CHITOSAN DERIVED FORMULATIONS AND EMZALOID™ TECHNOLOGY FOR MUCOSAL VACCINATION AGAINST DIPHTHERIA: NASAL EFFICACY IN MICE

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INTRODUCTION AND AIM OF STUDY

It is well known that the immunisation against infectious diseases and the effectiveness of these vaccines are one of the greatest breakthroughs in medicine to this day. Immunisation is by far the most cost-effective strategy to prevent needless morbidity and mortality. Currently it is estimated that immunisation saves the lives of three million children a year, but with the high cost of needles and syringes, the risk of infection with HIV due to vaccination with contaminated needles, refrigeration costs and labour it has become apparent that there is a great need for the development of new delivery systems for the delivery of vaccines (André, 2003:594).

In recent years, there has been an increasing interest in the development of novel vaccine systems for prophylactic and therapeutic purposes. New delivery systems and the use of adjuvants that can affect the immune response in both a qualitative as well as a quantitative way have been investigated by many researchers.

Mucosal routes of vaccination are attractive alternatives to parenteral immunisation since it is possible to stimulate both arms of the immune system and provide both humoral (anti-body) and cell mediated (cytotoxic lymphocytes) immune responses (Illum & Davis, 2001:1). It is also known that the majority of invading pathogens enter the body via the mucosal surfaces and it would therefore be beneficial if air and food borne pathogens could be neutralised upon arrival at the mucosal surfaces.

For mucosal vaccine delivery, the lymphoid tissue should be targeted. Access to the mucosal associated lymphoid tissue (MALT) is provided by antigen sampling cells. These microfold cells (M-cells) are located in between the epithelial cells and take up antigens and microparticles smaller than 10 μm (Van der Lubben *et al.*, 2001:201). Despite the need for an efficient mucosal vaccine, its development is still hindered by the degradation of antigens during transport to the mucosal associated lymphoid tissue

(MALT), as well as low uptake by the MALT. To avoid these problems, antigens for mucosal vaccination can be associated to an efficient delivery system.

Nasal vaccination is an attractive route of mucosal vaccination since it is easy to deliver the antigen to the target site, the nasal associated lymphoid tissue (NALT) situated mainly in the pharynx as a ring of lymphoid tissue, the Waldeyer's ring. Furthermore, it avoids degradation of the antigens in the gastrointestinal tract resulting from acidic or enzymatic degradation, which is a major disadvantage of oral vaccination. However, nasal vaccination is complicated by the fast clearance of antigens as well as the low and incomplete transport of antigens across the epithelial barrier. For a transient and reversible opening of tight junctions and permeation of antigens across the epithelial barrier, two different approaches can be pursued: co-administration of antigens with an absorption enhancer, or entrapment of antigens into a microparticulate or nanoparticulate system to stimulate M-cells present in the NALT, which will then subsequently lead to the production of an immune response (Van der Lubben *et al.,* 2001:142).

Chitosan [(1→4)-2-amino-2-deoxy-β-D-glucan] is regarded as a biocompatible, biodegradable polymer of natural origin that is widely used in the food industry. Recently, chitosan has been considered for pharmaceutical formulations and drug delivery applications in which attention has been focussed on its absorption-enhancing, controlled release and bioadhesive properties (Dodane & Vilivalam, 1998:246). Chitosan is known to improve peptide and protein transport across the epithelial barrier, including nasal epithelia (Illum *et al.,* 1994:1186). However, this polymer is only soluble in an acidic environment.

N-Trimethyl chitosan chloride (TMC) is a partially quaternised derivative of chitosan obtained by reductive methylation of chitosan in a strong basic environment. This polymer exhibits greater water solubility than chitosan, especially in neutral and basic environments (Kotzé *et al.,* 1997:244), making it applicable as an absorption-enhancing agent over a broader pH range. TMC is a potent absorption enhancer for peptide and

protein drugs by opening the tight junctions between epithelial cells (Kotzé *et al.*, 1997:245).

It has previously been demonstrated by Van der Lubben *et al.*, (2001:140) that microparticles can easily be obtained from chitosan and TMC and nanoparticles can easily be obtained from chitosan. Chitosan and TMC particulate delivery systems are very efficient and non-toxic absorption enhancers for nasally administered peptide drugs and vaccines.

