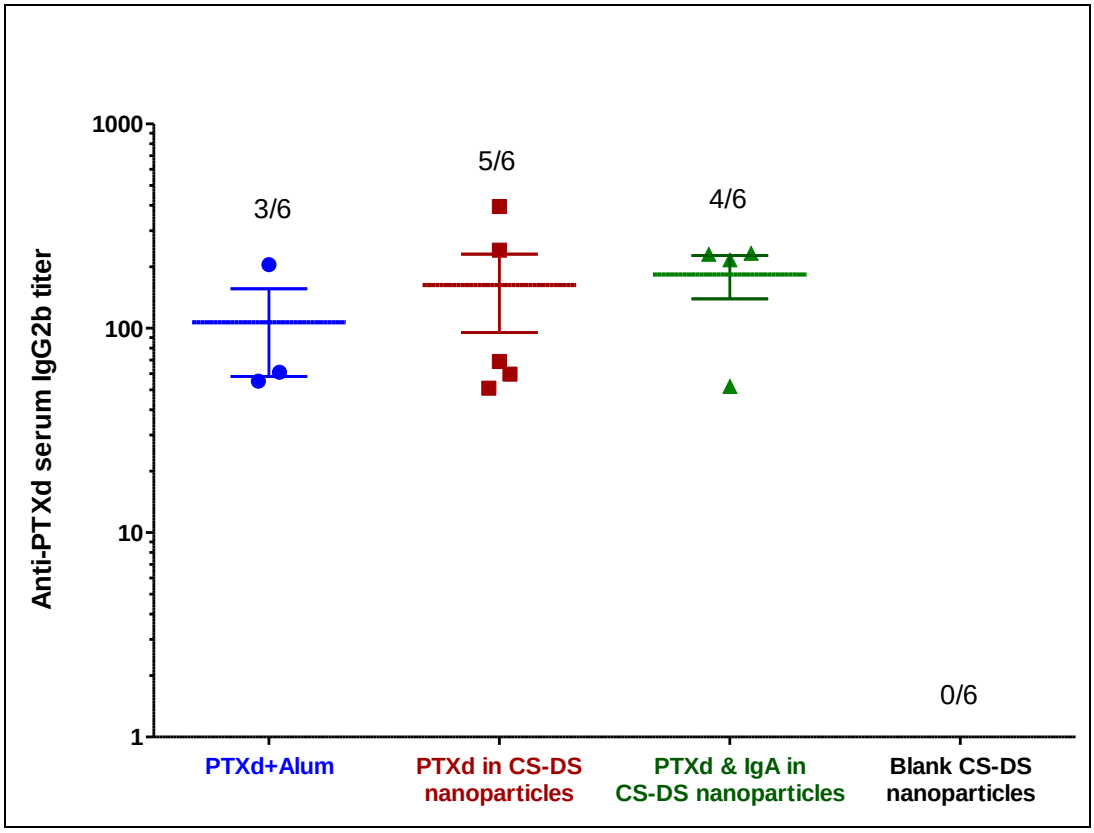


Figure 4.5. Comparative antigen specific serum IgG2b antibodies level induced by different formulations administered by subcutaneous route (error bar represents standard error; fractional numbers in graph represent the number of animals in a group showed measurable antibody titer as numerator and total number of animals in a group as denominator).

The quantification of anti-PTXd sIgA was performed on lung homongenate supernatants. The subcutaneous administration of the vaccine formulations was unable to induce the production of anti-PTXd IgA as expected[360] (raw data included in appendix 7.6).

It is noted that various studies in last two decades[361-363] have investigated utility of chitosan based particles through mucosal routes for vaccine delivery but there are fewer studies[364, 365] reporting chitosan particulate delivery systems for parenteral vaccine administration. Zhu et al[365] developed the chitosan microspheres and used to deliver a fusion protein through subcutaneous route. The group reported that the fusion protein incorporated in chitosan microspheres elicited strong humoral and cell-mediated immune response. The authors have explained the enhancement in immune response as a result of readily uptake of chitosan microspheres with appropriate size (~ 1-10μm) by APCs and release of antigen within the cell when chitosan is degraded by the lysozymes. It is interesting to note here that though various studies have used particulate delivery systems for vaccine delivery and reported the effect of particle size on induction of immune responses, there is still a lack of clear understanding on this formulation parameter. Xiang et al.[23] suggested that particles of different particles size ranges may follow different endocytic pathways and be preferentially taken up by different phagocytic cells. If uptake and processing of different sized particles occurs via different mechanisms, these may bias immune responses to generate a specific type of immunity. Particles in the size range of 20–200 nm are usually taken up by receptor-mediated endocytosis and elicit a cellular biased response, whereas particles with the size between 0.5 μm and 5.0 μm are predominantly taken up by phagocytosis and/or macropinocytosis and elicit a humoral response.[23, 339]

Borges et al[65] studied the alginate coated chitosan nanoparticles adsorbed with hepatitis B surface antigen (HBsAg) through subcutaneous route as vaccine delivery system. The authors reported higher anti-HBsAg serum IgG titer compared to control group, with the majority of antibodies being of Th2 type, when animals vaccinated with HBsAg loaded onto coated nanoparticles. These authors further reported that the co-delivery of antigen-loaded chitosan nanoparticles with or without alginate coating in the presence of the immunopotentiator (CpG ODN 1826) resulted in an increase of anti-HBsAg IgG titers that was not statistically different from the group receiving HBsAg loaded alginate coated nanoparticles; however, an increase of the IgG2a/IgG1 ratio from 0.1 to 1.0 and an increase ($p < 0.01$) of the

IFN-γ production by the splenocytes stimulated with the HBV antigen was observed. This study indicates that presence of an immunostimulator for Th1 cells in the formulation is probably crucial for induction of high cell-mediated immune responses except that the antigen itself has the Th1-inducing immunogenic properties.

In addition, various studies[366, 367] have reported efficient delivery of vaccines using chitosan solution or gel formulations administerd through parenteral route of administration. Interestingly, Gordon et al[53] compared the chitosan gel formulation against chitosan nanoparticle formulation using ovalbumin as model protein antigen through subcutaneous route of administration. The research group reported that ovalbumin-loaded chitosan nanoparticles showed no significant *in-vivo* immunogenicity inspite of their ability to activate APCs *in-vitro* but formulations of ovalbumin in chitosan gel were able to stimulate both cell-mediated and humoral immunity *in-vivo*. These unexpected results are explained by the authors as a result of difference in release profile of antigen from nanoparticle and gel formulations and different *in-vivo* conditions for different route of administration.

Overall, the *in-vivo* results of the study embodied in this thesis and the results of previously reported studies permit postulation of the hypothesis that depot formation by CS-DS nanoparticles at the site of injection with slow and sustained release of antigen from formulation as well as immunogenic properties of chitosan may have resulted in enhanced immune responses compared to alum-ajuvanted parenteral formulation. In addition, incorporation of IgA in CS-DS nanoparticulate formulation may have provided synergestic immune effect especially in induction of Th2 immune responses. However, futher investigations to test this hypothesis are warranted.

4.3.4. *In-vivo* immunological comparative evaluation of formulations through intranasal route of administration

The developed PTXd loaded CS-DS nanoparticle formulations with or without mouse IgA were compared against a PTXd solution formulation for

induction of systemic, mucosal and cell-mediated immune responses through intranasal route of administration. Intranasal route has been extensively studied in recent past as mucosal route of administration for vaccines with even few commercial successes (Flumist® influenza vaccine). In this study, the intention was to assess the effect of incorporation of a M cell targeting ligand (mouse IgA) in CS-DS nanoparticles on induction of immune responses against the co-administered antigen by intranasal route.

Table 4.8 and Figure 4.6 shows comparative antigen specific serum IgG levels induced by different formulations administered through intranasal route. All the PTXd formulations showed significantly higher serum IgG immune response compared to negative control groups (administered with buffer only or IgA loaded CS-DS nanoparticles) but no significant difference was found in serum IgG levels induced by different PTXd loaded CS-DS nanoparticles compared to PTXd solution. In addition, the antigen specific serum IgG titers induced by intranasal administration of PTXd-formulations were found about 10 to 100 times lower compared to subcutaneous administration of similar formulations. Furthermore, the other serum and mucosal immune responses (serum IgG1, IgG2a, IgG2b and lung homogenate IgA) were found almost negligible (raw data included in Appendix 7.6).

Table 4.7. Antigen specific serum IgG antibodies titer induced in animals by different formulations administered by intranasal route

Animal	PTXd solution	PTXd in CS-DS nanoparticles	PTXd and IgA in CS-DS nanoparticles	IgA in CS-DS nanoparticles	Buffer only
1	28.43	24.57	22.05	Negligible	Negligible
2	78.13	28.37	61.31	Negligible	Negligible
3	21.41	61.72	19.10	Negligible	Negligible
4	30.12	46.59	71.39	Negligible	Negligible
5	22.48	31.93	19.32	Negligible	Negligible
6	24.94	29.71	64.98	Negligible	Negligible
MEAN	**34.25**	**37.15**	**43.03**	**N/A**	**N/A**
SE	**9.73**	**5.80**	**10.32**	**N/A**	**N/A**

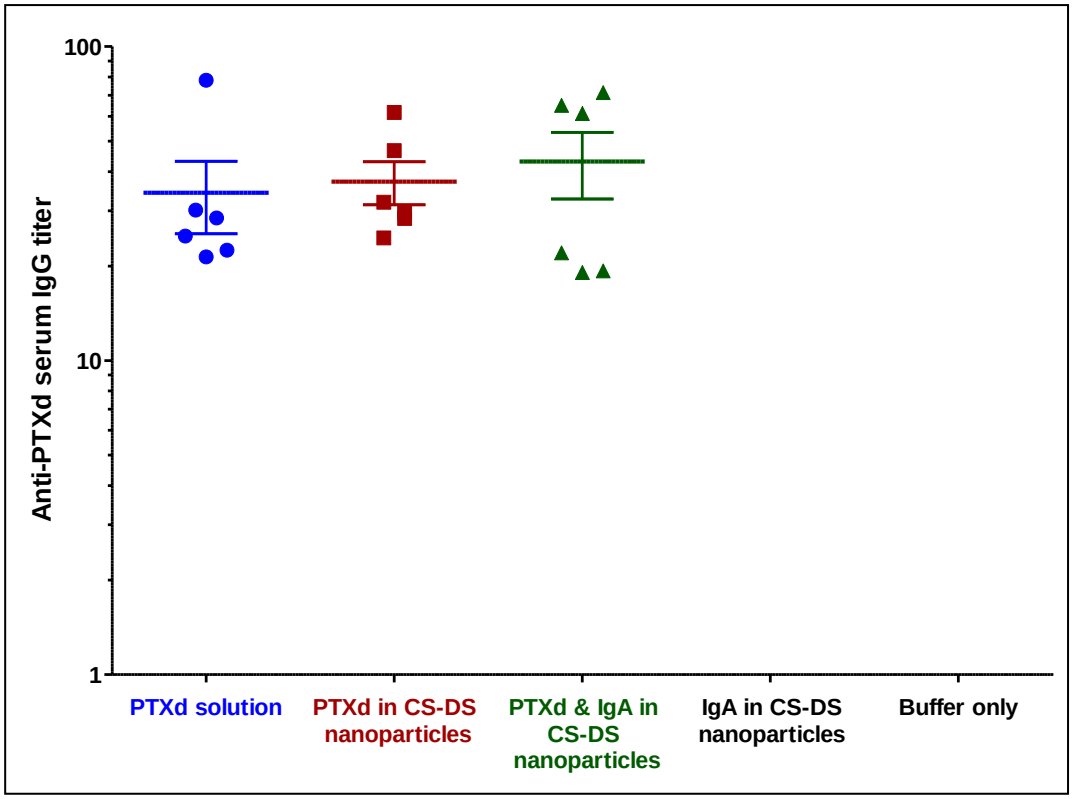


Figure 4.6. Comparative antigen specific serum IgG antibodies level induced by different formulations administered by intranasal route (error bar represents standard error).

The overall low immune responses and lack of enhancement in immune responses by PTXd-loaded CS-DS nanoparticle formulations may be explained as follows:

- The same dose of PTXd (10µg per mouse) was used for both subcutaneous and intranasal administration and it is generally accepted that at same dose mucosal administration of vaccines induce comparatively lower immune responses than parenteral administration. It is very common with mucosal administration especially through intranasal administration that a proportion of dose either get swallowed in GIT or expelled out of noses which result in lower effective dose compared to parenteral administration where 100% of administered dose is

effective.[78, 368] Newer delivery spray-devices (e.g. Optinose BD device[368] and Becton-Dickenson Accuspray™ device) have been developed and suggested to be used for improved efficiency in intranasal delivery in human beings. Currently, there is no commercial device available for an accurate dose administration to mouse model via the nasal route.

- The ELISA method used estimation of immune responses may not be sufficiently sensitive for detecting small differences between different animal groups and techniques with higher sensitivity such as use of fluorescent substrates or radioimmuno assays might be useful in comparative immunological studies.

- The slow and sustained release of PTXd from CS-DS nanoparticles may have been beneficial in case of subcutaneous administration because of the possibility of depot formation at the site of injection but in case of intranasal administration slow and sustained release of PTXd may have slowed substantial exposure of PTXd to immune system. Sharma et al[78] reviewed the effect of release characterstics of a vaccine delivery sytem on induction of immune response and emphasized that the relationship between the rate of antigen availability and induction of immunogenic responses is inadequately understood. However, it has been suggested that antigen presentation by APCs to naïve and effector T-cells may only be required over the first few days for an efficient induction of T-cell expansion and differentiation, and that the prolonging of antigen presentation for weeks or months may lead to T-cell death, decreased effector expansion and decreased cytokine production by recovered effectors.[369]

To summarize, the *in-vivo* studies conducted in this investigation indicate that CS-DS nanoparticulate delivery system could be useful in the delivery of vaccines through subcutaneous route of administration as this system significantly enhances the Th2 type immune responses against antigen. In addition, IgA

incorporation along with antigen in CS-DS nanoparticles may further enhance the induction of humoral immune response. Studies embodied in this thesis signified the importance of route of administration and probably also the release profile of antigen from CS-DS nanoparticles in the induction of strong immune responses against antigen. Studies embodied in this thesis, along with previously reported studies[1, 132, 276, 370] on the development of antigen delivery systems confirm the need to investigate both the pharmaceutical as well as immunological parameters such as release profile of antigen, the effect of the targeting ligands, route of administration, type of immune response desired and required antigen dose for particular routes of administration, for different vaccine candidate antigens.

CHAPTER 5

GENERAL DISCUSSION AND SCOPE OF FUTURE RESEARCH

5.1. GENERAL DISCUSSION

There is an ever increasing need for the development of delivery systems capable of meeting the challenge presented by the increasing proportion of new therapeutically active molecules of biological or biotechnology origin rather than conventional synthetic chemical entities. In addition there is a drive to replace the conventional injection with less invasive techniques that in the case of vaccines may also offer a broader immunological response. Research and development of particulate vaccine delivery systems, especially for newer sub-unit protein and nucleic acid based antigens has received significant attention recently due to their potential to co-deliver multiple active ingredients and induce a higher immune response compared to simple vaccine liquid formulations.

In this research project, CS-DS nanoparticles were prepared using a complex coacervation (polyelectrolyte complexation) technique. Particle size and zeta potential of the nanoparticles were used as initial optimization parameters. The optimized nanoparticle formulation was loaded with a model protein antigen (pertussis toxoid) and/or a potential immunological adjuvant with M cell targeting prospective (IgA). This nanoparticle system was evaluated for *in-vitro* parameters using various techniques including photon correlation spectroscopy, ELISA and SDS-PAGE. *In-vivo* studies were performed to compare and evaluate the immune responses induced by the developed formulations administered via the nasal and subcutaneous route.

CS-DS blank nanoparticles of size and zeta potential in the range of 150 - 400 nm and -40 to +60 mV respectively, were obtained by the simple method of preparation. The effect of pH change on particle size and zeta potential of CS-DS nanoparticles was studied by adjusting the pH of final formulations and most of the CS-DS nanoparticle formulations were found to be unstable at basic pH values. The instability of CS-DS nanoparticles in a basic pH environment can be explained by neutralization of charge over nanoparticles with increase in pH. This increases the probability of agglomeration of the nanoparticles as electrostatic repulsive forces between the nanoparticles were reduced, as supported by the zeta-potential approaching zero. The ratio of CS to DS,

order of mixing and pH of nanoparticle formulation were identified as important formulation factors governing the size and zeta potential of nanoparticles, which in turn affect the stability of the formulation. An optimized blank CS-DS nanoparticle formulation with particle size 314.7 ± 9.2 nm, zeta potential +53.2 ± 4.4 mV and isoelectric pH 8.9 (prepared with CS to DS weight ratio of 3:1) was used to load the antigen and/or IgA. This choice was based on the pH stability study and potential immunological efficiency. The entrapment efficiency of IgA and model protein in the CS-DS nanoparticles was significantly higher on addition in CS solution compared to DS solution during the preparation procedure. This might be due to the higher molecular weight of the chitosan compared to dextran sulfate, resulting in an increased possibility of intermingling of macromolecules with the chitosan compared to dextran sulfate. All loaded nanoparticles showed an initial release of < 15% followed by no significant release of IgA or PTX. This is important in ensuring that most of the active ingredients in the formulation remain entrapped in nanoparticles at the time of administration. However it is also important to mention here that it is not clearly known what kind of release profile of antigen from particulate vaccine delivery systems is most suitable for induction of strong immune responses. Furthermore, the route of administration could be another factor which should be considered along with release profile because it is highly likely that a similar *in-vitro* release profile of an antigen may lead to different type or magnitude of immune response through a different *in-vivo* route of administration. The *in-vitro* studies highlight the importance of pharmaceutical factors in the successful formulation of a vaccine. But, further research is warranted in the possibility of large scale production; antigen protective ability and long term storage stability of the nanoparticulate formulations. Freeze drying (lyophilization) has been reported by various recent studies[371, 372] as a promising technique for long-term stable storage of protein-loaded chitosan nanoparticle formulations. These research studies provide the platform on which scale-up technologies required for industrial production of nanoparticulate vaccines could be developed in future.

Visualization of IgA loaded nanoparticles in nasal epithelia after administration through the nasal route to Balb/c mice was performed using confocal microscopy. The *in-vivo* uptake study of IgA-loaded nanoparticles in the nasal epithelia of Balb/c mice

indicated the preferential uptake of nanoparticles in nasal associated lymphoid tissue (NALT) probably by M-cells. The immunological evaluation of developed formulations in Balb/c mice groups showed that CS-DS nanoparticle formulations induced higher serum IgG and IgG1 titers compared to a conventional alum adjuvanted PTXd formulation, administered by the subcutaneous route. The absence of mucosal immune responses and induction of relatively weaker cellular immune responses indicated preferential enhancement of Th2 type immune responses by CS-DS nanoparticle formulations. Intranasal administration of various PTXd formulations (CS-DS nanoparticle or solution formulations) failed to induce immune responses comparable to subcutaneous administration of the same formulations incorporating the same quatities of PTXd. It can be speculated that this may be due to the lack of availability of efficient delivery techniques for intranasal administration of vaccines to small animal models. It is also hypothesized that the release profile of the antigen from the CS-DS nanoparticulate formulations may have played an important role in the magnitude of immune responses induced on intranasal vs sub-cutaneous administration of vaccine formulations. The results of the immunological studies also signifed the importance of the route of administration in induction of immune responses against antigen and pointed toward the need for individualised formulations for different antigens. But, this thesis did not explored the molecular or receptor level mechanisms involved with immunological adjuvant properties of CS-DS nanoparticles and IgA through different routes of administration which create a need of further investigations in this field.