Emzaloid™ technology has proven in the past to be an effective delivery system for numerous drugs. Emzaloid is a patented system comprised of a unique submicron emulsion formulation. An Emzaloid is a stable structure within a system that can be manipulated in terms of morphology, structure, size and function. Emzaloids consist mainly of plant and essential fatty acids, emulsified in water saturated with nitrous oxide. These Emzaloids can entrap, transport and delver pharmacologically active compounds and other useful molecules. The use of Emzaloid technology in the mucosal delivery of antigens have not yet been studied, but the hypothesis for the nasal delivery of vaccines using the Emzaloid as delivery system is based on the same principle as that of microparticulate and nanoparticulate delivery systems.

The aim of this study is to investigate the efficacy of chitosan derived formulations and Emzloid™ technology for nasal vaccination against diphtheria toxoid in mice. The specific objectives of this study are:

1) To conduct a literature study on the history and development of vaccinology.
2) To conduct a literature study on chitosan, *N*-Trimethyl chitosan chloride and Emzaloid™ technology as novel delivery systems for nasal vaccination.
3) To synthesise and characterise a TMC polymer
4) To prepare and characterise chitosan microparticles and nanoparticles, TMC microparticles as well as micrometer and nanometer range Emzaloids.

5) To determine diphtheria toxoid loading and release from the microparticles and nanoparticles
6) To conduct stability studies on microparticles and nanoparticles loaded with diphtheria toxoid.
7) To evaluate the efficacy of chitosan derived formulations and Emzloid™ technology for nasal vaccination against diphtheria toxoid, by *in vivo* studies in mice.

Chapter 1 will provide an overview of the history and development of vaccination, while chapter 2 will focus on chitosan, *N*-Trimethyl chitosan chloride (TMC) and Emzaloid™ technology as novel delivery systems for nasal vaccination. In chapter 3 the synthesis and characterisation of a TMC polymer will be described. The preparation and characterisation of chitosan microparticles and nanoparticles, TMC microparticles as well as micrometer and nanometer range Emzaloids will be described in chapter 3. In chapter 4, the *in vivo* studies conducted on mice will be described and the results obtained from ELISA assays will be discussed.

ABSTRACT

Previous studies have demonstrated that chitosan and its derivative, *N*-trimethyl chitosan chloride (TMC) are effective and safe absorption enhancers to improve mucosal delivery of macromolecular drugs including vaccines. Furthermore, chitosan and TMC can easily form microparticles and nanoparticles, which have the ability to encapsulate large amounts of antigens. Emzaloid™ technology has proven in the past to be an effective delivery system for numerous drugs. Emzaloids can entrap, transport and deliver large amounts of drugs including vaccines.

In this study, the ability of chitosan microparticles and nanoparticles, TMC microparticles as well as micrometer and nanometer range Emzaloids to enhance both the systemic and mucosal (local) immune response against diphtheria toxoid (DT) after nasal administration in mice was investigated.

The above mentioned formulations were prepared and characterised according to size and morphology. DT was then associated to the chitosan microparticles and nanoparticles as well as TMC microparticles to determine the antigen loading and release. It was found that the loading efficacy of the formulations was 88.9 %, 27.74 % and 63.1 % respectively, and the loading capacity of the formulations was 25.7 %, 8.03 % and 18.3 %.

DT loaded and unloaded (empty) chitosan microparticles and nanoparticles, TMC microparticles, micrometer and nanometer range Emzaloids as well as DT in phosphate buffered saline (PBS) were administered nasally to mice. Mice were also vaccinated subcutaneous with DT associated to alum as a positive control. All mice were vaccinated on three consecutive days in week 1 and boosted in week 3. Sera was analysed for anti-DT IgG and nasal lavages were analysed for anti-DT IgA using an enzyme linked immunosorbent assay (ELISA).

In the study conducted to determine the systemic (IgG) and local (IgA) immune responses it was seen that DT associated to all the experimental formulations produced a systemic immune response. The said formulations produced a significantly higher systemic immune response when compared to the formulation of DT in PBS. Furthermore, the mice vaccinated with DT associated to the TMC formulations showed a much higher systemic immune response than the mice that were vaccinated subcutaneously with DT associated to alum, whereas the other formulations produced systemic immune responses that were comparable to that of DT associated to alum. It was also found that DT associated to the experimental formulations produced a local immune response, however only DT associated to TMC microparticles produced a consistent local immune response.