5.2. CONCLUSIONS

This study highlights the potential of CS-DS nanoparticles as a simple and effective particulate delivery system for acellular subunit antigens. The particle size and zeta potential of CS-DS nanoparticles can be modulated by change in pH of formulation and ratio of CS to DS. The research embodied in this thesis also highlighted the importance of optimization of formulation and pharmaceutical factors such as entrapment efficiency, release profile and stability in the development of an efficient vaccine delivery system. The optimized positively charged nanoparticulate formulation loaded with IgA was preferentially taken up by the NALT area of the nasal mucosa. The

results indicate the potential use of IgA as a targeting agent for nanoparticulate vaccine delivery. The induction of enhanced humoral immune responses against antigen by CS-DS nanoparticle formulations with and without IgA administered by the subcutaneous route indicated the potential of CS-DS nanoparticles as a vaccine delivery system with in-built immunological adjuvant properties. The route of administration could be enlisted as one of important determinants in induction of type and magnitude of immune responses against antigen based on the findings of this research project. The immunological studies also indicated the potential relevance of IgA as a novel non-pathogen derived immunological adjuvant for particulate vaccine delivery systems. The previous reports[307,308] and this study point toward IgA as a potentially useful formulation ingredient in vaccine delivery systems serving different purposes (M-cell targeting agent and immunostimulant) depending on route of administation. Notably, further studies with in-depth in-vivo immunological evaluations are required to make any conclusion on the real potential of CS-DS nanoparticles as a vaccine delivery system and IgA as an immunolgical adjuvant.

5.3. SCOPE FOR FUTURE RESEARCH

This research project has successfully explored some of the unknown areas in the field of vaccine delivery system and opens new research leads which could be investigated in future. The important research leads which could be followed in further studies are:

- Development and validation of methods for *in-vitro* quantification of detoxified PTX with a view to analysing the immune responses using chemically and genetically detoxified PTX. Further investigations in the development of *in-vitro* quantification methods for PTXd would be important measuring antigen loading in nanoparticles.

- The short term stability (about 48 hours) of CS-DS nanoparticles in aqueous environment presents a need of further exploration in lyophilisation or other type of solidification/drying methods to get long-term storage stability for CS-DS nanoparticulate formulations.

- ❖ Future investigations are required in evaluation of the duration of the immune response induced by nanoparticulate formulations through different route of administration.

- ❖ Further immunological *in-vivo* studies through different routes of administration in larger size animal groups with higher doses of PTXd using CS-DS nanoparticles as vaccine delivery system. In addition, microbial challenge studies to see the protective efficacy of CS-DS nanoparticulate vaccine formulations should be undertaken *in-vivo*. In addition, quality of antibodies generated by the CS-DS formulations using neutralization assays is desirable.

- ❖ Development and evaluation (*in-vitro* and *in-vivo*) of CS-DS nanoparticulate formulations using other antigens including different type of newer antigens such as other protein antigens, nucleic acid based antigens, synthetic peptides and carbohydrate antigens.

- ❖ Further *in-vivo* studies to evaluate M-cell targeting and potential immunological adjuvant properties of IgA with different antigens with or without different particulate delivery systems. Further studies are warranted to investigate immunological adjuvant properties of IgA on parenteral administration with an emphasis on elucidation of mechanism of actions involved.

- ❖ The effect of formulation factors such as particle size, surface charge, release profile and presence of different ingredients in formulation on the induction of immune responses against incorporated antigen need to be investigated with an emphasis on the understanding of molecular mechanisms involved.

It is expected that the investigations in above research leads would result in the generation of sufficient information and knowledge that could guide in development of a successful vaccine product. Overall, this investigation has made a significant contribution to the knowledge base pertaining to the development of nanoparticle based vaccine formulations against infectious diseases.

CHAPTER 6

REFERENCES

1. Kersten G, Hirschberg H. Antigen delivery systems. Expert Rev Vaccines. 2004; 3(4):453-62.
2. Brandtzaeg P, Pabst R. Let's go mucosal: communication on slippery ground. Trends Immunol. 2004; 25(11):570-7.
3. Langman JM, Rowland R. The number and distribution of lymphoid follicles in the human large intestine. J Anat. 1986; 149:189-94.
4. Brandtzaeg P. Overview of the mucosal immune system. Curr Top Microbiol Immunol. 1989; 146:13-25.
5. McGhee JR, Czerkinsky C, Mestecky J. Mucosal vaccines: an overview. In: Ogra PL, Lamm ME, Bienenstock J, Mestecky J, Strober W, McGhee JR, editors. Mucosal Immunology. 2nd ed. San Diego: Academic Press; 1999. p. 741–757.
6. Reddy ST, Swartz MA, Hubbell JA. Targeting dendritic cells with biomaterials: developing the next generation of vaccines. Trends Immunol. 2006; 27(12):573-9.
7. Reddy ST, van der Vlies AJ, Simeoni E, Angeli V, Randolph GJ, O'Neil C P, et al. Exploiting lymphatic transport and complement activation in nanoparticle vaccines. Nat Biotechnol. 2007; 25(10):1159-64.
8. O'Hagan DT, Valiante NM. Recent advances in the discovery and delivery of vaccine adjuvants. Nat Rev Drug Discov. 2003; 2(9):727-35.
9. Jegerlehner A, Tissot A, Lechner F, Sebbel P, Erdmann I, Kundig T, et al. A molecular assembly system that renders antigens of choice highly repetitive for induction of protective B cell responses. Vaccine. 2002; 20(25-26):3104-3112.
10. Bachmann MF, Rohrer UH, Kundig TM, Burki K, Hengartner H, Zinkernagel RM. The influence of antigen organization on B cell responsiveness. Science. 1993; 262(5138):1448-51.
11. O'Hagan DT. Recent developments in vaccine delivery systems. Curr Drug Targets Infect Disord. 2001; 1(3):273-86.
12. Frey A, Giannasca KT, Weltzin R, Giannasca PJ, Reggio H, Lencer WI, et al. Role of the glycocalyx in regulating access of microparticles to apical plasma membranes of intestinal epithelial cells: implications for microbial attachment and oral vaccine targeting. J Exp Med. 1996; 184(3):1045-59.

13. Jones BD, Ghori N, Falkow S. Salmonella typhimurium initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. J Exp Med. 1994; 180(1):15-23.
14. Neutra MR, Giannasca PJ, Troidle K, Kraehenbuhl JP. M cells and microbial pathogens. In: Blaser MJ, Smith PD, Ravdin JI, Greenberg HB, Guerrant L, editors. Infections of the Gastrointestinal Tract. New York: Raven; 1995. p. 163-178.
15. Owen RL. Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine: an ultrastructural study. Gastroenterology. 1977; 72(3):440-51.
16. Eyles JE, Carpenter ZC, Alpar HO, Williamson ED. Immunological aspects of polymer microsphere vaccine delivery systems. J Drug Target. 2003; 11(8-10):509-14.
17. Sun H-X, Xie Y, Ye Y-P. ISCOMs and ISCOMATRIX(TM). Vaccine. 2009; 27(33):4388-4401.
18. Eldridge JH, Staas JK, Meulbroek JA, Tice TR, Gilley RM. Biodegradable and biocompatible poly(DL-lactide-co-glycolide) microspheres as an adjuvant for staphylococcal enterotoxin B toxoid which enhances the level of toxin-neutralizing antibodies. Infect Immun. 1991; 59(9):2978-86.
19. van der Lubben IM, Kersten G, Fretz MM, Beuvery C, Coos Verhoef J, Junginger HE. Chitosan microparticles for mucosal vaccination against diphtheria: oral and nasal efficacy studies in mice. Vaccine. 2003; 21(13-14):1400-1408.
20. Voltan R, Castaldello A, Brocca-Cofano E, Altavilla G, Caputo A, Laus M, et al. Preparation and characterization of innovative protein-coated poly(methylmethacrylate) core-shell nanoparticles for vaccine purposes. Pharm Res. 2007; 24(10):1870-82.
21. Vila A, Gill H, McCallion O, Alonso MJ. Transport of PLA-PEG particles across the nasal mucosa: effect of particle size and PEG coating density. J Control Release. 2004; 98(2):231-44.
22. Koping-Hoggard M, Sanchez A, Alonso MJ. Nanoparticles as carriers for nasal vaccine delivery. Expert Rev Vaccines. 2005; 4(2):185-96.

23. Xiang SD, Scholzen A, Minigo G, David C, Apostolopoulos V, Mottram PL, et al. Pathogen recognition and development of particulate vaccines: Does size matter? Methods. 2006; 40(1):1-9.
24. Storni T, Kundig TM, Senti G, Johansen P. Immunity in response to particulate antigen-delivery systems. Adv Drug Deliv Rev. 2005; 57(3):333-355.
25. Liang MT, Davies NM, Blanchfield JT, Toth I. Particulate systems as adjuvants and carriers for peptide and protein antigens. Curr Drug Deliv. 2006; 3(4):379-88.
26. Chiellini E, Orsini LM, Solaro R. Polymeric nanoparticles based on polylactide and related co-polymers. Macromol. Symp. 2003; 197:345-354.
27. Carcaboso AM, Hernandez RM, Igartua M, Rosas JE, Patarroyo ME, Pedraz JL. Potent, long lasting systemic antibody levels and mixed Th1/Th2 immune response after nasal immunization with malaria antigen loaded PLGA microparticles. Vaccine. 2004; 22(11-12):1423-1432.
28. Chen Y, Wang F, Benson HA. Effect of formulation factors on incorporation of the hydrophilic peptide dalargin into PLGA and mPEG-PLGA nanoparticles. Biopolymers. 2008; 90(5):644-50.
29. Vila A, Sanchez A, Tobio M, Calvo P, Alonso MJ. Design of biodegradable particles for protein delivery. J Control Release. 2002; 78(1-3):15-24.
30. Vajdy M, O'Hagan DT. Microparticles for intranasal immunization. Adv Drug Deliv Rev. 2001; 51(1-3):127-141.
31. Kersten GFA, Donders D, Akkermans A, Beuvery EC. Single shot with tetanus toxoid in biodegradable microspheres protects mice despite acid-induced denaturation of the antigen. Vaccine. 1996; 14(17-18):1627-1632.
32. Tobío M, Sánchez A, Vila A, Soriano I, Evora C, Vila-Jato JL, et al. The role of PEG on the stability in digestive fluids and in vivo fate of PEG-PLA nanoparticles following oral administration. Colloid Surf B: Biointer. 2000; 18(3-4):315-323.
33. Lavelle EC, Yeh MK, Coombes AGA, Davis SS. The stability and immunogenicity of a protein antigen encapsulated in biodegradable microparticles based on blends of lactide polymers and polyethylene glycol. Vaccine. 1999; 17(6):512-529.

34. Jabbal-Gill I, Lin W, Jenkins P, Watts P, Jimenez M, Illum L, et al. Potential of polymeric lamellar substrate particles (PLSP) as adjuvants for vaccines. Vaccine. 1999; 18(3-4):238-250.
35. Jabbal-Gill I, Lin W, Kistner O, Davis SS, Illum L. Polymeric lamellar substrate particles for intranasal vaccination. Adv Drug Deliv Rev. 2001; 51(1-3):97-111.
36. Coombes AGA, Major D, Wood JM, Hockley DJ, Minor PD, Davis SS. Resorbable lamellar particles of polylactide as adjuvants for influenza virus vaccines. Biomaterials. 1998; 19(11-12):1073-1081.
37. Bramwell VW, Perrie Y. Particulate delivery systems for vaccines: what can we expect? J Pharm Pharmacol. 2006; 58(6):717-28.
38. He X, Jiang L, Wang F, Xiao Z, Li J, Liu LS, et al. Augmented humoral and cellular immune responses to hepatitis B DNA vaccine adsorbed onto cationic microparticles. J Control Release. 2005; 107(2):357-372.
39. Mollenkopf H-J, Dietrich G, Fensterle J, Grode L, Diehl K-D, Knapp B, et al. Enhanced protective efficacy of a tuberculosis DNA vaccine by adsorption onto cationic PLG microparticles. Vaccine. 2004; 22(21-22):2690-2695.
40. Men Y, Tamber H, Audran R, Gander B, Corradin G. Induction of a cytotoxic T lymphocyte response by immunization with a malaria specific CTL peptide entrapped in biodegradable polymer microspheres. Vaccine. 1997; 15(12-13):1405-1412.
41. Moore A, McGuirk P, Adams S, Jones WC, McGee JP, O'Hagan DT, et al. Immunization with a soluble recombinant HIV protein entrapped in biodegradable microparticles induces HIV-specific CD8+ cytotoxic T lymphocytes and CD4+ Th1 cells. Vaccine. 1995; 13(18):1741-9.
42. Vajdy M, Srivastava I, Polo J, Donnelly J, O'Hagan D, Singh M. Mucosal adjuvants and delivery systems for protein-, DNA- and RNA-based vaccines. Immunol Cell Biol. 2004; 82(6):617-27.
43. Ruedl C, Bachmann MF, Kopf M. The antigen dose determines T helper subset development by regulation of CD40 ligand. Eur J Immunol. 2000; 30(7):2056-64.
44. Rogers PR, Croft M. Peptide dose, affinity, and time of differentiation can contribute to the Th1/Th2 cytokine balance. J Immunol. 1999; 163(3):1205-13.

45. Taghavi A, Allan B, Mutwiri G, Foldvari M, Van Kessel A, Willson P, et al. Enhancement of immunoprotective effect of CpG-ODN by formulation with polyphosphazenes against E. coli septicemia in neonatal chickens. Curr Drug Deliv. 2009; 6(1):76-82.
46. Buescher JM, Margaritis A. Microbial biosynthesis of polyglutamic acid biopolymer and applications in the biopharmaceutical, biomedical and food industries. Crit Rev Biotechnol. 2007; 27(1):1-19.
47. Uto T, Wang X, Akagi T, Zenkyu R, Akashi M, Baba M. Improvement of adaptive immunity by antigen-carrying biodegradable nanoparticles. Biochem Biophys Res Comm. 2009; 379(2):600-604.
48. Shih IL, Van YT, Shen MH. Biomedical applications of chemically and microbiologically synthesized poly(glutamic acid) and poly(lysine). Mini Rev Med Chem. 2004; 4(2):179-88.
49. Aono R. Characterization of structural component of cell walls of alkalophilic strain of Bacillus sp. C-125. Preparation of poly(gamma-L-glutamate) from cell wall component. Biochem J. 1987; 245(2):467-72.
50. Drysdale M, Heninger S, Hutt J, Chen Y, Lyons CR, Koehler TM. Capsule synthesis by Bacillus anthracis is required for dissemination in murine inhalation anthrax. EMBO J. 2005; 24(1):221-7.
51. Hamasaki T, Uto T, Akagi T, Akashi M, Baba M. Modulation of gene expression related to Toll-like receptor signaling in dendritic cells by poly(gamma-glutamic acid) nanoparticles. Clin Vaccine Immunol. 2010; 17(5):748-56.
52. Okamoto S, Matsuura M, Akagi T, Akashi M, Tanimoto T, Ishikawa T, et al. Poly([gamma]-glutamic acid) nano-particles combined with mucosal influenza virus hemagglutinin vaccine protects against influenza virus infection in mice. Vaccine. 2009; 27(42):5896-5905.
53. Gordon S, Saupe A, McBurney W, Rades T, Hook S. Comparison of chitosan nanoparticles and chitosan hydrogels for vaccine delivery. J Pharm Pharmacol. 2008; 60(12):1591-600.

54. Calvo P, Remunan-Lopez C, Vila-Jato JL, Alonso MJ. Chitosan and chitosan/ethylene oxide-propylene oxide block copolymer nanoparticles as novel carriers for proteins and vaccines. Pharm Res. 1997; 14(10):1431-1436.
55. Amidi M, Mastrobattista E, Jiskoot W, Hennink WE. Chitosan-based delivery systems for protein therapeutics and antigens. Adv Drug Deliv Rev. 2010; 62(1):59-82.
56. Soane RJ, Frier M, Perkins AC, Jones NS, Davis SS, Illum L. Evaluation of the clearance characteristics of bioadhesive systems in humans. Int J Pharm. 1999; 178(1):55-65.
57. Chen Y, Mohanraj V, Parkin J. Chitosan-dextran sulfate nanoparticles for delivery of an anti-angiogenesis peptide. Int J Pept Res Ther. 2003; 10(5-6):621-629.
58. Nagamoto T, Hattori Y, Takayama K, Maitani Y. Novel chitosan particles and chitosan-coated emulsions inducing immune response via intranasal vaccine delivery. Pharm Res. 2004; 21(4):671-4.
59. Janes KA, Fresneau MP, Marazuela A, Fabra A, Alonso MJ. Chitosan nanoparticles as delivery systems for doxorubicin. J Control Release. 2001; 73(2-3):255-267.
60. Sinha VR, Singla AK, Wadhawan S, Kaushik R, Kumria R, Bansal K, et al. Chitosan microspheres as a potential carrier for drugs. Int J Pharm. 2004; 274(1-2):1-33.
61. Yang X, Yuan X, Cai D, Wang S, Zong L. Low molecular weight chitosan in DNA vaccine delivery via mucosa. Int J Pharm. 2009; 375(1-2):123-132.
62. Ajdary S, Dobakhti F, Taghikhani M, Riazi-Rad F, Rafiei S, Rafiee-Tehrani M. Oral administration of BCG encapsulated in alginate microspheres induces strong Th1 response in BALB/c mice. Vaccine. 2007; 25(23):4595-4601.
63. Rebelatto MC, Guimond P, Bowersock TL, HogenEsch H. Induction of systemic and mucosal immune response in cattle by intranasal administration of pig serum albumin in alginate microparticles. Vet Immunol Immunopathol. 2001; 83(1-2):93-105.
64. Tafaghodi M, Sajadi Tabassi SA, Jaafari MR. Induction of systemic and mucosal immune responses by intranasal administration of alginate microspheres

encapsulated with tetanus toxoid and CpG-ODN. Int J Pharm. 2006 [cited 25 July 2007]; 319(1-2):37-43.

65. Borges O, Silva M, de Sousa A, Borchard G, Junginger HE, Cordeiro-da-Silva A. Alginate coated chitosan nanoparticles are an effective subcutaneous adjuvant for hepatitis B surface antigen. Int Immunopharmacol. 2008; 8(13-14):1773-1780.
66. Borges O, Tavares J, de Sousa A, Borchard G, Junginger HE, Cordeiro-da-Silva A. Evaluation of the immune response following a short oral vaccination schedule with hepatitis B antigen encapsulated into alginate-coated chitosan nanoparticles. Euro J Pharm Sci. 2007; 32(4-5):278-290.
67. McDermott MR, Heritage PL, Bartzoka V, Brook MA. Polymer-grafted starch microparticles for oral and nasal immunization. Immunol Cell Biol. 1998; 76(3):256-62.
68. Moynihan JS, Blair J, Coombes A, D'Mello F, Howard CR. Enhanced immunogenicity of a hepatitis B virus peptide vaccine using oligosaccharide ester derivative microparticles. Vaccine. 2002; 20(13-14):1870-1876.
69. Nakaoka R, Tabata Y, Ikada Y. Potentiality of gelatin microsphere as immunological adjuvant. Vaccine. 1995; 13(7):653-661.
70. Altin JG, Parish CR. Liposomal vaccines--targeting the delivery of antigen. Methods. 2006; 40(1):39-52.
71. Baca-Estrada ME, Foldvari M, Babiuk SL, Babiuk LA. Vaccine delivery: lipid-based delivery systems. J Biotechnol. 2000; 83(1-2):91-104.
72. Copland MJ, Rades T, Davies NM, Baird MA. Lipid based particulate formulations for the delivery of antigen. Immunol Cell Biol. 2005; 83(2):97-105.
73. Phillips NC, Gagné L, Ivanoff N, Riveau G. Influence of phospholipid composition on antibody responses to liposome encapsulated protein and peptide antigens. Vaccine. 1996; 14(9):898-904.
74. Allen TM, Austin GA, Chonn A, Lin L, Lee KC. Uptake of liposomes by cultured mouse bone marrow macrophages: influence of liposome composition and size. Biochim Biophys Acta - Biomembranes. 1991; 1061(1):56-64.
75. Fortin A, Therien HM. Mechanism of liposome adjuvanticity: an in vivo approach. Immunobiology. 1993; 188(3):316-22.