It can be concluded from the *in vivo* experiments that the TMC formulations, moreover, the TMC microparticles is the most effective and promising formulation for the nasal delivery of vaccines.

Key words: Nasal vaccination, Chitosan microparticles, Chitosan nanoparticles, *N*-trimethyl chitosan chloride (TMC) microparticles, Emzaloids, Diphtheria toxoid, Systemic immune response (IgG), Local immune response (IgA), ELISA assay.

UITTREKSEL

Dit is in vorige studies vasgestel dat kitosaan en sy derivaat, *N*-trimetiel kitosaan chloried (TMC), veilige en doeltreffende absorpsiebevorderaars is wat die mukosale aflewering van makromolekulêre geneesmiddels, insluitende vaksienes, verbeter. Mikropartikels en nanopartikels wat groot hoeveelhede antigeen kan enkapsuleer, kan maklik van kitosaan en TMC vervaardig word. Dit is in vorige studies bewys dat Emzaloid™ tegnologie 'n effektiewe afleweringsisteem vir baie geneesmiddels is. Emzaloids kan groot hoeveelhede geneesmiddels, insluitende vaksienes, enkapsuleer, vervoer en aflewer.

In hierdie studie is daar ondersoek ingestel na die vermoë van kitosaan mikro- en nanopartikels, TMC mikropartikels sowel as mikro- en nanometer Emzaloid vesikels om die sistemiese en lokale immuunrespons teen difterie toksoïed (DT) na nasale toediening te verbeter.

Die bogenoemde formulerings is voorberei en gekarakteriseer ten opsigte van deeltjiegrootte en morfologie. DT is met die kitosaan mikro- en nanopartikels sowel as aan die TMC mikropartikels geassosieer om die antigeen laaivermoë en vrystelling te bepaal. Uit hierdie studie is vasgestel dat die inkorporeringseffektiwiteit van die formulerings 88.9 %, 27.74 % en 63.1 % onderskeidelik is, terwyl die inkorporeringskapasiteit 25.7 %, 8.03 % en 18.3 % is.

Gelaaide en ongelaaide (leë) kitosaan mikro- en nanopartikels, TMC mikropartikels, mikro- en nanometer grootte Emzaloids so wel as DT in PBS is nasaal aan muise toegedien. DT geassosieer met alum is subkutaneus, as positiewe kontrole, aan muise toegedien. Die muise is op drie agtereenvolgende dae in week 1 en week 3 gevaksineer. Sera is geanaliseer vir anti-DT IgG en nasale sekresies is geanaliseer vir anti-DT IgA deur gebruik te maak van 'n ELISA analise metode.

Hierdie studie het aangetoon dat al die eksperimentele formulerings wat met DT gelaai is, 'n sistemiese immuunrespons veroorsaak het. Die bogenoemde formulerings het 'n aansienlik groter sistemiese immuunrespons in vergelyking met die formulering van DT in PBS veroorsaak. Verder is daar ook waargeneem dat die muise wat met die gelaaide TMC formulerings gevaksineer is 'n baie groter sistemiese immuunrespons as die muise wat subkutaneus met DT, geassosieer aan alum, gehad het. Die ander formulerings het dieselfde effek getoon as die subkutaneuse vaksien. Daar is ook waargeneem dat al die gelaaide eksperimentele formulerings 'n lokale immuunrespons veroorsaak het, maar dat net die TMC mikropartikels 'n deurlopende lokale immuunrespons veroorsaak het.

Hierdie studie toon duidelik aan dat die TMC formulerings, veral die TMC mikropartikels, die effektiefste formulering vir die nasale aflewering van vaksienes is.

Sleutel woorde: Nasale vaksinering, Kitosaan mikropartikels, Kitosaan nanopartikels, *N*-trimetiel kitosaan chloried (TMC) mikropartikels, Emzaloids, Difterie toksoïed, Sistemiese immuunrespons (IgG), Lokale immuunrespons (IgA), ELISA analise.