76. Kersten GFA, Crommelin DJA. Liposomes and ISCOMS as vaccine formulations. Biochim Biophys Acta - Reviews on Biomembranes. 1995; 1241(2):117-138.
77. Mann JF, Acevedo R, Campo JD, Perez O, Ferro VA. Delivery systems: a vaccine strategy for overcoming mucosal tolerance? Expert Rev Vaccines. 2009; 8(1):103-12.
78. Sharma S, Mukkur TK, Benson HA, Chen Y. Pharmaceutical aspects of intranasal delivery of vaccines using particulate systems. J Pharm Sci. 2009; 98(3):812-43.
79. Machluf M, Apte RN, Regev O, Cohen S. Enhancing the immunogenicity of liposomal hepatitis B surface antigen (HBsAg) by controlling its delivery from polymeric microspheres. J Pharm Sci. 2000; 89(12):1550-7.
80. Vyas SP, Khatri K, Mishra V. Vesicular carrier constructs for topical immunisation. Expert Opin Drug Deliv. 2007; 4(4):341-8.
81. Lee K-Y, Chun E, Seong BL. Investigation of Antigen Delivery Route in Vivo and Immune-Boosting Effects Mediated by pH-Sensitive Liposomes Encapsulated with Kb-Restricted CTL Epitope. Biochem Biophys Res Comm. 2002; 292(3):682-688.
82. Chang J-S, Choi M-J, Nejat D. pH-Sensitive Liposomes as Adjuvants for Peptide Antigens. In: Methods in Enzymology: Academic Press; 2003. p. 127-136.
83. Rao M, Alving CR. Delivery of lipids and liposomal proteins to the cytoplasm and Golgi of antigen-presenting cells. Adv Drug Deliv Rev. 2000; 41(2):171-188.
84. Estevez F, Carr A, Solorzano L, Valiente O, Mesa C, Barroso O, et al. Enhancement of the immune response to poorly immunogenic gangliosides after incorporation into very small size proteoliposomes (VSSP). Vaccine. 1999; 18(1-2):190-197.
85. Rodríguez T, Pérez O, Ménager N, Ugrinovic S, Bracho G, Mastroeni P. Interactions of proteoliposomes from serogroup B Neisseria meningitidis with bone marrow-derived dendritic cells and macrophages: adjuvant effects and antigen delivery. Vaccine. 2005; 23(10):1312-1321.
86. Perez O, Bracho G, Lastre M, Mora N, del Campo J, Gil D, et al. Novel adjuvant based on a proteoliposome-derived cochleate structure containing native

lipopolysaccharide as a pathogen-associated molecular pattern. Immunol Cell Biol. 2004; 82(6):603-10.

87. Treanor J, Nolan C, O'Brien D, Burt D, Lowell G, Linden J, et al. Intranasal administration of a proteosome-influenza vaccine is well-tolerated and induces serum and nasal secretion influenza antibodies in healthy human subjects. Vaccine. 2006; 24(3):254-262.
88. Langley JM, Halperin SA, McNeil S, Smith B, Jones T, Burt D, et al. Safety and immunogenicity of a Proteosome(TM)-trivalent inactivated influenza vaccine, given nasally to healthy adults. Vaccine. 2006; 24(10):1601-1608.
89. Plante M, Jones T, Allard F, Torossian K, Gauthier J, St-Félix N, et al. Nasal immunization with subunit proteosome influenza vaccines induces serum HAI, mucosal IgA and protection against influenza challenge. Vaccine. 2001; 20(1-2):218-225.
90. Cyr SL, Jones T, Stoica-Popescu I, Burt D, Ward BJ. C57Bl/6 mice are protected from respiratory syncytial virus (RSV) challenge and IL-5 associated pulmonary eosinophilic infiltrates following intranasal immunization with Protollin-eRSV vaccine. Vaccine. 2007; 25(16):3228-3232.
91. Jones T, Adamovicz JJ, Cyr SL, Bolt CR, Bellerose N, Pitt LM, et al. Intranasal Protollin(TM)/F1-V vaccine elicits respiratory and serum antibody responses and protects mice against lethal aerosolized plague infection. Vaccine. 2006; 24(10):1625-1632.
92. Jones T, Cyr S, Allard F, Bellerose N, Lowell GH, Burt DS. Protollin(TM): a novel adjuvant for intranasal vaccines. Vaccine. 2004; 22(27-28):3691-3697.
93. Boutriau D, Poolman J, Borrow R, Findlow J, Domingo JD, Puig-Barbera J, et al. Immunogenicity and safety of three doses of a bivalent (B:4:p1.19,15 and B:4:p1.7-2,4) meningococcal outer membrane vesicle vaccine in healthy adolescents. Clin Vaccine Immunol. 2007; 14(1):65-73.
94. Gluck R. Immunopotentiating reconstituted influenza virosomes (IRIVs) and other adjuvants for improved presentation of small antigens. Vaccine. 1992; 10(13):915-919.

95. Almeida JD, Edwards DC, Brand CM, Heath TD. Formation of virosomes from influenza subunits and liposomes. Lancet. 1975; 2(7941):899-901.
96. Daemen T, de Mare A, Bungener L, de Jonge J, Huckriede A, Wilschut J. Virosomes for antigen and DNA delivery. Adv Drug Deliv Rev. 2005; 57(3):451-463.
97. De Magistris MT. Mucosal delivery of vaccine antigens and its advantages in pediatrics. Adv Drug Deliv Rev. 2006; 58(1):52-67.
98. Glück R, Metcalfe IC. New technology platforms in the development of vaccines for the future. Vaccine. 2002; 20(Supplement 5):B10-B16.
99. Zurbriggen R. Immunostimulating reconstituted influenza virosomes. Vaccine. 2003; 21(9-10):921-924.
100. Bungener L, Serre K, Bijl L, Leserman L, Wilschut J, Daemen T, et al. Virosome-mediated delivery of protein antigens to dendritic cells. Vaccine. 2002; 20(17-18):2287-2295.
101. Mestecky J, Moldoveanu Z, Michalek SM, Morrow CD, Compans RW, Schafer DP, et al. Current options for vaccine delivery systems by mucosal routes. J Control Release. 1997; 48(2-3):243-257.
102. Scholl I, Boltz-Nitulescu G, Jensen-Jarolim E. Review of novel particulate antigen delivery systems with special focus on treatment of type I allergy. J Control Release. 2005; 104(1):1-27.
103. Benson HA. Transfersomes for transdermal drug delivery. Expert Opin Drug Deliv. 2006; 3(6):727-37.
104. Cevc G. Transfersomes, liposomes and other lipid suspensions on the skin: permeation enhancement, vesicle penetration, and transdermal drug delivery. Crit Rev Ther Drug Carrier Syst. 1996; 13(3-4):257-388.
105. Gupta PN, Mishra V, Rawat A, Dubey P, Mahor S, Jain S, et al. Non-invasive vaccine delivery in transfersomes, niosomes and liposomes: a comparative study. Int J Pharm. 2005; 293(1-2):73-82.
106. Mahor S, Gupta PN, Rawat A, Vyas SP. A needle-free approach for topical immunization: antigen delivery via vesicular carrier system(s). Curr Med Chem. 2007; 14(27):2898-910.

107. Walker SA, Kennedy MT, Zasadzinski JA. Encapsulation of bilayer vesicles by self-assembly. Nature. 1997; 387(6628):61-4.
108. Mishra V, Mahor S, Rawat A, Dubey P, Gupta PN, Singh P, et al. Development of novel fusogenic vesosomes for transcutaneous immunization. Vaccine. 2006; 24(27-28):5559-5570.
109. Patel GB, Sprott GD. Archaeobacterial ether lipid liposomes (archaeosomes) as novel vaccine and drug delivery systems. Crit Rev Biotechnol. 1999; 19(4):317-57.
110. Patel GB, Chen W. Archaeosome immunostimulatory vaccine delivery system. Curr Drug Deliv. 2005; 2(4):407-21.
111. Benvegnu T, Rethore G, Brard M, Richter W, Plusquellec D. Archaeosomes based on novel synthetic tetraether-type lipids for the development of oral delivery systems. Chem Commun (Camb). 2005; 28(44):5536-8.
112. Krishnan L, Sprott GD. Archaeosome adjuvants: Immunological capabilities and mechanism(s) of action. Vaccine. 2008; 26(17):2043-2055.
113. Patel GB, Agnew BJ, Deschatelets L, Fleming LP, Sprott GD. In vitro assessment of archaeosome stability for developing oral delivery systems. Int J Pharm. 2000; 194(1):39-49.
114. Sprott GD, Sad S, Fleming LP, Dicaire CJ, Patel GB, Krishnan L. Archaeosomes varying in lipid composition differ in receptor-mediated endocytosis and differentially adjuvant immune responses to entrapped antigen. Archaea. 2003; 1(3):151-64.
115. Krishnan L, Dicaire CJ, Patel GB, Sprott GD. Archaeosome vaccine adjuvants induce strong humoral, cell-mediated, and memory responses: comparison to conventional liposomes and alum. Infect Immun. 2000; 68(1):54-63.
116. Krishnan L, Dennis Sprott G. Archaeosomes as self-adjuvanting delivery systems for cancer vaccines. J Drug Target. 2003; 11(8-10):515-24.
117. Krishnan L, Sad S, Patel GB, Sprott GD. Archaeosomes induce enhanced cytotoxic T lymphocyte responses to entrapped soluble protein in the absence of interleukin 12 and protect against tumor challenge. Cancer Res. 2003; 63(10):2526-34.

118. Hao S, Moyana T, Xiang J. Review: cancer immunotherapy by exosome-based vaccines. Cancer Biother Radiopharm. 2007; 22(5):692-703.
119. Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ, et al. B lymphocytes secrete antigen-presenting vesicles. J Exp Med. 1996; 183(3):1161-72.
120. Blanchard N, Lankar D, Faure F, Regnault A, Dumont C, Raposo G, et al. TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex. J Immunol. 2002; 168(7):3235-41.
121. Wolfers J, Lozier A, Raposo G, Regnault A, Thery C, Masurier C, et al. Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. Nat Med. 2001; 7(3):297-303.
122. Caby MP, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C. Exosomal-like vesicles are present in human blood plasma. Int Immunol. 2005; 17(7):879-87.
123. Thery C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. Nat Rev Immunol. 2002; 2(8):569-79.
124. Stoorvogel W, Kleijmeer MJ, Geuze HJ, Raposo G. The biogenesis and functions of exosomes. Traffic. 2002; 3(5):321-30.
125. Wubbolts R, Leckie RS, Veenhuizen PT, Schwarzmann G, Mobius W, Hoernschemeyer J, et al. Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation. J Biol Chem. 2003; 278(13):10963-72.
126. Le Pecq J-B. Dexosomes as a therapeutic cancer vaccine: From bench to bedside. Blood Cells, Molecules, and Diseases. 2005; 35(2):129-135.
127. Zitvogel L, Regnault A, Lozier A, Wolfers J, Flament C, Tenza D, et al. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. Nat Med. 1998; 4(5):594-600.
128. Delcayre A, Shu H, Le Pecq JB. Dendritic cell-derived exosomes in cancer immunotherapy: exploiting nature's antigen delivery pathway. Expert Rev Anticancer Ther. 2005; 5(3):537-47.

129. Morse MA, Garst J, Osada T, Khan S, Hobeika A, Clay TM, et al. A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer. J Transl Med. 2005; 3(1):9.
130. Escudier B, Dorval T, Chaput N, Andre F, Caby MP, Novault S, et al. Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of thefirst phase I clinical trial. J Transl Med. 2005; 3(1):10.
131. Brewer JM, Alexander J. Studies on the adjuvant activity of non-ionic surfactant vesicles: adjuvant-driven IgG2a production independent of MHC control. Vaccine. 1994; 12(7):613-9.
132. Bramwell VW, Perrie Y. Particulate delivery systems for vaccines. Crit Rev Ther Drug Carrier Syst. 2005; 22(2):151-214.
133. Rentel CO, Bouwstra JA, Naisbett B, Junginger HE. Niosomes as a novel peroral vaccine delivery system. Int J Pharm. 1999; 186(2):161-167.
134. Shukla A, Khatri K, Gupta PN, Goyal AK, Mehta A, Vyas SP. Oral immunization against hepatitis B using bile salt stabilized vesicles (bilosomes). J Pharm Pharm Sci. 2008; 11(1):59-66.
135. Coulter A, Harris R, Davis R, Drane D, Cox J, Ryan D, et al. Intranasal vaccination with ISCOMATRIX® adjuvanted influenza vaccine. Vaccine. 2003; 21(9-10):946-949.
136. Morein B, Sundquist B, Hoglund S, Dalsgaard K, Osterhaus A. Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses. Nature. 1984; 308(5958):457-60.
137. Ryan EJ, Daly LM, Mills KH. Immunomodulators and delivery systems for vaccination by mucosal routes. Trends Biotechnol. 2001; 19(8):293-304.
138. Gould-Fogerite S, Kheiri MT, Zhang F, Wang Z, Scolpino AJ, Feketeova E, et al. Targeting immune response induction with cochleate and liposome-based vaccines. Adv Drug Deliv Rev. 1998; 32(3):273-287.
139. Gould-Fogerite S, Mannino RJ. Mucosal and systemic immunization using cochleate and liposome vaccines. J Liposom Res. 1996; 6(2):357-379.
140. Chen X, Doffek K, Sugg SL, Shilyansky J. Phosphatidylserine regulates the maturation of human dendritic cells. J Immunol. 2004; 173(5):2985-94.

141. Zarif L, Mannino R. Cochleates: Lipid based vehicles for Gene Delivery-Concept, Achievements and Future Development. In: Habib N, editor. Cancer Gene Therapy: Past Achievements and Future Challenges. 1st ed. New York: Kluwer Academic/Plenum publishers; 2000. p. 83-93.
142. Hoffmann PR, Kench JA, Vondracek A, Kruk E, Daleke DL, Jordan M, et al. Interaction between phosphatidylserine and the phosphatidylserine receptor inhibits immune responses in vivo. J Immunol. 2005; 174(3):1393-404.
143. Mannino RJ, Gould-Fogerite S. Lipid matrix-based vaccines for mucosal and systemic immunization. In: Powell MF, Newman MJ, Burdman JR, editors. Vaccine design : the subunit and adjuvant approach. 1st ed. New York: Plenum Press; 1995. p. 363-387.
144. Gil D, Bracho G, Zayas C, del Campo J, Acevedo R, Toledo A, et al. Strategy for determination of an efficient Cochleate particle size. Vaccine. 2006; 24(Supplement 2):S92-S93.
145. Perez O, Bracho G, Lastre M, Zayas C, Gonzalez D, Gil D, et al. Proteliposome-derived Cochleate as an immunomodulator for nasal vaccine. Vaccine. 2006; 24(Supplement 2):S52-S53.
146. Zayas C, Bracho G, Lastre M, Gonzalez D, Gil D, Acevedo R, et al. Scale up of proteoliposome derived Cochleate production. Vaccine. 2006; 24(Supplement 2):S94-S95.
147. Boisgerault F, Moron G, Leclerc C. Virus-like particles: a new family of delivery systems. Expert Rev Vaccines. 2002; 1(1):101-9.
148. Scheerlinck J-PY, Greenwood DLV. Virus-sized vaccine delivery systems. Drug Discov Tod. 2008; 13(19-20):882-887.
149. Chackerian B. Virus-like particles: flexible platforms for vaccine development. Expert Rev Vaccines. 2007; 6(3):381-90.
150. Jennings GT, Bachmann MF. Designing recombinant vaccines with viral properties: a rational approach to more effective vaccines. Curr Mol Med. 2007; 7(2):143-55.
151. Jennings GT, Bachmann MF. The coming of age of virus-like particle vaccines. Biol Chem. 2008; 389(5):521-36.

152. Jennings GT, Bachmann MF. Immunodrugs: therapeutic VLP-based vaccines for chronic diseases. Annu Rev Pharmacol Toxicol. 2009; 49:303-26.
153. von Hoegen P. Synthetic biomimetic supra molecular Biovector(TM) (SMBV(TM)) particles for nasal vaccine delivery. Adv Drug Deliv Rev. 2001; 51(1-3):113-125.
154. Debin A, Kravtzoff R, Santiago JV, Cazales L, Sperandio S, Melber K, et al. Intranasal immunization with recombinant antigens associated with new cationic particles induces strong mucosal as well as systemic antibody and CTL responses. Vaccine. 2002; 20(21-22):2752-2763.
155. Halperin SA, Smith B, Clarke K, Treanor J, Mabrouk T, Germain M. Phase I, randomized, controlled trial to study the reactogenicity and immunogenicity of a nasal, inactivated trivalent influenza virus vaccine in healthy adults. Hum Vaccin. 2005; 1(1):37-42.
156. Kravtzoff R, Fisher A, De Miguel I, Perkins A, Major M, Betbeder D Nasal residence time evaluation of cationic biovector in human volunteers. Proceedings of the International Symposium on Controlled Release of Bioactive Materials; 1998 Controlled Release Society Inc p. 818-19.
157. Umashankar Shankar MS, Sachdeva RK, Gulati M. Aquasomes: a promising carrier for peptides and protein delivery. Nanomedicine: Nanotechnology, Biology and Medicine. In Press, Corrected Proof.
158. Goyal AK, Khatri K, Mishra N, Mehta A, Vaidya B, Tiwari S, et al. Aquasomes--a nanoparticulate approach for the delivery of antigen. Drug Dev Ind Pharm. 2008; 34(12):1297-305.
159. Kossovsky N, Gelman A, Rajguru S, Nguyen R, Sponsler E, Hnatyszyn HJ, et al. Control of molecular polymorphisms by a structured carbohydrate / ceramic delivery vehicle -- aquasomes. J Control Release. 1996; 39(2-3):383-388.
160. Kossovsky N, Gelman A, Hnatyszyn HJ, Rajguru S, Garrell RL, Torbati S, et al. Surface-modified diamond nanoparticles as antigen delivery vehicles. Bioconjug Chem. 1995; 6(5):507-11.

161. Goyal AK, Rawat A, Mahor S, Gupta PN, Khatri K, Vyas SP. Nanodecoy system: A novel approach to design hepatitis B vaccine for immunopotentiation. Int J Pharm. 2006; 309(1-2):227-233.
162. Heegaard PM, Boas U, Sorensen NS. Dendrimers for Vaccine and Immunostimulatory Uses. A Review. Bioconjug Chem. 2009; 4:4.
163. Calvo-Calle JM, Oliveira GA, Watta CO, Soverow J, Parra-Lopez C, Nardin EH. A linear peptide containing minimal T- and B-cell epitopes of Plasmodium falciparum circumsporozoite protein elicits protection against transgenic sporozoite challenge. Infect Immun. 2006; 74(12):6929-39.
164. Dupuis M, Denis-Mize K, LaBarbara A, Peters W, Charo IF, McDonald DM, et al. Immunization with the adjuvant MF59 induces macrophage trafficking and apoptosis. Eur J Immunol. 2001; 31(10):2910-8.
165. Dupuis M, McDonald DM, Ott G. Distribution of adjuvant MF59 and antigen gD2 after intramuscular injection in mice. Vaccine. 1999; 18(5-6):434-439.
166. Higgins DA, Carlson JR, Van Nest G. MF59 adjuvant enhances the immunogenicity of influenza vaccine in both young and old mice. Vaccine. 1996; 14(6):478-484.
167. Singh M, Carlson JR, Briones M, Ugozzoli M, Kazzaz J, Barackman J, et al. A comparison of biodegradable microparticles and MF59 as systemic adjuvants for recombinant gD from HSV-2. Vaccine. 1998; 16(19):1822-1827.
168. Singh M, Ugozzoli M, Kazzaz J, Chesko J, Soenawan E, Mannucci D, et al. A preliminary evaluation of alternative adjuvants to alum using a range of established and new generation vaccine antigens. Vaccine. 2006; 24(10):1680-1686.
169. Valensi JP, Carlson JR, Van Nest GA. Systemic cytokine profiles in BALB/c mice immunized with trivalent influenza vaccine containing MF59 oil emulsion and other advanced adjuvants. J Immunol. 1994; 153(9):4029-39.
170. Wack A, Baudner BC, Hilbert AK, Manini I, Nuti S, Tavarini S, et al. Combination adjuvants for the induction of potent, long-lasting antibody and T-cell responses to influenza vaccine in mice. Vaccine. 2008; 26(4):552-561.

171. Schultze V, D'Agosto V, Wack A, Novicki D, Zorn J, Hennig R. Safety of MF59(TM) adjuvant. Vaccine. 2008; 26(26):3209-3222.
172. Tomasi M, Dertzbaugh MT, Hearn T, Hunter RL, Elson CO. Strong mucosal adjuvanticity of cholera toxin within lipid particles of a new multiple emulsion delivery system for oral immunization. Eur J Immunol. 1997; 27(10):2720-5.
173. Shata MT, Stevceva L, Agwale S, Lewis GK, Hone DM. Recent advances with recombinant bacterial vaccine vectors. Mol Med Today. 2000; 6(2):66-71.
174. Polo JM, Dubensky TW. Virus-based vectors for human vaccine applications. Drug Discovery Today. 2002; 7(13):719-727.
175. Brun A, Albina E, Barret T, Chapman DAG, Czub M, Dixon LK, et al. Antigen delivery systems for veterinary vaccine development: Viral-vector based delivery systems. Vaccine. 2008; 26(51):6508-6528.
176. Lee JS, Hadjipanayis AG, Parker MD. Viral vectors for use in the development of biodefense vaccines. Adv Drug Deliv Rev. 2005; 57(9):1293-1314.
177. Daniell H, Singh ND, Mason H, Streatfield SJ. Plant-made vaccine antigens and biopharmaceuticals. Trends in Plant Science. 2009; 14(12):669-679.
178. Rybicki EP. Plant-produced vaccines: promise and reality. Drug Discover Today. 2009; 14(1-2):16-24.
179. Streatfield SJ, Howard JA. Plant-based vaccines. Int J Parasitol. 2003; 33(5-6):479-493.
180. Carstens MG. Opportunities and challenges in vaccine delivery. Euro J Pharm Sci. 2009; 36(4-5):605-608.
181. Edelman R. The development and use of vaccine adjuvants. Mol Biotechnol. 2002; 21(2):129-48.
182. Clements CJ, Griffiths E. The global impact of vaccines containing aluminium adjuvants. Vaccine. 2002; 20 Suppl 3(20):S24-33.
183. Ulanova M, Tarkowski A, Hahn-Zoric M, Hanson LA. The Common vaccine adjuvant aluminum hydroxide up-regulates accessory properties of human monocytes via an interleukin-4-dependent mechanism. Infect Immun. 2001; 69(2):1151-9.

184. Reed SG, Bertholet S, Coler RN, Friede M. New horizons in adjuvants for vaccine development. Trends Immunol. 2009; 30(1):23-32.
185. Burgess MA, McIntyre PB. Vaccines and the cold chain: is it too hot ... or too cold? Med J Aust. 1999; 171(2):82.
186. Gupta RK, Siber GR. Comparison of adjuvant activities of aluminium phosphate, calcium phosphate and stearyl tyrosine for tetanus toxoid. Biologicals. 1994; 22(1):53-63.
187. Rimaniol AC, Gras G, Verdier F, Capel F, Grigoriev VB, Porcheray F, et al. Aluminum hydroxide adjuvant induces macrophage differentiation towards a specialized antigen-presenting cell type. Vaccine. 2004; 22(23-24):3127-35.
188. Wilson-Welder JH, Torres MP, Kipper MJ, Mallapragada SK, Wannemuehler MJ, Narasimhan B. Vaccine adjuvants: current challenges and future approaches. J Pharm Sci. 2009; 98(4):1278-316.
189. Hunter RL. Overview of vaccine adjuvants: present and future. Vaccine. 2002; 20 Suppl 3(20):S7-12.
190. Aguilar JC, Rodriguez EG. Vaccine adjuvants revisited. Vaccine. 2007; 25(19):3752-62.
191. Mutsch M, Zhou W, Rhodes P, Bopp M, Chen RT, Linder T, et al. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. N Engl J Med. 2004; 350(9):896-903.
192. Couch RB. Nasal vaccination, Escherichia coli enterotoxin, and Bell's palsy. N Engl J Med. 2004; 350(9):860-1.
193. Childers NK, Miller KL, Tong G, Llarena JC, Greenway T, Ulrich JT, et al. Adjuvant activity of monophosphoryl lipid A for nasal and oral immunization with soluble or liposome-associated antigen. Infect Immun. 2000; 68(10):5509-16.
194. Klinman DM. CpG DNA as a vaccine adjuvant. Expert Rev Vaccines. 2003; 2(2):305-15.
195. Audibert F, Chedid L, Lefrancier P, Choay J. Distinctive adjuvanticity of synthetic analogs of mycobacterial water-soluble components. Cell Immunol. 1976; 21(2):243-9.

196. McCluskie MJ, Weeratna RD, Payette PJ, Davis HL. The potential of CpG oligodeoxynucleotides as mucosal adjuvants. Crit Rev Immunol. 2001; 21(1-3):103-20.
197. Baldridge JR, Crane RT. Monophosphoryl lipid A (MPL) formulations for the next generation of vaccines. Methods. 1999; 19(1):103-7.
198. McCormack S, Tilzey A, Carmichael A, Gotch F, Kepple J, Newberry A, et al. A phase I trial in HIV negative healthy volunteers evaluating the effect of potent adjuvants on immunogenicity of a recombinant gp120W61D derived from dual tropic R5X4 HIV-1ACH320. Vaccine. 2000; 18(13):1166-77.
199. Keam SJ, Harper DM. Human papillomavirus types 16 and 18 vaccine (recombinant, AS04 adjuvanted, adsorbed) [Cervarix]. Drugs. 2008; 68(3):359-72.
200. Audibert FM, Lise LD. Adjuvants: current status, clinical perspectives and future prospects. Immunol Today. 1993; 14(6):281-4.
201. Gebert A. The role of M cells in the protection of mucosal membranes. Histochem Cell Biol. 1997; 108(6):455-70.
202. Jepson MA, Clark MA, Hirst BH. M cell targeting by lectins: a strategy for mucosal vaccination and drug delivery. Advanced Drug Delivery Reviews. 2004; 56(4):511-525.
203. Manocha M, Pal PC, Chitralekha KT, Thomas BE, Tripathi V, Gupta SD, et al. Enhanced mucosal and systemic immune response with intranasal immunization of mice with HIV peptides entrapped in PLG microparticles in combination with Ulex Europaeus-I lectin as M cell target. Vaccine. 2005; 23(48-49):5599-5617.
204. Giannasca PJ, Boden JA, Monath TP. Targeted delivery of antigen to hamster nasal lymphoid tissue with M-cell-directed lectins. Infect Immun. 1997; 65(10):4288-98.
205. Clark MA, Jepson MA, Simmons NL, Booth TA, Hirst BH. Differential expression of lectin-binding sites defines mouse intestinal M-cells. J Histochem Cytochem. 1993; 41(11):1679-87.
206. Brayden DJ, Jepson MA, Baird AW. Keynote review: Intestinal Peyer's patch M cells and oral vaccine targeting. Drug Discover Today. 2005; 10(17):1145-1157.

207. Hopkins PA, Sriskandan S. Mammalian Toll-like receptors: to immunity and beyond. Clin Exp Immunol. 2005; 140(3):395-407.
208. Pandey S, Agrawal DK. Immunobiology of Toll-like receptors: emerging trends. Immunol Cell Biol. 2006; 84(4):333-41.
209. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol. 2004; 5(10):987-95.
210. Perrie Y, Mohammed AR, Kirby DJ, McNeil SE, Bramwell VW. Vaccine adjuvant systems: enhancing the efficacy of sub-unit protein antigens. Int J Pharm. 2008; 364(2):272-80.
211. Lapa JA, Sincock SA, Ananthakrishnan M, Porter CK, Cassels FJ, Brinkley C, et al. Randomized clinical trial assessing the safety and immunogenicity of oral microencapsulated enterotoxigenic Escherichia coli surface antigen 6 with or without heat-labile enterotoxin with mutation R192G. Clin Vaccine Immunol. 2008; 15(8):1222-8.
212. Mills KH, Cosgrove C, McNeela EA, Sexton A, Giemza R, Jabbal-Gill I, et al. Protective levels of diphtheria-neutralizing antibody induced in healthy volunteers by unilateral priming-boosting intranasal immunization associated with restricted ipsilateral mucosal secretory immunoglobulin a. Infect Immun. 2003; 71(2):726-32.
213. Hasegawa A, Fu Y, Koyama K. Nasal immunization with diphtheria toxoid conjugated-CD52 core peptide induced specific antibody production in genital tract of female mice. Am J Reprod Immunol. 2002; 48(5):305-11.
214. Fries LF, Montemarano AD, Mallett CP, Taylor DN, Hale TL, Lowell GH. Safety and immunogenicity of a proteosome-Shigella flexneri 2a lipopolysaccharide vaccine administered intranasally to healthy adults. Infect Immun. 2001; 69(7):4545-53.
215. Stephenson I, Zambon MC, Rudin A, Colegate A, Podda A, Bugarini R, et al. Phase I evaluation of intranasal trivalent inactivated influenza vaccine with nontoxigenic Escherichia coli enterotoxin and novel biovector as mucosal adjuvants, using adult volunteers. J Virol. 2006; 80(10):4962-70.

216. Angelakopoulos H, Hohmann EL. Pilot study of phoP/phoQ-deleted Salmonella enterica serovar typhimurium expressing Helicobacter pylori urease in adult volunteers. Infect Immun. 2000; 68(4):2135-41.
217. Fries LF, Gordon DM, Richards RL, Egan JE, Hollingdale MR, Gross M, et al. Liposomal malaria vaccine in humans: a safe and potent adjuvant strategy. Proc Natl Acad Sci U S A. 1992; 89(1):358-62.
218. Wedege E, Hoiby EA, Rosenqvist E, Bjune G. Immune responses against major outer membrane antigens of Neisseria meningitidis in vaccinees and controls who contracted meningococcal disease during the Norwegian serogroup B protection trial. Infect Immun. 1998; 66(7):3223-31.
219. Poovorawan Y, Theamboonlers A, Chumdermpadetsuk S, Glück R, Cryz SJ. Safety, immunogenicity, and kinetics of the immune response to a single dose of virosome-formulated hepatitis A vaccine in Thais. Vaccine. 1995; 13(10):891-893.
220. Rimmelzwaan GF, Nieuwkoop N, Brandenburg A, Sutter G, Beyer WEP, Maher D, et al. A randomized, double blind study in young healthy adults comparing cell mediated and humoral immune responses induced by influenza ISCOM(TM) vaccines and conventional vaccines. Vaccine. 2000; 19(9-10):1180-1187.
221. Harper DM, Franco EL, Wheeler C, Ferris DG, Jenkins D, Schuind A, et al. Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial. The Lancet. 2004; 364(9447):1757-1765.
222. Sindoni D, La Fauci V, Squeri R, Cannavo G, Bacilieri S, Panatto D, et al. Comparison between a conventional subunit vaccine and the MF59-adjuvanted subunit influenza vaccine in the elderly: an evaluation of the safety, tolerability and immunogenicity. J Prev Med Hyg. 2009; 50(2):121-6.
223. Frey S, Poland G, Percell S, Podda A. Comparison of the safety, tolerability, and immunogenicity of a MF59-adjuvanted influenza vaccine and a non-adjuvanted influenza vaccine in non-elderly adults. Vaccine. 2003; 21(27-30):4234-7.
224. Mestecky J, Michalek SM, Moldoveanu Z, Russell MW. Routes of immunization and antigen delivery systems for optimal mucosal immune responses in humans. Behring Inst Mitt. 1997; (98):33-43.

225. Mukkur TKS, Walker KH, Baker P, Jones D. Systemic and mucosal intestinal antibody response of sheep immunized with aromatic-dependent live or killed Salmonella typhimurium. Comparative Immunology, Microbiology and Infectious Diseases. 1995; 18(1):27-39.
226. Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. Nat Med. 2005; 11(4 Suppl):S45-53.
227. Davis SS. Nasal vaccines. Adv Drug Deliv Rev. 2001; 51(1-3):21-42.
228. Kallenius G, Pawlowski A, Brandtzaeg P, Svenson S. Should a new tuberculosis vaccine be administered intranasally? Tuberculosis. 2007; 87(4):257-266.
229. Brandtzaeg P. Induction of secretory immunity and memory at mucosal surfaces. Vaccine. 2007; 25(30):5467-5484.
230. Kiyono H, Fukuyama S. NALT- versus PEYER'S-patch-mediated mucosal immunity. Nat Rev Immunol. 2004; 4(9):699-710.
231. van Kempen MJ, Rijkers GT, Van Cauwenberge PB. The immune response in adenoids and tonsils. Int Arch Allergy Immunol. 2000; 122(1):8-19.
232. Kresse H. Medimmune: Flumist sales to heat up. Buisness Review Ltd.; 2007 [cited 04-02-2008]. Available from: http://www.pharmaceutical-business-review.com/article_feature_print.asp?guid=F62A1684-B18F-4287-95E9-2985BB717995.
233. Kotzé AF, Lueßen HL, de Boer AG, Verhoef JC, Junginger HE. Chitosan for enhanced intestinal permeability: Prospects for derivatives soluble in neutral and basic environments. Euro J Pharm Sci. 1999; 7(2):145-151.
234. Lehr C-M, Bouwstra JA, Schacht EH, Junginger HE. In vitro evaluation of mucoadhesive properties of chitosan and some other natural polymers. Int J Pharm. 1992; 78(1-3):43-48.
235. Bivas-Benita M, Laloup M, Versteyhe S, Dewit J, Braekeleer JD, Jongert E, et al. Generation of Toxoplasma gondii GRA1 protein and DNA vaccine loaded chitosan particles: preparation, characterization, and preliminary in vivo studies. Int J Pharm. 2003; 266(1-2):17-27.

236. Li GP, Liu ZG, Liao B, Zhong NS. Induction of Th1-type immune response by chitosan nanoparticles containing plasmid DNA encoding house dust mite allergen Der p 2 for oral vaccination in mice. Cell Mol Immunol. 2009; 6(1):45-50.
237. Chew JL, Wolfowicz CB, Mao H-Q, Leong KW, Chua KY. Chitosan nanoparticles containing plasmid DNA encoding house dust mite allergen, Der p 1 for oral vaccination in mice. Vaccine. 2003; 21(21-22):2720-2729.
238. Svirshchevskaya EV, Alekseeva LG, Reshetov PD, Phomicheva NN, Parphenyuk SA, Ilyina AV, et al. Mucoadjuvant properties of lipo- and glycoconjugated derivatives of oligochitosans. Euro J Med Chem. 2009; 44(5):2030-2037.
239. Roldo M, Hornof M, Caliceti P, Bernkop-Schnürch A. Mucoadhesive thiolated chitosans as platforms for oral controlled drug delivery: synthesis and in vitro evaluation. Euro J Pharm Biopharm. 2004; 57(1):115-121.
240. Hornof MD, Kast CE, Bernkop-Schnürch A. In vitro evaluation of the viscoelastic properties of chitosan-thioglycolic acid conjugates. Euro J Pharm Biopharm. 2003; 55(2):185-190.
241. Bernkop-Schnürch A, Hornof M, Zoidl T. Thiolated polymers--thiomers: synthesis and in vitro evaluation of chitosan-2-iminothiolane conjugates. Int J Pharm. 2003; 260(2):229-237.
242. Thanou M, Nihot MT, Jansen M, Verhoef JC, Junginger HE. Mono-N-carboxymethyl chitosan (MCC), a polyampholytic chitosan derivative, enhances the intestinal absorption of low molecular weight heparin across intestinal epithelia in vitro and in vivo. J Pharm Sci. 2001; 90(1):38-46.
243. Jia Z, shen D, Xu W. Synthesis and antibacterial activities of quaternary ammonium salt of chitosan. Carbohdrate Res. 2001; 333(1):1-6.
244. Thanou MM, Kotzé AF, Scharringhausen T, Lueßen HL, de Boer AG, Verhoef JC, et al. Effect of degree of quaternization of N-trimethyl chitosan chloride for enhanced transport of hydrophilic compounds across intestinal Caco-2 cell monolayers. J Control Release. 2000; 64(1-3):15-25.
245. Amidi M, Romeijn SG, Verhoef JC, Junginger HE, Bungener L, Huckriede A, et al. N-Trimethyl chitosan (TMC) nanoparticles loaded with influenza subunit

antigen for intranasal vaccination: Biological properties and immunogenicity in a mouse model. Vaccine. 2007; 25(1):144-153.

246. Nishimura K, Nishimura S-i, Nishi N, Numata F, Tone Y, Tokura S, et al. Adjuvant activity of chitin derivatives in mice and guinea-pigs. Vaccine. 1985; 3(5):379-384.
247. Boonyo W, Junginger HE, Waranuch N, Polnok A, Pitaksuteepong T. Chitosan and trimethyl chitosan chloride (TMC) as adjuvants for inducing immune responses to ovalbumin in mice following nasal administration. J Control Release. 2007; 121(3):168-175.
248. Sayin B, Somavarapu S, Li XW, Thanou M, Sesardic D, Alpar HO, et al. Mono-N-carboxymethyl chitosan (MCC) and N-trimethyl chitosan (TMC) nanoparticles for non-invasive vaccine delivery. Int J Pharm. 2008; 363(1-2):139-148.
249. Bal SM, Slütter B, van Riet E, Kruithof AC, Ding Z, Kersten GFA, et al. Efficient induction of immune responses through intradermal vaccination with N-trimethyl chitosan containing antigen formulations. J Control Release. 2010; 142(3):374-383.
250. Pandit S, Cevher E, Zariwala MG, Somavarapu S, Alpar HO. Enhancement of immune response of HBsAg loaded poly (L-lactic acid) microspheres against hepatitis B through incorporation of alum and chitosan. J Microencapsul. 2007; 24(6):539-52.
251. Janes KA, Calvo P, Alonso MJ. Polysaccharide colloidal particles as delivery systems for macromolecules. Adv Drug Deliv Rev. 2001; 47(1):83-97.
252. Xu Y, Du Y. Effect of molecular structure of chitosan on protein delivery properties of chitosan nanoparticles. Int J Pharm. 2003; 250(1):215-226.
253. De S, Robinson D. Polymer relationships during preparation of chitosan-alginate and poly-l-lysine-alginate nanospheres. J Control Release. 2003; 89(1):101-112.
254. Chen Y, Mohanraj VJ, Wang F, Benson HA. Designing chitosan-dextran sulfate nanoparticles using charge ratios. AAPS PharmSciTech. 2007; 8(4):E98.
255. Csaba N, Köping-Höggård M, Alonso MJ. Ionically crosslinked chitosan/tripolyphosphate nanoparticles for oligonucleotide and plasmid DNA delivery. Int J Pharm. 2009; 382(1-2):205-214.

256. Fernandez-Urrusuno R, Calvo P, Remunan-Lopez C, Vila-Jato JL, Alonso MJ. Enhancement of nasal absorption of insulin using chitosan nanoparticles. Pharm Res. 1999; 16(10):1576-81.
257. Gan Q, Wang T, Cochrane C, McCarron P. Modulation of surface charge, particle size and morphological properties of chitosan-TPP nanoparticles intended for gene delivery. Colloids Surf B Biointerfaces. 2005; 44(2-3):65-73.
258. Lin YH, Chung CK, Chen CT, Liang HF, Chen SC, Sung HW. Preparation of nanoparticles composed of chitosan/poly-gamma-glutamic acid and evaluation of their permeability through Caco-2 cells. Biomacromolecules. 2005; 6(2):1104-12.
259. Naessens M, Cerdobbel A, Soetaert W, Vandamme EJ. Leuconostoc dextransucrase and dextran: production, properties and applications. J Chem Technol Biotechnol. 2005; 80:845-860.
260. Baldwin AD, Kiick KL. Polysaccharide-modified synthetic polymeric biomaterials. Biopolymers. 2010; 94(1):128-40.
261. Rosenfeld EL, Lukomskaya IS. The splitting of dextran and isomaltose by animal tissues. Clinica Chimica Acta. 1957; 2(2):105-114.
262. Sery TW, Hehre EJ. Degradation of dextrans by enzymes of intestinal bacteria. J Bacteriol. 1956; 71(3):373-80.
263. Ogawa T, Asai Y, Sakamoto H, Yasuda K. Oral immunoadjuvant activity of Lactobacillus casei subsp. casei in dextran-fed layer chickens. Br J Nutr. 2006; 95(2):430-4.
264. Shu Q, Hillard MA, Bindon BM, Duan E, Xu Y, Bird SH, et al. Effects of various adjuvants on efficacy of a vaccine against Streptococcus bovis and Lactobacillus spp in cattle. Am J Vet Res. 2000; 61(7):839-43.
265. Diwan M, Misra A, Khar RK, Talwar GP. Long-term high immune response to diphtheria toxoid in rodents with diphtheria toxoid conjugated to dextran as a single contact point delivery system. Vaccine. 1997; 15(17-18):1867-1871.
266. Diwan M, Khar RK, Talwar GP. Tetanus toxoid loaded [`]preformed microspheres' of cross-linked dextran. Vaccine. 2001; 19(28-29):3853-3859.