CHAPTER 1

THE DEVELOPMENT OF VACCINOLOGY: PAST TO PRESENT

1.1 INTRODUCTION

The use of vaccines to prevent infectious diseases may be considered among medicine's greatest achievements (Glück & Metcalfe, 2002:B10). Of all the branches of modern medicine, vaccinology can claim to be the one to have contributed most to the relief of human misery and the spectacular increase in life expectancy in the last two centuries. It is currently estimated that immunisation saves the lives of 3 million children every year. (André, 2003:593). However, infectious diseases remain the leading cause of death worldwide. Thus, the development of vaccines to prevent diseases for which no vaccine currently exists such as AIDS and malaria as well as the improvement of efficacy and safety of existing vaccines remains a high priority (Leclerc, 2003:329).

The recent developments in vaccinology are directly related to major breakthroughs in the fields of immunology, molecular biology, genomics, proteomics, physico-chemistry and computers. These developments promise a bright future for prevention, not only of acute infectious diseases, but also treatment of conditions like chronic infections, allergy, autoimmune diseases and cancer (André, 2003:593).

In this chapter, the history of vaccinology and the achievements made in vaccinology during the last century will be discussed in short, as well as delivery systems and future trends for vaccines.

1.2 THE HISTORY OF VACCINOLOGY

The ancient Chinese practice of preventing severe natural smallpox by inoculating puss from smallpox patients was introduced into Europe in the early eighteenth century. Laypersons, such as farmer Benjamin Justy, inoculated his family with the cowpox puss to prevent smallpox long before the time of Jenner. It was with this background knowledge that the English practitioner, Edward Jenner, conducted the first scientific investigations of smallpox prevention by human experimentation in 1796 (Hilleman, 2000:1437).

The science of vaccinology took off on 14 May 1796 when Edward Jenner inoculated James Phipps, a 13-year-old boy, with the vaccinia virus obtained from a young woman named Sarah Nelmes who had been accidentally infected by a cow named Rosebud. James Phipps was then found to be "secure" (immune) to smallpox as demonstrated by an unsuccessful challenge with the variola virus some months later. Soon afterwards, in 1798, Jenner predicted that the systemic use of his "vaccine", a term proposed many years later by Louis Pasteur to describe Jenner's invention, would result in the "annihilation" of smallpox. Jenner's prediction was finally realised on 9 December 1979 when the World Health Organisation (WHO) certified that one of the worst scourges of humanity had been wiped out by a vaccine developed nearly 200 years before (André, 2003:593).

1.3 ADVANCES IN VACCINOLOGY DURING THE PAST CENTURY

1.3.1 The era of grand expectations

From the vantage point of the 1890s, there was reason to believe that a remarkable series of vaccine innovation would follow the scientific breakthroughs of Louis Pasteur, Robert Koch, Emil van Behring and Paul Ehrlich (Galambos, 1999:S7).

Having noted attenuation of fowl plague bacteria by laboratory cultivation, Pasteur also observed that they induced resistance to subsequent challenge with virulent bacteria. Further studies gave rise to his development of credibly useful vaccines against anthrax, cholera and virus-caused rabies (Hilleman, 2000:1438).

Robert Koch, in Berlin, was the master of pure culture technology and was heralded for his discovery of both the cholera and tubercle bacilli. Koch's postulates gave rigid definition to establishing specific etiology in disease, and his discovery of clinical hypersensitivity ranked with Metchnikoff's discovery of phagocytic cells in relation to innate immunity. Emil van Behring, the first recipient of the Nobel Prize, utilised Roux's and Yersin's discoveries of the soluble toxins of diphtheria and tetanus bacilli that could be detoxified for purpose of immunisation, and established the field of passive immunotherapy. This was to dominate therapeutic medicine against infectious diseases for decades to come. The most far-reaching discoveries of that era, however, were those of Paul Ehrlich who found specific affinities of dyes and other chemicals for cell components. Based on the principles of selectivity, he developed the world's first synthetic pharmaceutical drug, that of compound 606, or Salvarsan, for treating syphilis (Hilleman, 2000:1438).