267. Hiemstra C, Aa LJ, Zhong Z, Dijkstra PJ, Feijen J. Rapidly in situ-forming degradable hydrogels from dextran thiols through Michael addition. Biomacromolecules. 2007; 8(5):1548-56.
268. Hiemstra C, Zhong Z, van Steenbergen MJ, Hennink WE, Feijen J. Release of model proteins and basic fibroblast growth factor from in situ forming degradable dextran hydrogels. J Control Release. 2007; 122(1):71-78.
269. Zhang Y, Chu CC. Biodegradable dextran-polylactide hydrogel networks: their swelling, morphology and the controlled release of indomethacin. J Biomed Mater Res. 2002; 59(2):318-28.
270. de Jong SJ, van Eerdenbrugh B, van Nostrum CF, Kettenes-van den Bosch JJ, Hennink WE. Physically crosslinked dextran hydrogels by stereocomplex formation of lactic acid oligomers: degradation and protein release behavior. J Control Release. 2001; 71(3):261-275.
271. Bhumkar DR, Pokharkar VB. Studies on effect of pH on cross-linking of chitosan with sodium tripolyphosphate: a technical note. AAPS PharmSciTech. 2006; 7(2):E50.
272. Reis CP, Ribeiro AJ, Veiga F, Neufeld RJ, Damge C. Polyelectrolyte biomaterial interactions provide nanoparticulate carrier for oral insulin delivery. Drug Deliv. 2008; 15(2):127-39.
273. Tiyaboonchai W, Limpeanchob N. Formulation and characterization of amphotericin B-chitosan-dextran sulfate nanoparticles. Int J Pharm. 2007; 329(1-2):142-149.
274. Pan Y, Li Y-j, Zhao H-y, Zheng J-m, Xu H, Wei G, et al. Bioadhesive polysaccharide in protein delivery system: chitosan nanoparticles improve the intestinal absorption of insulin in vivo. Int J Pharm. 2002; 249(1-2):139-147.
275. Wang F. Micro and Nanoparticles for Site-Specific Drug Delivery in the Skin [thesis]. Perth, Australia: Curtin University of Technology; 2005.
276. Agnihotri SA, Mallikarjuna NN, Aminabhavi TM. Recent advances on chitosan-based micro- and nanoparticles in drug delivery. J Control Release. 2004; 100(1):5-28.

277. Tiyaboonchai W, Woiszwillo J, Middaugh CR. Formulation and characterization of DNA-polyethylenimine-dextran sulfate nanoparticles. Eur J Pharm Sci. 2003; 19(4):191-202.
278. Schatz C, Lucas JM, Viton C, Domard A, Pichot C, Delair T. Formation and properties of positively charged colloids based on polyelectrolyte complexes of biopolymers. Langmuir. 2004; 20(18):7766-78.
279. Fukuda H, Kikuchi Y. Polyelectrolyte complexes of sodium dextran sulfate with chitosan, 2. Die Makromol Chem. 1977; 178(10):2895-2899.
280. Gamzazade AI, Nasibov SM. Formation and properties of polyelectrolyte complexes of chitosan hydrochloride and sodium dextransulfate. Carbohydrate Polymers. 2002; 50(4):339-343.
281. Drogoz A, David L, Rochas C, Domard A, Delair T. Polyelectrolyte complexes from polysaccharides: formation and stoichiometry monitoring. Langmuir. 2007; 23(22):10950-8.
282. Sogias IA, Williams AC, Khutoryanskiy VV. Why is chitosan mucoadhesive? Biomacromolecules. 2008; 9(7):1837-42.
283. Sarmento B, Ribeiro A, Veiga F, Ferreira D. Development and characterization of new insulin containing polysaccharide nanoparticles. Colloids Surf B Biointerfaces. 2006; 53(2):193-202.
284. Gerelli Y, Barbieri S, Di Bari MT, Deriu A, Cantu L, Brocca P, et al. Structure of self-organized multilayer nanoparticles for drug delivery. Langmuir. 2008; 24(20):11378-84.
285. Csaba N, Garcia-Fuentes M, Alonso MJ. Nanoparticles for nasal vaccination. Adv Drug Deliv Rev. 2009; 61(2):140-57.
286. Keegan ME, Whittum-Hudson JA, Mark Saltzman W. Biomimetic design in microparticulate vaccines. Biomaterials. 2003; 24(24):4435-43.
287. Vogel FR. Improving vaccine performance with adjuvants. Clin Infect Dis. 2000; 30 Suppl 3(3):S266-70.
288. Huang H, Yuan Q, Yang X. Preparation and characterization of metal-chitosan nanocomposites. Colloids Surf B Biointerfaces. 2004; 39(1-2):31-7.

289. WHO. Pertussis vaccines WHO position paper. Geneva: World health organization; 2005 28, Jan 2005.
290. Khelef N, Zychlinsky A, Guiso N. Bordetella pertussis induces apoptosis in macrophages: role of adenylate cyclase-hemolysin. Infect Immun. 1993; 61(10):4064-71.
291. Parton R. New perspectives on Bordetella pathogenicity. J Med Microbiol. 1996; 44(4):233-5.
292. Relman D, Tuomanen E, Falkow S, Golenbock DT, Saukkonen K, Wright SD. Recognition of a bacterial adhesion by an integrin: macrophage CR3 (alpha M beta 2, CD11b/CD18) binds filamentous hemagglutinin of Bordetella pertussis. Cell. 1990; 61(7):1375-82.
293. Schipper H, Krohne GF, Gross R. Epithelial cell invasion and survival of Bordetella bronchiseptica. Infect Immun. 1994; 62(7):3008-11.
294. Weiss AA, Hewlett EL. Virulence factors of Bordetella pertussis. Annu Rev Microbiol. 1986; 40:661-86.
295. Marzouqi I, Richmond P, Fry S, Wetherall J, Mukkur T. Development of improved vaccines against whooping cough: Current status. Hum Vaccin. 2010; 6(6):6.
296. Tamura M, Nogimori K, Murai S, Yajima M, Ito K, Katada T, et al. Subunit structure of islet-activating protein, pertussis toxin, in conformity with the A-B model. Biochemistry. 1982; 21(22):5516-22.
297. Barbieri JT, Rappuoli R, Collier RJ. Expression of the S-1 catalytic subunit of pertussis toxin in Escherichia coli. Infect Immun. 1987; 55(5):1321-3.
298. Hornef MW, Wick MJ, Rhen M, Normark S. Bacterial strategies for overcoming host innate and adaptive immune responses. Nat Immunol. 2002; 3(11):1033-40.
299. Mooi FR, van Loo IH, van Gent M, He Q, Bart MJ, Heuvelman KJ, et al. Bordetella pertussis strains with increased toxin production associated with pertussis resurgence. Emerg Infect Dis. 2009; 15(8):1206-13.
300. Mooi FR, van Loo IH, King AJ. Adaptation of Bordetella pertussis to vaccination: a cause for its reemergence? Emerg Infect Dis. 2001; 7(3 Suppl):526-8.

301. Cherry JD. The science and fiction of the "resurgence" of pertussis. Pediatrics. 2003; 112(2):405-6.
302. von Konig CH, Halperin S, Riffelmann M, Guiso N. Pertussis of adults and infants. Lancet Infect Dis. 2002; 2(12):744-50.
303. Butler NR, Wilson BD, Benson PF, Dudgeon JA, Ungar J, Beale AJ. Response of infants to pertussis vaccine at one week and to poliomyelitis, diphtheria, and tetanus vaccine at six months. Lancet. 1962; 2(7247):112-4.
304. Roy MJ, Varvayanis M. Development of dome epithelium in gut-associated lymphoid tissues: association of IgA with M cells. Cell Tissue Res. 1987; 248(3):645-51.
305. Kato T. A study of secretory immunoglobulin A on membranous epithelial cells (M cells) and adjacent absorptive cells of rabbit Peyer's patches. Gastroenterol Jpn. 1990; 25(1):15-23.
306. Weltzin R, Lucia-Jandris P, Michetti P, Fields BN, Kraehenbuhl JP, Neutra MR. Binding and transepithelial transport of immunoglobulins by intestinal M cells: demonstration using monoclonal IgA antibodies against enteric viral proteins. J Cell Biol. 1989; 108(5):1673-85.
307. Mantis NJ, Cheung MC, Chintalacharuvu KR, Rey J, Corthesy B, Neutra MR. Selective adherence of IgA to murine Peyer's patch M cells: evidence for a novel IgA receptor. J Immunol. 2002; 169(4):1844-51.
308. Corthesy B. Roundtrip ticket for secretory IgA: role in mucosal homeostasis? J Immunol. 2007; 178(1):27-32.
309. Favre L, Spertini F, Corthesy B. Secretory IgA possesses intrinsic modulatory properties stimulating mucosal and systemic immune responses. J Immunol. 2005; 175(5):2793-800.
310. Zhou F, Kraehenbuhl J-P, Neutra MR. Mucosal IgA response to rectally administered antigen formulated in IgA-coated liposomes. Vaccine. 1995; 13(7):637-644.
311. Dixit CK, Vashist SK, O'Neill FT, O'Reilly B, MacCraith BD, O'Kennedy R. Development of a high sensitivity rapid sandwich ELISA procedure and its comparison with the conventional approach. Anal Chem. 2010; 82(16):7049-52.

312. Munoz JJ, Arai H, Cole RL. Mouse-protecting and histamine-sensitizing activities of pertussigen and fimbrial hemagglutinin from Bordetella pertussis. Infect Immun. 1981; 32(1):243-50.
313. Hewlett EL, Sauer KT, Myers GA, Cowell JL, Guerrant RL. Induction of a novel morphological response in Chinese hamster ovary cells by pertussis toxin. Infect Immun. 1983; 40(3):1198-203.
314. Amidi M, Romeijn SG, Borchard G, Junginger HE, Hennink WE, Jiskoot W. Preparation and characterization of protein-loaded N-trimethyl chitosan nanoparticles as nasal delivery system. J Control Release. 2006; 111(1-2):107-116.
315. Rabilloud T, Brodard V, Peltre G, Righetti PG, Ettori C. Modified silver staining for immobilized pH gradients. Electrophoresis. 1992; 13(4):264-6.
316. Isbrucker RA, Bliu A, Prior F. Modified binding assay for improved sensitivity and specificity in the detection of residual pertussis toxin in vaccine preparations. Vaccine. 2010; 28(15):2687-2692.
317. Corbel MJ, Xing DKL, Bolgiano B, Hockley DJ. Approaches to the Control of Acellular Pertussis Vaccines. Biologicals. 1999; 27(2):133-141.
318. Gillenius P, Jaatmaa E, Askelof P, Granstrom M, Tiru M. The standardization of an assay for pertussis toxin and antitoxin in microplate culture of Chinese hamster ovary cells. J Biol Stand. 1985; 13(1):61-6.
319. Corbel MJ, Xing DK. Toxicity and potency evaluation of pertussis vaccines. Expert Rev Vaccines. 2004; 3(1):89-101.
320. Wong WS, Rosoff PM. Pharmacology of pertussis toxin B-oligomer. Can J Physiol Pharmacol. 1996; 74(5):559-64.
321. Isbrucker RA, Bliu A, Prior F. Modified binding assay for improved sensitivity and specificity in the detection of residual pertussis toxin in vaccine preparations. Vaccine. 28(15):2687-92.
322. Ibsen PH. The effect of formaldehyde, hydrogen peroxide and genetic detoxification of pertussis toxin on epitope recognition by murine monoclonal antibodies. Vaccine. 1996; 14(5):359-68.

323. Burns DL, Kenimer JG, Manclark CR. Role of the A subunit of pertussis toxin in alteration of Chinese hamster ovary cell morphology. Infect Immun. 1987; 55(1):24-8.
324. Fowler S, Byron O, Jumel K, Xing D, Corbel MJ, Bolgiano B. Novel configurations of high molecular weight species of the pertussis toxin vaccine component. Vaccine. 2003; 21(19-20):2678-88.
325. Nencioni L, Pizza MG, Volpini G, De Magistris MT, Giovannoni F, Rappuoli R. Properties of the B oligomer of pertussis toxin. Infect Immun. 1991; 59(12):4732-4.
326. Shahin RD, Brennan MJ, Li ZM, Meade BD, Manclark CR. Characterization of the protective capacity and immunogenicity of the 69-kD outer membrane protein of Bordetella pertussis. J Exp Med. 1990; 171(1):63-73.
327. Huang M, Vitharana SN, Peek LJ, Coop T, Berkland C. Polyelectrolyte complexes stabilize and controllably release vascular endothelial growth factor. Biomacromolecules. 2007; 8(5):1607-14.
328. Gan Q, Wang T. Chitosan nanoparticle as protein delivery carrier--systematic examination of fabrication conditions for efficient loading and release. Colloids Surf B Biointerfaces. 2007; 59(1):24-34.
329. Kabanov VA, Zezin AB. A new class of complex water-soluble polyelectrolytes. Die Makromol Chem. 1984; 6(S1):259-276.
330. Mincheva R, Bougard F, Paneva D, Vachaudez M, Manolova N, Rashkov I, et al. Natural polyampholyte-based core-shell nanoparticles with N-carboxyethylchitosan-containing core and poly(ethylene oxide) shell. Biomacromolecules. 2009; 10(4):838-44.
331. Ma Z, Yeoh HH, Lim LY. Formulation pH modulates the interaction of insulin with chitosan nanoparticles. J. Pharm. Sci. 2002; 91(6):1396-1404.
332. Borges O, Cordeiro-da-Silva A, Romeijn SG, Amidi M, de Sousa A, Borchard G, et al. Uptake studies in rat Peyer's patches, cytotoxicity and release studies of alginate coated chitosan nanoparticles for mucosal vaccination. J Control Release. 2006; 114(3):348-58.

333. Ehlers S. Commentary: adaptive immunity in the absence of innate immune responses? The un-Tolled truth of the silent invaders. Eur J Immunol. 2004; 34(7):1783-8.
334. Harlan DM, Karp CL, Matzinger P, Munn DH, Ransohoff RM, Metzger DW. Immunological concerns with bioengineering approaches. Ann N Y Acad Sci. 2002; 961:323-30.
335. Coico R, Sunshine G, Benjamini E. IMMUNOLOGY A Short Course. 5th ed. Hoboken, New Jersey: John Wiley & Sons, Inc.; 2003.
336. Iwasaki A. Mucosal dendritic cells. Annu Rev Immunol. 2007; 25:381-418.
337. Gogolak P, Rethi B, Hajas G, Rajnavolgyi E. Targeting dendritic cells for priming cellular immune responses. J Mol Recognit. 2003; 16(5):299-317.
338. R Coico, Sunshine G. Immunology: a short course. 6th ed. New Jersey, USA: Wiley-Blackwell; 2009.
339. Slutter B, Hagenaars N, Jiskoot W. Rational design of nasal vaccines. J Drug Target. 2008; 16(1):1-17.
340. Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, et al. Phenotypic and functional features of human Th17 cells. J Exp Med. 2007; 204(8):1849-61.
341. Villadangos JA, Schnorrer P. Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. Nat Rev Immunol. 2007; 7(7):543-55.
342. Rogers PR, Croft M. CD28, Ox-40, LFA-1, and CD4 modulation of Th1/Th2 differentiation is directly dependent on the dose of antigen. J Immunol. 2000; 164(6):2955-63.
343. McNeela EA, Mills KH. Manipulating the immune system: humoral versus cell-mediated immunity. Adv Drug Deliv Rev. 2001; 51(1-3):43-54.
344. Finkelman FD, Urban JF, Jr. Cytokines: making the right choice. Parasitol Today. 1992; 8(9):311-4.
345. Brewer JM, Pollock KGJ. Adjuvant-induced Th2 and Th-1 dominated immune responses. In: Kaufmann SHE, editor. Novel vaccination strategies. 1st ed. Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA; 2004. p. 147-163.

346. Giudice EL, Campbell JD. Needle-free vaccine delivery. Adv Drug Deliv Rev. 2006; 58(1):68-89.
347. O'Hagan DT, Rappuoli R. Novel approaches to pediatric vaccine delivery. Adv Drug Deliv Rev. 2006; 58(1):29-51.
348. Shahin R, Leef M, Eldridge J, Hudson M, Gilley R. Adjuvanticity and protective immunity elicited by Bordetella pertussis antigens encapsulated in poly(DL-lactide-co-glycolide) microspheres. Infect Immun. 1995; 63(4):1195-200.
349. Shahin RD, Cowell JL. Mouse respiratory infection models for pertussis. Methods Enzymol. 1994; 235:47-58.
350. Sharma R, Schumacher U, Adam E. Lectin histochemistry reveals the appearance of M-cells in Peyer's patches of SCID mice after syngeneic normal bone marrow transplantation. J Histochem Cytochem. 1998; 46(2):143-8.
351. Porta C, James PS, Phillips AD, Savidge TC, Smith MW, Cremaschi D. Confocal analysis of fluorescent bead uptake by mouse Peyer's patch follicle-associated M cells. Exp Physiol. 1992; 77(6):929-32.
352. Fujimura Y, Akisada T, Harada T, Haruma K. Uptake of microparticles into the epithelium of human nasopharyngeal lymphoid tissue. Med Mol Morphol. 2006; 39(4):181-6.
353. Eyles JE, Bramwell VW, Williamson ED, Alpar HO. Microsphere translocation and immunopotentiation in systemic tissues following intranasal administration. Vaccine. 2001; 19(32):4732-4742.
354. Zeng M, Xu Q, Pichichero ME. Protection against anthrax by needle-free mucosal immunization with human anthrax vaccine. Vaccine. 2007; 25(18):3588-3594.
355. Huang Y, Hajishengallis G, Michalek SM. Induction of protective immunity against Streptococcus mutans colonization after mucosal immunization with attenuated Salmonella enterica serovar typhimurium expressing an S. mutans adhesin under the control of in vivo-inducible nirB promoter. Infect Immun. 2001; 69(4):2154-61.
356. Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunology Today. 1996; 17(3):138-146.

357. Watanabe M, Nagai M. Reciprocal protective immunity against Bordetella pertussis and Bordetella parapertussis in a murine model of respiratory infection. Infect Immun. 2001; 69(11):6981-6.
358. Watanabe M, Komatsu E, Sato T, Nagai M. Evaluation of efficacy in terms of antibody levels and cell-mediated immunity of acellular pertussis vaccines in a murine model of respiratory infection. FEMS Immunol Med Microbiol. 2002; 33(3):219-25.
359. Rosoff PM, Walker R, Winberry L. Pertussis toxin triggers rapid second messenger production in human T lymphocytes. J Immunol. 1987; 139(7):2419-23.
360. Borges O, Cordeiro-da-Silva A, Tavares J, Santarém N, de Sousa A, Borchard G, et al. Immune response by nasal delivery of hepatitis B surface antigen and codelivery of a CpG ODN in alginate coated chitosan nanoparticles. Eur J Pharm Biopharm. 2008; 69(2):405-416.
361. Yoo MK, Kang SK, Choi JH, Park IK, Na HS, Lee HC, et al. Targeted delivery of chitosan nanoparticles to Peyer's patch using M cell-homing peptide selected by phage display technique. Biomaterials. 2010; 23:23.
362. Slutter B, Plapied L, Fievez V, Sande MA, des Rieux A, Schneider YJ, et al. Mechanistic study of the adjuvant effect of biodegradable nanoparticles in mucosal vaccination. J Control Release. 2009; 138(2):113-21.
363. Yuan X, Yang X, Cai D, Mao D, Wu J, Zong L, et al. Intranasal immunization with chitosan/pCETP nanoparticles inhibits atherosclerosis in a rabbit model of atherosclerosis. Vaccine. 2008; 26(29-30):3727-34.
364. Ghendon Y, Markushin S, Vasiliev Y, Akopova I, Koptiaeva I, Krivtsov G, et al. Evaluation of properties of chitosan as an adjuvant for inactivated influenza vaccines administered parenterally. J Med Virol. 2009; 81(3):494-506.
365. Zhu B, Qie Y, Wang J, Zhang Y, Wang Q, Xu Y, et al. Chitosan microspheres enhance the immunogenicity of an Ag85B-based fusion protein containing multiple T-cell epitopes of Mycobacterium tuberculosis. Eur J Pharm Biopharm. 2007; 66(3):318-26.