This was the era of grand expectations, and there was a lot of progress, but it fell far short of the promise that seemed to exist in the 1890s and this was certainly true in vaccinology. There were indeed accomplishments: the conservative opposition to smallpox vaccination was steadily beaten back in the developed world. Innovators in the public and private sectors produced a variety of new serum antitoxins, a few of which were actually effective. There were experimental vaccines as well, not all of which were effective and safe against diphtheria, pertussis, tuberculosis (BCG), tetanus, yellow fever and typhus *(Rickettsia)* (Galambos, 1999:S8).

Although the business in biologicals continued to expand, the public health authorities in Europe and the United States were helpless when the great influenza pandemic of 1918-1919 struck down millions. Having neither an understanding of the source of the

influenza nor a vaccine, physicians could do nothing to prevent the spread of the disease or the onset of secondary bacterial pneumonia. It was the 1930s before the etiology of influenza was understood, and it took years of additional research to produce truly effective vaccines (Galambos, 1999:S8).

1.3.2 The era of fulfilment (1930-1957)

The two decades between 1930 and 1950, which covered World War II, was a time of transition for what was to become the era of vaccines. The large breakthrough of the era was Goodpasture's demonstration in 1931 of viral growth in embryonated hen's eggs. From this came Theiler's safe and effective minced chick tissue vaccine 17D against yellow fever that found enormous application in tropical countries (Hilleman, 2000:1439). During this era vaccinology gradually became the same kind of exciting frontier that bacteriology had been to the scientific community in the 1890s.

Table 1.1: Vaccinology: 1930- 1958 (Hilleman, 2000:1438).

1931	*Goodpasture* – Virus propagation on membranes of embryonated hens eggs
1935	*Theiler* – Safe and effective yellow fever vaccine attenuated by passage in minced chick embryo cultures
Early 1940s	*Cox* – Formalin- inactivated embryonated hen's egg (yolk sac) typhus vaccine for European invasion
1944	Formalin- inactivated mouse brain Japanese B encephalitis vaccine for Far East invasion
1945	*Wendell Stanley* – Sharpless- purified chick embryo allantoic fluid-derived influenza virus vaccine
1948-1958	Discovery of progressive antigenic change (drift) and major change (shift) in influenza viruses by prospective and retrospective viral and seroepidemiologic studies
1953-1957	Discovery of Adenovirus – 1953 Killed virus vaccine developed and proved effective (98%) – 1956 Vaccine went commercial – 1958

1.3.3 The golden age of vaccine development

A strong institutional base, generous funding, and new scientific knowledge led vaccinology into a long era of fulfilment extending from the 1950s through the 1970s. This was as Susan and Stanley Plotkin have explained 'the golden age of vaccine development ...' (Galambos, 1999:S8).

Following the development of vaccines for measles, mumps and rubella, a combination of the three vaccines greatly simplified the process of immunisation. Like DPT – the combination of a diphtheria toxoid, pertussis vaccine and tetanus toxoid – the measles, mumps and rubella combination has had a dramatic impact on the mortality and the morbidity in the developed counties where immunisation rates are extremely high (Galambos, 1999:S8).

Golden age science also yielded effective, safe pneumococcal vaccines, whose history tells us a somewhat different story. In this case, the first significant steps towards the development of a multivalent vaccine based on the polysaccharide capsule of the bacterium took place in the late 1930s and 1940s. However, the penicillin mystique was so powerful that both products were withdrawn and further progress was delayed for two decades. Then, thanks to the hard-minded determination of Drs Robert Austrian and Jerome Gold, who demonstrated the figures on the morbidity and mortality from pneumonia, research began anew in the 1960s (Galambos, 1999:S8).
Decades of research lay behind the first successful subunit vaccine (1981), produced from plasma. Additional research resulted in the world's first recombinant DNA vaccine for use in humans in 1986 (Galambos, 1999:S8).

1.3.4 Challenges of the present day

Appropriate to the times is the leading challenges of the day: to develop a successful vaccine against malaria, tuberculosis and HIV infection. Substantial resources have been

concentrated on the efforts to produce an effective vaccine, and the history thus far has been one of early enthusiasm, followed by depressing defeats, followed in turn by determination to pursue the vaccine path. Only a vaccine, it appears today, will provide a solution to the problems in African countries where the rates of infection and mortality from AIDS are staggering (Galambos, 1999:S9).