366. Ghendon Y, Markushin S, Krivtsov G, Akopova I. Chitosan as an adjuvant for parenterally administered inactivated influenza vaccines. Arch Virol. 2008; 153(5):831-7.
367. Zaharoff DA, Rogers CJ, Hance KW, Schlom J, Greiner JW. Chitosan solution enhances both humoral and cell-mediated immune responses to subcutaneous vaccination. Vaccine. 2007; 25(11):2085-94.
368. Djupesland PG, Skretting A, Winderen M, Holand T. Bi-directional nasal delivery of aerosols can prevent lung deposition. J Aerosol Med. 2004; 17(3):249-59.
369. Jelley-Gibbs DM, Lepak NM, Yen M, Swain SL. Two distinct stages in the transition from naive CD4 T cells to effectors, early antigen-dependent and late cytokine-driven expansion and differentiation. J Immunol. 2000; 165(9):5017-26.
370. Bramwell VW, Perrie Y. The rational design of vaccines. Drug Discover Today. 2005; 10(22):1527-1534.
371. Sonaje K, Chen YJ, Chen HL, Wey SP, Juang JH, Nguyen HN, et al. Enteric-coated capsules filled with freeze-dried chitosan/poly(gamma-glutamic acid) nanoparticles for oral insulin delivery. Biomaterials. 2010; 31(12):3384-94.
372. Prego C, Paolicelli P, Díaz B, Vicente S, Sánchez A, González-Fernández Á, et al. Chitosan-based nanoparticles for improving immunization against hepatitis B infection. Vaccine. 2010; 28(14):2607-2614.

CHAPTER 7

APPENDIX

7.1. COMPOSITION AND PREPARATION OF BUFFERS USED FOR ELISA

The following buffer systems were used in estimation of IgA, PTX, PTXd, serum anti-PTXd IgG, serum anti-PTXd IgG1, serum anti-PTXd IgG2a, serum anti-PTXd IgG2b and lung homogenate anti-PTXd IgA by ELISA method:

- **Blocking buffer**

Tris buffered saline (0.05M tris, 0.138M NaCl, 0.0027M KCl) (Catalog no. T6664, Sigma Aldrich, Australia)	15.25 g
Bovine serum albumin (BSA)	10 g

Dissolved in 900 mL of distilled water, pH adjusted to 8.0 and made to 1L with distilled water.

- **Wash buffer**

Tris buffered saline (0.05M tris, 0.138M NaCl, 0.0027M KCl) (Catalog no. T6664, Sigma Aldrich, Australia)	15.25 g
Tween 20	0.5 g

Dissolved in 900 mL of distilled water, pH adjusted to 8.0 and made to 1L with distilled water.

- **Dilution buffer**

Tris buffered saline (0.05M tris, 0.138M NaCl, 0.0027M KCl) (Catalog no. T6664, Sigma Aldrich, Australia)	15.25 g
Tween 20	0.5 g
BSA	10 g

Dissolved in 900 mL of distilled water, pH adjusted to 8.0 and made to 1L with distilled water.

Note:

For estimation of IFN-γ in splenocyte culture supernatant by ELISA, phosphate bufferd saline (0.1M phosphate buffer, 0.137M NaCl, 0.0027M KCl) was used in place of Tris buffered saline (0.05M tris, 0.138M NaCl, 0.0027M KCl; Catalog no. P4417; Sigma Aldrich, Australia) for making of blocking, wash and dilution buffers as amine group containing buffers cannot be used with biotinylated anti-mouse IFN-γ monoclonal secondary antibody and streptavidin-horseradish peroxidase conjugate.

7.2. ROUTINE CELL CULTURE AND STORAGE OF CELL LINES

❖ **Routine passaging / splitting of cells**

Cell lines were routinely maintained and sub-cultured aseptically. To passage the cells, 75 cm^2 cell-culture flask containing 80-90% confluent cells were removed from the 37 °C CO_2 incubator and placed in a bio-safety cabinet. The complete nutrient medium containing 100 µg/mL penicillin-streptomycin and 10% FCS was removed from the flask and cells were trysinized by addition of 2.5% 1 mL trypsin/EDTA followed by incubation at 37 °C in a CO_2 incubator for 3-5 minutes. The trypsinized cells were pipetted up and down in the flask to loosen the cells. 50-200 µL of the trypsinized cells were added to a new 75 cm^2 flask containing 10 mL complete nutrient medium and incubated until next sub-culturing.

❖ **Freezing of cells**

One or more 75 cm^2 flasks containing cells of 80% confluence were normally used for as a source of cells for freezing. The flasks were trypsinized and 5 mL complete nutrient medium was added to each flask. The cell suspension from each flask was combined into a 15 mL centrifuge tube and centrifuged at 1000 rpm for 5 min. The supernatant medium was aspirated and cells were resuspended uniformly in 1 mL or appropriate volume of medium for cell counting. Cell counting was performed using a haemocytometer with conversion factor (CF) of 10,000 for a counting chamber of depth 0.1 mm. The 20 µL cell suspension aliquot was added to 20 µL of 0.14% trypan blue. The mixture was added to the haemocytometer to fill the chamber. The chamber consists of 25 small squares separated by triple border lines in a large central square. The cells were counted in 25 small squares of the large square. The equation (equation 7.1) for viable cells per mL is,

(7.1)

The cells were added with chilled complete nutrient medium to obtain approximately 10^6 cells/mL. 900 µL of cell suspension was added to each Nunc cryotube containing 100 µL chilled DMSO. All the cryotubes were then placed in the cryo freezing container and stored at -70 °C for 24 hours followed by transferto liquid nitrogen for long term storage.

❖ **Thawing and culturing of cells**

The cryotubes containing cells were removed from liquid nitrogen and thawed in a 37 °C water bath. The contents of a cryotube were then transferred to a 15 mL centrifuge tube containing 9 mL warm complete nutrient medium and centrifuged at 1000 rpm for 5 min. The supernatant was removed and the cells were resuspended in 1 mL warm complete nutrient medium. 0.5 mL of cell suspension was added to each 75 cm^2 flask containing 10 mL complete nutrient medium and incubated in a 37°C CO_2 incubator.

7.3. COMPOSITION AND PREPARATION OF GEL MIXTURES USED FOR SDS-PAGE

❖ **Resolving Gel Mixture (10%)**

1.5M Tris HCl buffer, pH 8.8	4.0 mL
10% w/v SDS solution	100 µL
30% acrylamide/bis-acrylamide	3.3 mL
10% ammonium persulfate	100 µL
N, N, N', N'-tetramethylethylenediamine (TEMED)	10 µL
Distilled water	4.0 mL

The mixture was made up and degassed for 5 minutes at room temperature before the addition of ammonium persulfate and TEMED.

❖ **Stacking Gel Mixture (4%)**

0.5M Tris HCl buffer, pH 6.8	2.5 mL
10% w/v SDS solution	100 µL
30% acrylamide/bis-acrylamide	1.3 mL
10% ammonium persulfate	100 µL
TEMED	10 µL
Distilled water	6.1 mL

The mixture was made up and degassed for 5 minutes at room temperature before addition of ammonium persulfate and TEMED.

7.4. ESTIMATION OF PTXd USING SANDWICH ELISA METHOD (RAW DATA)

It was tried to develop a sensitive, accurate and reproducible sandwich ELISA method for PTXd as explained in chapter 3. But the developed method failed to provide any reproducible data with a meaningful correlation with concentration of PTXd, necessary to develop a standard (calibration) curve. Table 7.1 represents variability in absorbances (raw data) for same PTXd concentration in different ELISA runs. Even runs with higher concentrations of PTXd (4 and 10 µg/mL) in sandwich ELISA could not produce reproducibility or correlation in absorbance and concentration. The results of sandwich ELISA for PTXd led to the use of PTX for entrapment efficiency and release studies as explained in chapter 3.

Table 7.1 Representative raw data obtained for PTXd estimation by sandwich ELISA method.

PTXd concentration (ng/mL)	Average absorbance (Run 1)	Average absorbance (Run 2)
125	0.239	0.168
250	0.294	0.207
500	0.270	0.328
1000	0.234	0.294
2000	0.268	0.421
Negative controls (Ag and Ab free wells)	0.189	0.169

7.5. Z-SCAN CLSM PICTURE SERIES OF NASAL TISSUES

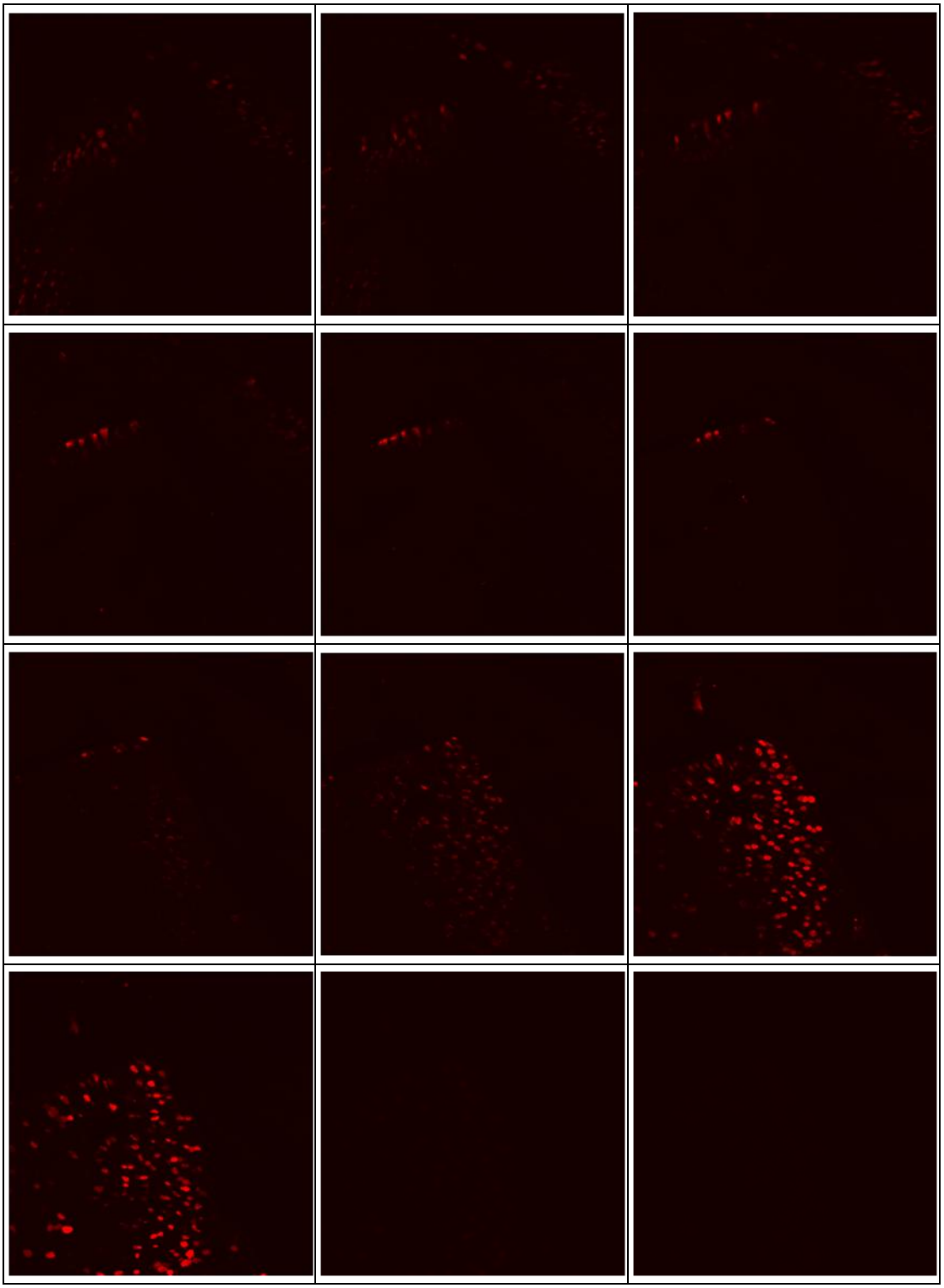
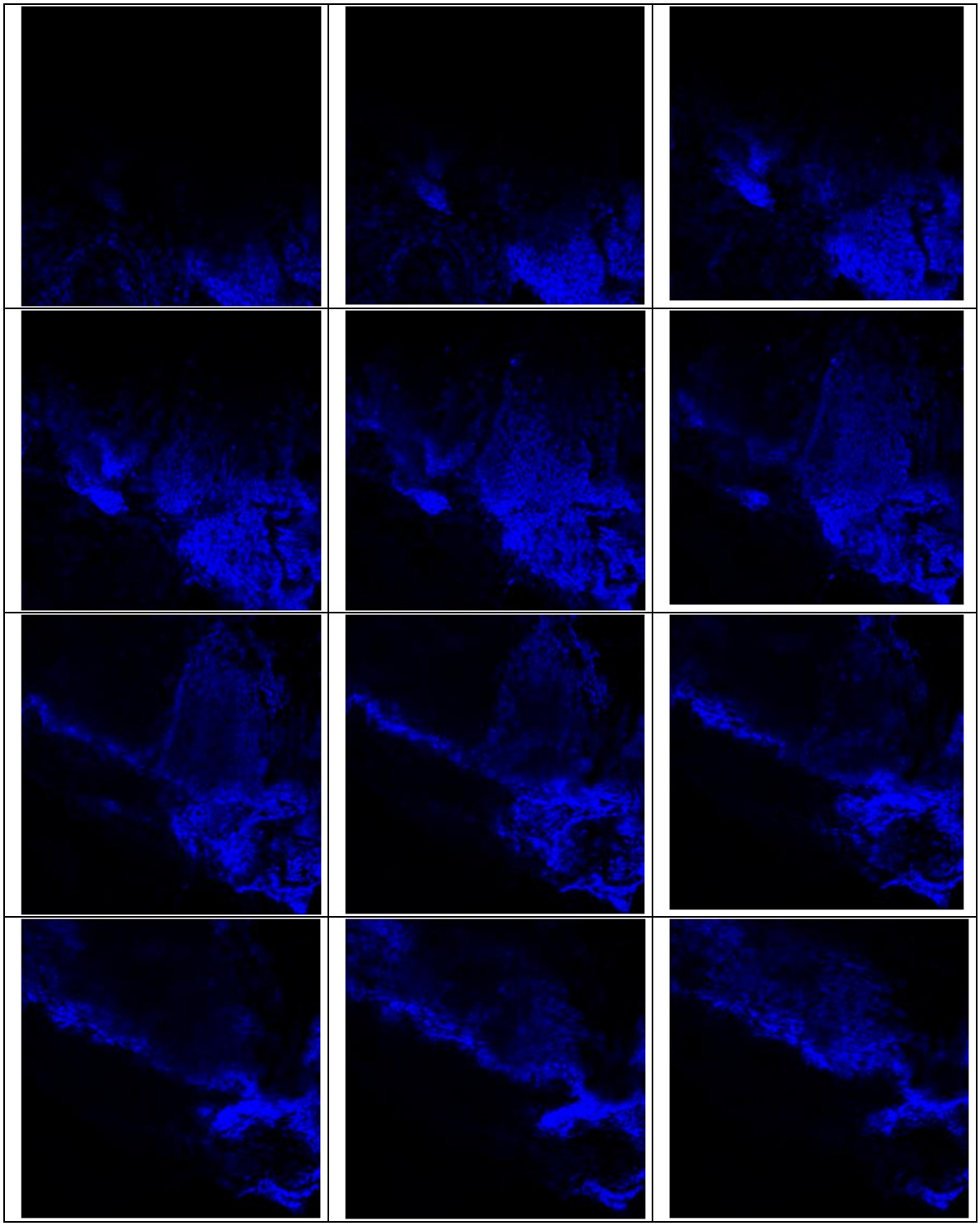


Figure 7.1 Z-scan confocal pictures of nasal tissue treated with UEA-1 - TRITC



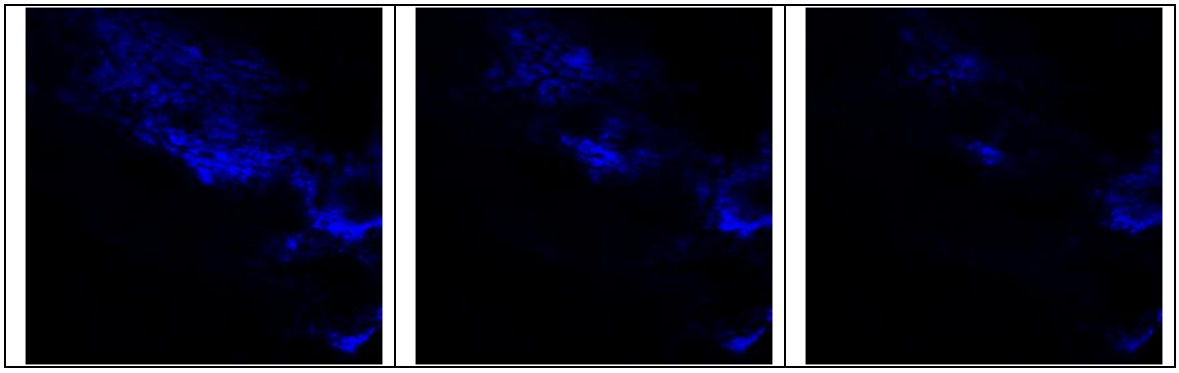
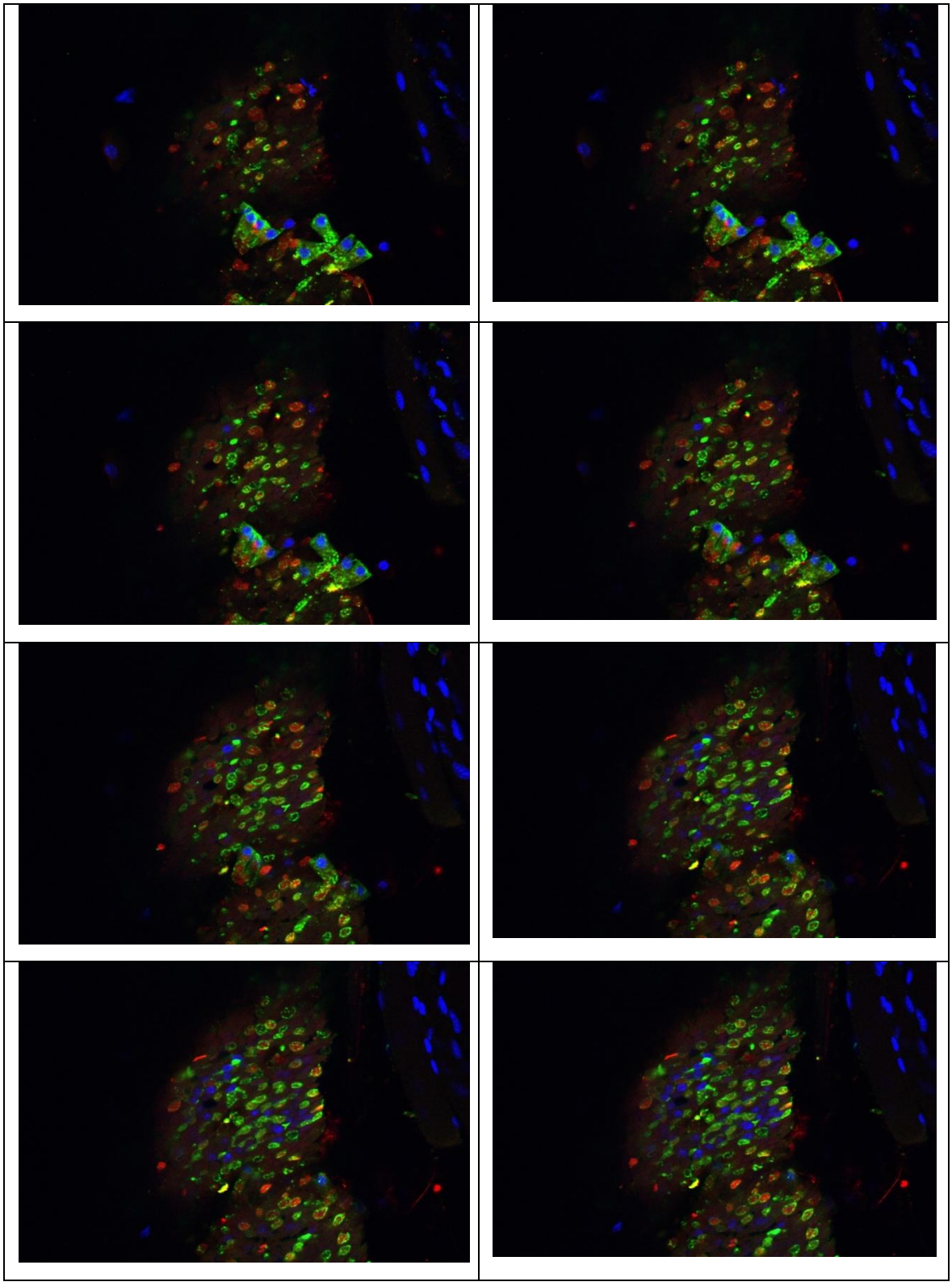
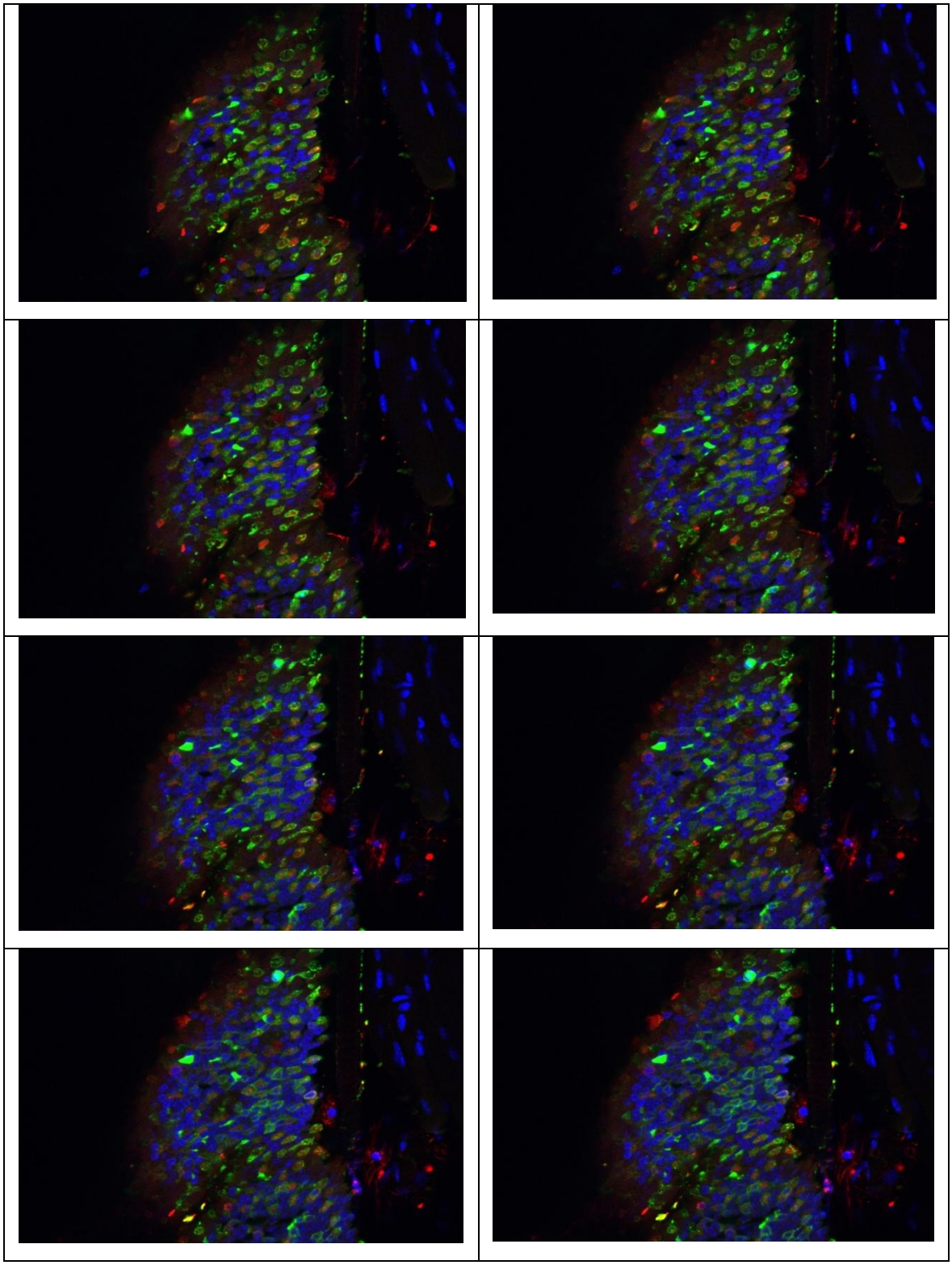


Figure 7.2 Z-scan confocal pictures of nasal tissue treated with Hoechst 33342





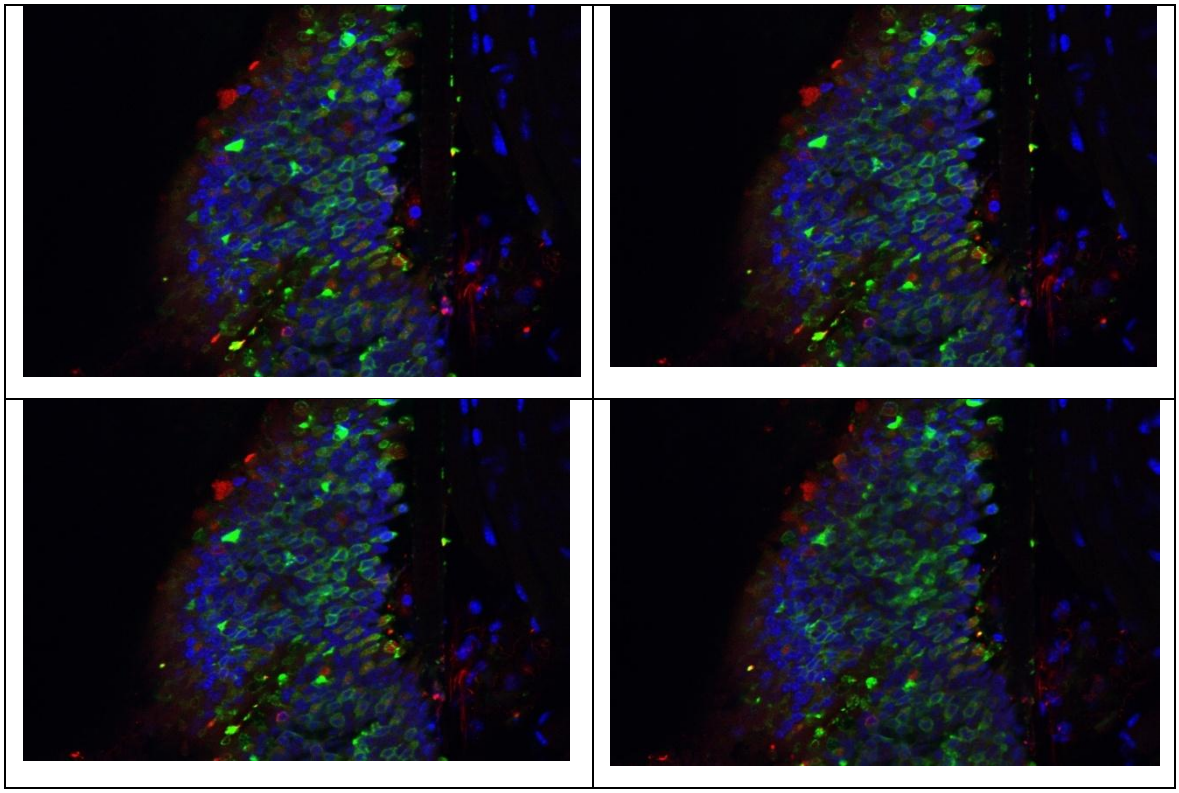


Figure 7.3 Z-scan confocal pictures of nasal tissue treated with Hoechst 33342, UEA-1 conjugated with TRITC and anti-mouse IgA conjugated with FITC

7.6. *IN-VIVO* IMMUNOLOGICAL DATA AND CALCULATIONS

The mean of blanks (antigen and antibody free wells) was subtracted from normalized absorbances of samples and graphs were plotted with absorbance on Y axis versus dilution factor for sample on the X axis. A linear regression fit was performed for the absorbance values between 0.1 and 1.0 and linear trendline was extrapolated to X-axis to find the corresponding dilution factor as antibody titer. If no two processed absorbace values (after subtraction of mean absorbance value for blanks) for any dilution of a sample were found above 0.1, then titer was assumed negligible (zero) for that particular sample. In other words, the cut-off point was 0.1 optical density. The data for all immunological parameters (serum IgG, serum IgG1, serum IgG2a, serum IgG2b, lung homogenate IgA and splenocyte culture supernatant IFN-γ) is attached below. The calculations for titer determination have been examplified here for serum IgG1 responses induced by subcutaneous administration of PTXd formulations. Same calculation procedures have been followed for all parameters and final comparative graphs and tables are included in chapter 4.

Table 7.2 ELISA data (absorbance at 450nm) for serum anti-PTXd IgG1 induced by subcutaneous administration of PTXd formulations

Animal	Dilution factor	PTXd + Alum	PTXd in CS-DS nanoparticles	PTXd & IgA in CS-DS nanoparticles	Blank CS-DS nanoparticles
1	40	1.640	3.345	3.381	0.327
	100	0.854	3.388	3.016	0.285
	200	0.602	1.511	1.450	0.301
	400	0.401	1.111	1.103	0.26
	800	0.211	0.888	0.845	0.271
	1600	0.202	0.611	0.548	0.258
2	40	3.445	3.415	2.376	0.388
	100	2.245	3.434	1.160	0.341
	200	1.112	1.700	0.988	0.310
	400	0.854	1.210	0.745	0.332
	800	0.540	0.808	0.580	0.320
	1600	0.310	0.584	0.401	0.305
3	40	0.585	1.938	3.363	0.415
	100	0.480	0.734	2.204	0.354
	200	0.310	0.545	1.218	0.344
	400	0.258	0.408	0.858	0.324
	800	0.165	0.314	0.561	0.288
	1600	0.175	0.211	0.403	0.311
4	40	2.373	3.436	3.411	0.285
	100	1.100	2.204	1.788	0.277
	200	0.805	1.917	1.106	0.215
	400	0.602	1.661	0.885	0.202
	800	0.405	0.845	0.588	0.189

	1600	0.301	0.512	0.405	0.178
5	40	3.321	3.328	3.404	0.444
	100	1.708	2.101	2.458	0.405
	200	0.884	1.549	1.957	0.380
	400	0.555	1.081	1.257	0.354
	800	0.405	0.696	0.882	0.365
	1600	0.301	0.463	0.607	0.371
6	40	2.173	2.599	3.414	0.361
	100	1.175	1.696	2.888	0.314
	200	0.602	0.943	1.854	0.325
	400	0.405	0.622	1.152	0.322
	800	0.401	0.526	0.888	0.345
	1600	0.388	0.410	0.620	0.323

Average absorbance for blanks (antigen free and antibody wells) = 0.378

Table 7.3 Corrected absorbance data for serum anti-PTXd IgG1 (obtained by subtraction of average absorbance for blanks)

Animal	Dilution factor	PTXd + Alum	PTXd in CS-DS nanoparticles	PTXd & IgA in CS-DS nanoparticles	Blank CS-DS nanoparticles
1	40	1.262	2.968	3.004	-0.051
	100	0.476	3.010	2.638	-0.093
	200	0.224	1.133	1.072	-0.077
	400	0.023	0.733	0.725	-0.118
	800	-0.166	0.510	0.467	-0.107
	1600	-0.176	0.233	0.170	-0.120
2	40	3.067	3.037	1.998	0.010
	100	1.867	3.057	0.782	-0.037
	200	0.734	1.322	0.610	-0.068
	400	0.476	0.832	0.367	-0.046
	800	0.162	0.430	0.202	-0.058
	1600	-0.068	0.206	0.023	-0.073
3	40	0.207	1.561	2.985	0.037
	100	0.102	0.356	1.826	-0.024
	200	-0.068	0.167	0.840	-0.034
	400	-0.120	0.030	0.480	-0.054
	800	-0.213	-0.064	0.184	-0.090
	1600	-0.202	-0.167	0.025	-0.067
4	40	1.995	3.058	3.033	-0.093
	100	0.722	1.826	1.410	-0.101
	200	0.427	1.539	0.728	-0.163
	400	0.224	1.283	0.507	-0.176
	800	0.027	0.467	0.210	-0.189
	1600	-0.077	0.134	0.027	-0.200
5	40	2.943	2.951	3.026	0.066
	100	1.330	1.723	2.080	0.027

	200	0.506	1.171	1.579	0.002
	400	0.177	0.703	0.880	-0.024
	800	0.027	0.319	0.504	-0.013
	1600	-0.077	0.086	0.229	-0.007
6	40	1.795	2.221	3.036	-0.017
	100	0.797	1.319	2.510	-0.064
	200	0.224	0.566	1.476	-0.053
	400	0.027	0.244	0.774	-0.056
	800	0.023	0.148	0.510	-0.033
	1600	0.010	0.032	0.242	-0.055

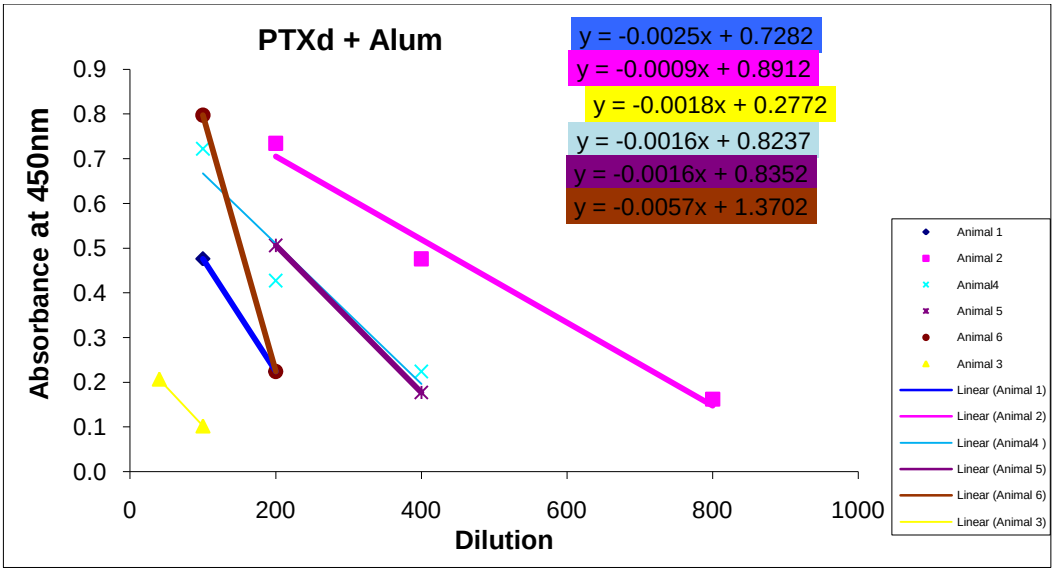


Figure 7.4 A plot between corrected absorbances (0.1 - 1.0) and dilution factor with linear model fitting for calculation of titer for mice immunised with PTXd and alum by subcutaneous route.

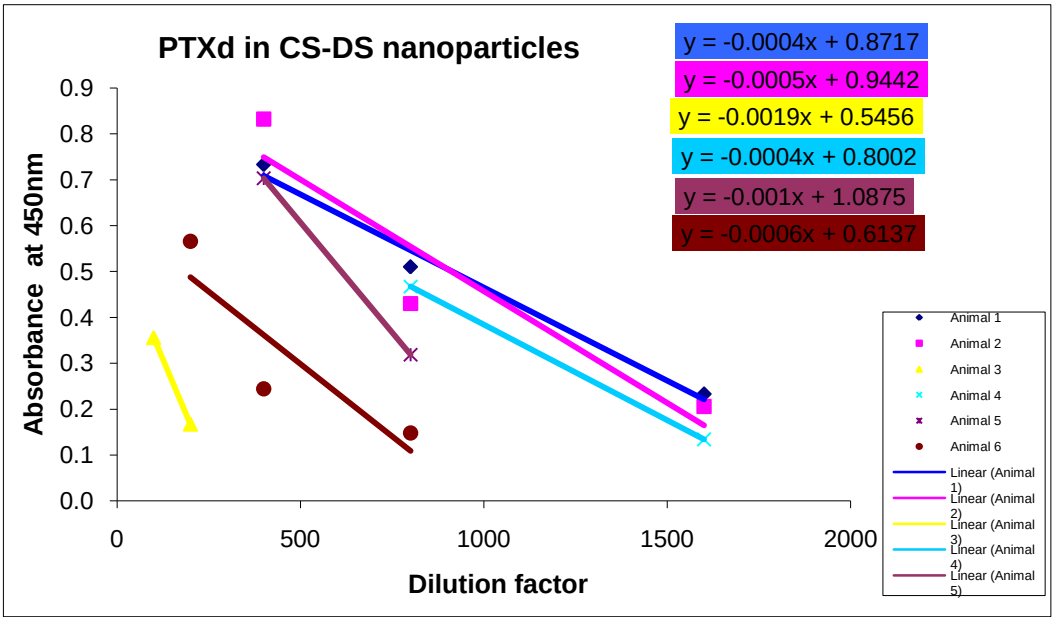


Figure 7.5 A plot between corrected absorbances (0.1 – 1.0) and dilution factor with linear model fitting for calculation of titer for mice immunised with PTXd-loaded CS-DS nanoparticles by subcutaneous route.

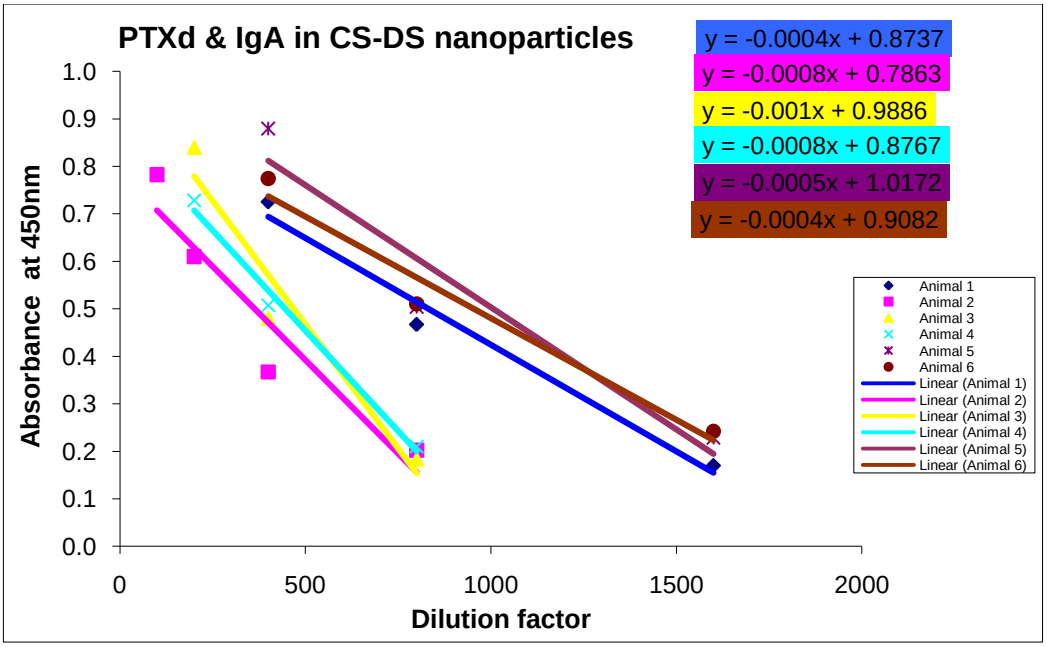


Figure 7.6 A plot between corrected absorbances (0.1 – 1.0) and dilution factor with linear model fitting for calculation of titer for mice immunised with PTXd and IgA-loaded CS-DS nanoparticles by subcutaneous route.