1.4 TYPES OF VACCINES

- **Live attenuated vaccines** → The effectiveness of live attenuated vaccines is due to their ability to stimulate both the humoral and cell-mediated immune response, the latter typified by the generation of cytotoxic T lymphocytes (CTL). Cytotoxic T lymphocytes are required not only for the eradication of entrenched viral infections and tumours, but also in the early stages of the primary infection or neoplasia to impede the establishment of the infection or tumour. Despite the high degree of efficacy and safety observed with live attenuated vaccines, some pathogens, such as HIV, may be too risky for use as an attenuated organism for routine vaccination (Shroff *et al.,* 1999:205).

- **Inactivated vaccines**→ These vaccines include killed organisms and isolated non-replicated sub-cellular components. Inactivated vaccines stimulate a lower level and shorter duration of immunity than that elicited by live vaccines (Marciani, 2003:934).

- **Recombinant sub-unit vaccines (peptide vaccines)** → These vaccines stimulate antibodies to the pathogen by mimicking proteins on the pathogen's surface.

- **Recombinant vectored vaccines** → These vaccines consist of genes or fragments of genes of the pathogen incorporated into vectors. Vector vaccines have been shown to produce pathogen-specific cytotoxic T cell responses in subjects.

- **DNA vaccines and replicons** → These vaccines contain the gene or genes, coding for an antigenic portion of a virus, parasite or cancer. The genetic sequences are injected into subjects to induce the expression of antigen cells. In the case of replicons, these genes are wrapped in the outer coat of an unrelated virus (Mor, 1998:1151).

- **Combination vaccines or 'prime and boost vaccines'** → This is a combination of two or more different vaccines to broaden or intensify immune responses. Examples include a vector with antigen to prime a T cell response with a subunit booster to produce antibodies, or delivery of DNA followed by a vector with genes or gene sequences expressing the same gene(s) or gene sequences.

1.5 CLASSIFICATION OF VACCINES AVAILABLE TO THE PUBLIC

Table 1.2: Dates of introduction of commonly used vaccines (André, 2003:594).

Vaccine	Date	Vaccine	Date
Smallpox	1796	Hepatitis B (HB)	1981
Rabies	1885	Varicella (V)	1984
Cholera	1896	RDNA HB	1986
Typhoid	1896	*H. influenzae b* (Hib)	1988
Plague	1896	Hepatitis A (HA)	1991
Diphtheria (D)	1923	DTPwIPVHib	1993
Pertussis (Pw)	1926	DTPa	1994
Tetanus (T)	1927	DTPwHB	1996
Tuberculosis (BCG)	1927	HBHA	1996
Yellow fever	1935	DTPaHib	1997
Influenza	1936	DTPaIPVHib	1997
Polio (IPV)	1955	Lyme	1998
DTPw	1957	Rotavirus	1998
Polio (OPV)	1958	Dtpa	1999
DTIPV	1961	HATy	1999
Measles (M)	1963	DTPaHBIPV	2000
DTPIPV	1966	DTPaHBIPVHib	2000
Mumps (M)	1967	MCCV[b]	2000
Rubella (R)	1969	PCV[a]	2000
MMR	1971		

1.6 DELIVERY SYSTEMS AND FUTURE TRENDS

1.6.1 DNA vaccines

A series of publications in the early 1990s demonstrated that plasma DNA (pDNA), alone or in a combination with transfecting agents, could be taken up by muscle cells and that the encoded proteins can be expressed by mouse skeletal muscle, or could elicit antibody responses, and that DNA vaccination could protect mice against lethal virus challenge. Numerous publications followed these observations, demonstrating that many different pDNA-expressed antigens of viral, bacterial, parasitic and tumour origin could provoke immune responses in various species (Shroff *et al.,* 1999:205).