Table 7.4 ELISA data (absorbance at 450nm) for serum anti-PTXd IgG2a induced by subcutaneous administration of PTXd formulations

Animal	Dilution factor	PTXd + Alum group	PTXd in CS-DS nanoparticles	PTXd & IgA in CS-DS nanoparticles	Blank CS-DS nanoparticles
1	10	0.288	0.956	0.238	0.192
	40	0.257	0.648	0.238	0.170
	160	0.241	0.417	0.208	0.163
2	10	0.586	0.521	0.283	0.203
	40	0.446	0.343	0.218	0.182
	160	0.299	0.301	0.204	0.179
3	10	0.340	0.210	0.345	0.198
	40	0.301	0.210	0.302	0.213
	160	0.221	0.196	0.250	0.185
4	10	0.263	0.537	1.106	0.202
	40	0.212	0.346	0.654	0.133
	160	0.209	0.299	0.414	0.129
5	10	0.391	0.520	0.940	0.26
	40	0.305	0.314	0.544	0.202
	160	0.277	0.249	0.362	0.170
6	10	0.760	1.616	0.550	0.224
	40	0.419	0.820	0.357	0.19
	160	0.291	0.512	0.298	0.185

Average absorbance for blanks (antigen free and antibody wells) = 0.148

Table 7.5 ELISA data (absorbance at 450nm) for serum anti-PTXd IgG2b induced by subcutaneous administration of PTXd formulations

Animal	Dilution factor	PTXd + Alum group	PTXd in CS-DS nanoparticles	PTXd & IgA in CS-DS nanoparticles	Blank CS-DS nanoparticles
1	10	0.358	3.300	0.281	0.312
	40	0.179	1.221	0.209	0.188
	160	0.178	0.888	0.186	0.198
2	10	2.223	1.336	0.225	0.345
	40	1.183	0.851	0.191	0.182
	160	0.484	0.475	0.152	0.179
3	10	0.282	0.224	0.574	0.288
	40	0.178	0.179	0.329	0.213
	160	0.174	0.175	0.247	0.185
4	10	0.730	0.605	3.284	0.202
	40	0.396	0.376	1.222	0.133
	160	0.223	0.209	0.602	0.129
5	10	0.815	0.758	2.370	0.350
	40	0.470	0.488	1.221	0.301
	160	0.311	0.219	0.545	0.245
6	10	0.482	0.670	1.818	0.224
	40	0.251	0.345	0.942	0.190
	160	0.168	0.192	0.498	0.185

Average absorbance for blanks (antigen free and antibody wells) = 0.228

Table 7.6 ELISA data (absorbance at 450nm) for serum anti-PTXd IgG induced by subcutaneous administration of PTXd formulations

Animal	Dilution factor	PTXd + Alum	PTXd in CS-DS nanoparticles	PTXd & IgA in CS-DS nanoparticles	Blank CS-DS nanoparticles
1	25	0.538	2.905	2.217	0.242
	50	0.430	2.104	1.498	0.170
	100	0.323	1.511	0.988	0.163
	200	0.298	1.111	0.597	0.161
	400	0.211	0.800	0.415	0.168
	800	0.233	0.611	0.345	0.154
	1600	0.222	0.482	0.285	0.144
2	25	0.793	0.771	1.540	0.203
	50	0.655	0.632	1.235	0.182
	100	0.485	0.503	0.988	0.179
	200	0.320	0.335	0.745	0.165
	400	0.280	0.264	0.580	0.156
	800	0.202	0.244	0.401	0.155
	1600	0.154	0.211	0.301	0.156
3	25	0.548	0.885	1.522	0.476
	50	0.405	0.684	1.110	0.213
	100	0.310	0.545	1.000	0.185
	200	0.258	0.408	0.858	0.170
	400	0.165	0.314	0.561	0.155
	800	0.175	0.211	0.403	0.158
	1600	0.161	0.170	0.338	0.154
4	25	0.345	1.102	2.845	0.202
	50	0.282	0.888	2.057	0.133
	100	0.211	0.682	1.917	0.129

	200	0.185	0.505	1.661	0.111
	400	0.142	0.410	1.294	0.112
	800	0.120	0.305	0.846	0.112
	1600	0.116	0.260	0.501	0.102
5	25	0.854	2.584	2.804	0.329
	50	0.688	1.885	2.463	0.266
	100	0.528	1.549	1.957	0.224
	200	0.377	1.081	1.257	0.202
	400	0.296	0.696	0.882	0.184
	800	0.198	0.463	0.607	0.168
	1600	0.166	0.295	0.334	0.170
6	25	0.982	0.943	2.888	0.185
	50	0.774	0.727	2.154	0.158
	100	0.534	0.692	1.184	0.144
	200	0.406	0.526	0.888	0.130
	400	0.354	0.458	0.62	0.134
	800	0.298	0.384	0.544	0.138
	1600	0.271	0.285	0.410	0.121

Average absorbance for blanks (antigen free and antibody wells) = 0.182

Table 7.7 ELISA data (absorbance at 450nm) for lung homogenate anti-PTXd IgA induced by subcutaneous administration of PTXd formulations

Animal	Dilution factor	PTXd + Alum group	PTXd in CS-DS nanoparticles	PTXd & IgA in CS-DS nanoparticles	Blank CS-DS nanoparticles
1	5	0.338	0.345	0.363	0.334
	10	0.209	0.242	0.336	0.307
2	5	0.277	0.214	0.333	0.285
	10	0.227	0.207	0.209	0.224
3	5	0.297	0.310	0.488	0.295
	10	0.283	0.266	0.333	0.263
4	5	0.330	0.377	0.293	0.315
	10	0.281	0.282	0.329	0.351
5	5	0.252	0.237	0.208	0.233
	10	0.190	0.220	0.208	0.230
6	5	0.411	0.430	0.456	0.466
	10	0.329	0.333	0.329	0.318

Average absorbance for blanks (antigen free and antibody wells) = 0.259

Table 7.8 Data (absorbances at 450nm) obtained from estimation of IFN-γ in splenocyte culture supernatants of subcutaneously immunized animal groups using ELISA method.

Formulation used for immunization of animal group ↓	**Splenocytes stimulated with Con-A**	**Splenocytes stimulated with PBS**	**Splenocytes stimulated with PTX**
PTXd + Alum	2.979	0.695	3.304
PTXd and IgA in CS-DS nanoparticles	3.374	0.622	2.344
PTXd in CS-DS nanoparticles	3.407	0.837	2.559
Blank nanoparticles	3.345	0.633	2.750

Average absorbance for blanks (antigen free and antibody wells) = 0.305

Table 7.9 Data (absorbance at 450nm) obtained for IFN- γ standards in ELISA method.

IFN- γ standards Conc. (pg/mL)	Absorbance
2000	3.233
1000	2.385
500	1.585
250	1.124
125	1.056

Table 7.10 ELISA data (absorbance at 450nm) for serum anti-PTXd IgG1 induced by intranasal administration of PTXd formulations

Animal	Dilution factor	PTXd solution	PTXd in CS-DS nanoparticles	PTXd & IgA in CS-DS nanoparticles	IgA in CS-DS nanoparticles	Buffer only
1	10	0.271	0.185	0.158	0.130	0.167
	20	0.162	0.142	0.112	0.109	0.129
2	10	0.186	0.157	0.170	0.200	0.119
	20	0.146	0.135	0.127	0.198	0.105
3	10	0.168	0.193	0.148	0.100	0.101
	20	0.173	0.135	0.123	0.103	0.126
4	10	0.208	0.134	0.117	0.126	0.122
	20	0.169	0.118	0.111	0.118	0.113
5	10	0.171	0.148	0.243	0.121	0.116
	20	0.117	0.131	0.162	0.113	0.103
6	10	0.158	0.258	0.238	0.104	0.224
	20	0.127	0.169	0.173	0.121	0.188

Average absorbance for blanks (antigen free and antibody wells) = 0.109

Table 7.11 ELISA data (absorbance at 450nm) for serum anti-PTXd IgG2a induced by intranasal administration of PTXd formulations

Animal	Dilution factor	PTXd solution	PTXd in CS-DS nanoparticles	PTXd & IgA in CS-DS nanoparticles	IgA in CS-DS nanoparticles	Buffer only
1	5	0.170	0.139	0.168	0.156	0.188
	20	0.159	0.125	0.162	0.131	0.144
	80	0.171	0.126	0.134	0.148	0.148
2	5	0.235	0.155	0.182	0.198	0.121
	20	0.177	0.139	0.156	0.188	0.112
	80	0.162	0.122	0.168	0.154	0.103
3	5	0.169	0.156	0.153	0.185	0.145
	20	0.152	0.145	0.137	0.188	0.161
	80	0.169	0.146	0.162	0.145	0.125
4	5	0.180	0.157	0.175	0.144	0.170
	20	0.137	0.133	0.161	0.121	0.162
	80	0.146	0.121	0.153	0.104	0.104
5	5	0.161	0.152	0.141	0.112	0.145
	20	0.153	0.128	0.133	0.107	0.158
	80	0.137	0.130	0.133	0.109	0.128
6	5	0.169	0.177	0.161	0.158	0.184
	20	0.152	0.154	0.148	0.148	0.158
	80	0.137	0.158	0.131	0.155	0.161

Average absorbance for blanks (antigen free and antibody wells) = 0.183

Table 7.12 ELISA data (absorbance at 450nm) for serum anti-PTXd IgG2b induced by intranasal administration of PTXd formulations

Animal	Dilution factor	PTXd solution	PTXd in CS-DS nanoparticles	PTXd & IgA in CS-DS nanoparticles	IgA in CS-DS nanoparticles	Buffer only
1	5	0.312	0.425	0.160	0.172	0.420
	10	0.215	0.180	0.154	0.130	0.200
2	5	0.598	0.463	0.413	0.158	0.310
	10	0.228	0.318	0.322	0.157	0.285
3	5	0.170	0.526	0.172	0.175	0.245
	10	0.138	0.246	0.156	0.144	0.232
4	5	0.498	0.356	0.411	0.246	0.202
	10	0.181	0.167	0.215	0.180	0.215
5	5	0.388	0.528	0.380	0.266	0.354
	10	0.251	0.266	0.270	0.228	0.301
6	5	0.342	0.487	0.335	0.254	0.314
	10	0.160	0.194	0.169	0.235	0.228

Average absorbance for blanks (antigen free and antibody wells) = 0.230

Table 7.13 ELISA data (absorbance at 450nm) for serum anti-PTXd IgG induced by intranasal administration of PTXd formulations

Animal	Dilution factor	PTXd solution	PTXd in CS-DS nanoparticles	PTXd & IgA in CS-DS nanoparticles	IgA in CS-DS nanoparticles	Buffer only
1	3	0.375	0.460	0.443	0.255	0.311
	12	0.297	0.331	0.306	0.222	0.247
	48	0.228	0.219	0.200	0.212	0.220
2	3	0.482	0.393	0.590	0.188	0.238
	12	0.346	0.307	0.397	0.198	0.209
	48	0.275	0.216	0.252	0.154	0.131
3	3	0.363	0.668	0.526	0.230	0.170
	12	0.260	0.417	0.317	0.202	0.134
	48	0.210	0.273	0.210	0.212	0.148
4	3	0.463	0.352	0.521	0.301	0.195
	12	0.360	0.311	0.356	0.206	0.207
	48	0.250	0.226	0.272	0.199	0.161
5	3	0.399	0.400	0.630	0.144	0.265
	12	0.285	0.323	0.366	0.137	0.233
	48	0.185	0.214	0.225	0.141	0.214
6	3	0.553	0.408	0.519	0.193	0.171
	12	0.389	0.393	0.371	0.165	0.135
	48	0.246	0.232	0.252	0.150	0.128

Average absorbance for blanks (antigen free and antibody wells) = 0.151

Table 7.14 ELISA data (absorbance at 450nm) for lung homogenate anti-PTXd IgA induced by intranasal administration of PTXd formulations

Animal	Dilution factor	PTXd solution	PTXd in CS-DS nanoparticles	PTXd & IgA in CS-DS nanoparticles	IgA in CS-DS nanoparticles	Buffer only
1	5	0.276	0.284	0.265	0.310	0.288
	10	0.259	0.265	0.270	0.291	0.244
	20	0.231	0.260	0.234	0.248	0.248
2	5	0.227	0.284	0.222	0.298	0.221
	10	0.202	0.242	0.207	0.288	0.212
	20	0.225	0.235	0.219	0.254	0.203
3	5	0.306	0.313	0.262	0.285	0.292
	10	0.278	0.285	0.262	0.274	0.261
	20	0.199	0.246	0.262	0.245	0.225
4	5	0.288	0.294	0.294	0.302	0.360
	10	0.215	0.267	0.351	0.300	0.334
	20	0.206	0.221	0.253	0.254	0.304
5	5	0.207	0.208	0.221	0.233	0.237
	10	0.197	0.225	0.210	0.224	0.220
	20	0.177	0.212	0.203	0.190	0.177
6	5	0.372	0.374	0.370	0.346	0.392
	10	0.301	0.354	0.314	0.331	0.358
	20	0.337	0.358	0.331	0.255	0.261

Average absorbance for blanks (antigen free and antibody wells) = 0.300

Table 7.15 Data (absorbances at 450nm) obtained from estimation of IFN-γ in splenocyte culture supernatants of animal groups immunized by intranasal route using ELISA method.

Formulation used for immunization of animal group ↓	Splenocytes stimulated with Con-A	Splenocytes stimulated with PBS	Splenocytes stimulated with PTX
Buffer only	3.327	0.781	3.367
Blank nanoparticles	3.411	0.721	3.384
PTXd solution	3.447	0.686	3.385
PTXd and IgA in CS-DS nanoparticles	3.395	0.757	2.180
IgA in CS-DS nanoparticles	3.353	0.571	3.161
PTXd in CS-DS nanoaparticles	3.244	0.538	1.661

Average absorbance for blanks (antigen free and antibody wells) = 0.305

7.7 APPROVAL FROM PUBLISHERS FOR USE OF FIGURES

ELSEVIER LICENSE
TERMS AND CONDITIONS

Jan 02, 2011

This is a License Agreement between Sameer Sharma ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Sameer Sharma
Customer address	63 Jackson Road Perth, other 6152
License number	2580581079530
License date	Jan 02, 2011
Licensed content publisher	Elsevier
Licensed content publication	Vaccine
Licensed content title	Immunostimulating reconstituted influenza virosomes
Licensed content author	Rinaldo Zurbriggen
Licensed content date	14 February 2003
Licensed content volume number	21
Licensed content issue number	9-10
Number of pages	4
Start Page	921
End Page	924
Type of Use	reuse in a thesis/dissertation

Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Order reference number	
Title of your thesis/dissertation	Development and Evaluation of Novel Biodegradable Nanoparticles for Vaccine Delivery
Expected completion date	Jan 2011
Estimated size (number of pages)	250
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
Value added tax 0.0%	0.0 USD / 0.0 GBP
Total	0.00 USD

ELSEVIER LICENSE
TERMS AND CONDITIONS

Sep 14, 2010

This is a License Agreement between Sameer Sharma ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Sameer Sharma
Customer address	63 Jackson Road Perth, other 6152
License number	2496940560042
License date	Aug 27, 2010
Licensed content publisher	Elsevier
Licensed content publication	Advanced Drug Delivery Reviews
Licensed content title	Targeting immune response induction with cochleate and liposome-based vaccines
Licensed content author	Susan Gould-Fogerite, Masoumeh T. Kheiri, Fan Zhang, Zheng Wang, Anthony J. Scolpino, Eleonora Feketeova, Mario Canki, Raphael J. Mannino
Licensed content date	6 July 1998
Licensed content volume number	32
Licensed content issue number	3
Number of pages	15
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No

Will you be translating?	No
Order reference number	
Title of your thesis/dissertation	Development and Evaluation of Novel Biodegradable Nanoparticles for Vaccine Delivery
Expected completion date	Sep 2010
Estimated size (number of pages)	220
Elsevier VAT number	GB 494 6272 12

ELSEVIER LICENSE
TERMS AND CONDITIONS

Sep 14, 2010

This is a License Agreement between Sameer Sharma ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Sameer Sharma
Customer address	63 Jackson Road Perth, other 6152
License number	2496941097660
License date	Aug 27, 2010
Licensed content publisher	Elsevier
Licensed content publication	Advanced Drug Delivery Reviews
Licensed content title	Synthetic biomimetic supra molecular Biovector™ (SMBV™) particles for nasal vaccine delivery
Licensed content author	Paul von Hoegen
Licensed content date	23 September 2001
Licensed content volume number	51
Licensed content issue number	1-3
Number of pages	13
Type of Use	reuse in a thesis/dissertation
Intended publisher of new work	other
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic

Are you the author of this Elsevier article?	No
Will you be translating?	No
Order reference number	
Title of your thesis/dissertation	Development and Evaluation of Novel Biodegradable Nanoparticles for Vaccine Delivery
Expected completion date	Sep 2010
Estimated size (number of pages)	220
Elsevier VAT number	GB 494 6272 12

Terms and Conditions

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and

conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article. If this license is to re-use 1 or 2 figures then permission is granted for non-exclusive world rights in all languages.

16. **Website**: The following terms and conditions apply to electronic reserve and author websites:
Electronic reserve: If licensed material is to be posted to website, the web site is to be password-protected

and made available only to bona fide students registered on a relevant course if:
This license was made in connection with a course,
This permission is granted for 1 year only. You may obtain a license for future website posting,
All content posted to the web site must maintain the copyright information line on the bottom of each image,
A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com , and
Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

17. **Author website** for journals with the following additional clauses:

All content posted to the web site must maintain the copyright information line on the bottom of each image, and
he permission granted is limited to the personal version of your paper. You are not allowed to download and post the published electronic version of your article (whether PDF or HTML, proof or final version), nor may you scan the printed edition to create an electronic version,
A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx , As part of our normal production process, you will receive an e-mail notice when your article appears on Elsevier's online service ScienceDirect (www.sciencedirect.com). That e-mail will include the article's Digital Object Identifier (DOI). This number provides the electronic link to the published article and should be included in the posting of your personal version. We ask that you wait until you receive this e-mail and have the DOI to do any posting.
Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

18. **Author website** for books with the following additional clauses:
Authors are permitted to place a brief summary of their work online only.
A hyper-text must be included to the Elsevier homepage at http://www.elsevier.com

All content posted to the web site must maintain the copyright information line on the bottom of each image
You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version.
Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

19. **Website** (regular and for author): A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx. or for books to the Elsevier homepage at http://www.elsevier.com

20. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

21. **Other Conditions**:

v1.6

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK10838817.
Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006

If you find copyrighted material related to this license will not be used and wish to cancel, please contact us referencing this license number 2496941097660 and noting the reason for cancellation.

Questions? customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

7.8 PUBLICATIONS AND PRESENTATIONS

- Sameer Sharma, TKS Mukkur, Heather AE Benson, Yan Chen. Pharmaceutical aspects of intranasal delivery of vaccines using particulate systems. Journal of Pharmaceutical Sciences, 2009, 98(3), 812-843.
- Development and optimization of chitosan-dextran sulfate nanoparticulate vaccine delivery system. In preparation.
- *In-vivo* immunological evaluation of a polyelectrolyte complex nanoparticulate vaccine formulation incorporating IgA antibody as novel immunomodulator. In preparation.
- Sameer Sharma, TKS Mukkur, Heather AE Benson, Yan Chen. Development and evaluation of a potential M-Cell targeted nanoparticulate vaccine delivery system. The 37th Annual Meeting and Exposition of the Controlled Release Society 2010, Portland, Oregon, U.S.A.
- Sameer Sharma, Heather AE Benson, TKS Mukkur, Yan Chen. Optimization and evaluation of a potential Immunomodulator-loaded Nanoparticulate nasal vaccine. APSA 2009 conference, Hobart, Tasmania, Australia.
- Jilna Bhatt, Sameer Sharma, Yan Chen, Heather AE Benson. Optimization and comparison of immunoassay methods for estimation of mouse immunoglobulin. APSA 2008 conference, Canberra, ACT, Australia.
- Development and evaluation of a nanoparticulate vaccine delivery system. ASMR Medical Research Week Scientific Symposium 2010, Perth, Australia. (Oral presentation)
- Intranasal delivery of vaccines using particulate systems. WABRI Postgraduate Symposium 2008, Perth, Australia. (Oral presentation)