1.6.1.1 How does the DNA vaccine work?

DNA vaccines contain the gene or genes coding for an antigenic portion of a virus (the viral core or envelope proteins), parasite or cancer. It has been proposed that following intramuscular injection, plasmid DNA is endocytosed by the myocytes located at the injection site. These host cells are then thought to take up the foreign DNA, express the viral gene, and make the corresponding viral protein. An important advantage of this system is that the foreign protein enters the cell's major histocompatibility complex (MHC) class I pathway (only proteins originating inside a cell are processed in this manner). Major histocompatibility complex (MHC) class I molecules then carry the peptide fragments of the foreign protein to the cell surface, where they evoke cell-mediated immunity by stimulating $CD8^+$ cytotoxic T cells. This is in contrast to standard vaccines antigens, which are taken up into cells via phagocytosis or endocytosis and are processed through the major histocompatibility complex (MHC) class II system pathway, thereby primarily stimulating antibody responses (Mor, 1998:1151).

1.6.1.2 The immune response following DNA vaccination

Numerous studies have described the kinetics, intensity, character and longevity of the immune response to pDNA-expressed antigens. In small animals, the immune response follows a time course similar to that observed with parentally administered protein antigens. pDNA results in a long lasting response and the level of response is proportional to the amount of pDNA and the number of injections administered. Depending on the antigen, both humoral and cellular immune responses are observed. In mice the immune response to pDNA can be readily characterised as T helper cell type 1 (Th1), rather than T helper cell type 2 (Th2). The difference in these two pathways is that the Th2 response leads to a more pronounced antibody response where as the Th1 response drives a more prominent cell-based inflammatory response. The Th1 response containing a major CTL (cytotoxic T lymphocytes) component is particularly important for killing virally infected cells. In the mouse, these two different pathways are readily identified by a characteristic pattern of antibody isotypes and cytokines. In humans, these two subsets are not well defined, but initial clinical trails indicate that pDNA immunisation induces human immune responses biased toward cell-mediated immunity (Shroff *et al.,* 1999:207).

1.6.1.3 DNA delivery methods

Plasmid DNA has elicited immune responses to the expressed antigen using a variety of routes and delivery methods. To be expressed in the host cell, plasmids must cross the plasma membrane, escape endosomal degratory pathways, and access the cytoplasm. Plasmid DNA coated onto gold beads can be delivered directly into the cytoplasm of the skin cells using a 'gene gun' driven by compressed helium. By any method of administration, pDNA must finally enter the nucleus before gene expression can take place, but once the pDNA is in the nucleus normal cellular transcriptional and translational pathways are exploited for the production of gene products (Shroff *et al.,* 1999:208).

To elicit B cell and T cell immune responses, pDNA and / or their encoded proteins must access the necessary antigen presenting cells (APCs) that are essential for initiating immune responses. Muscles cells clearly harbour pDNA and express protein following intramuscular injections, and therefore probably serve as important antigen reservoirs. However, professional antigen presenting cells (APCs) of haematopoietic origin, not muscle cells, appear to be the dominant cell type presenting antigen, as revealed by bone marrow chimera experiments. Antigen-presenting cells may acquire protein or protein complexes shed by muscle cells and/or phagocytose apoptotic cells and 'cross present' the antigens, as demonstrated for viral systems. Furthermore, evidence exists to support the direct acquisition of pDNA by professional antigen presenting cells (APCs) known as dendritic cells (DCs) and the subsequent expression of antigen by these cells (Shroff *et al.,* 1999:208).

During the very early stages of DNA vaccine development, experiments using a reporter gene activity led to the belief that only muscle cells could take up pDNA. As a result, pDNA was administered most often by intramuscular injection using a needle and syringe. It has been shown that DNA injection into other sites, such as intravenous, intratracheal, intraorbital, intradermal (ID) and subcutaneous produces detectable immune responses (Shroff *et al.,* 1999:208). A study was conducted by Shroff *et al.,* (1999:208), where a 'gene gun' was used to deliver a vaccine to the epidermis. This study revealed that 24 hours post-delivery, 50–100 dendritic cells expressing reporter genes were found in the proximal draining lymph node and robust responses to the expressed antigen was observed.

In general, parenteral routes of antigen delivery fail to elicit protective mucosal immunity. However, it has been observed that there is an excellent level of protection from a lethal mucosal challenge in mice and guinea pigs immunised intramuscularly with a pDNA vaccine against herpes simplex virus. Plasmid DNA delivered via the intranasal (IN) route has also been shown to generate distal mucosal immunity, particularly when the vaccine is co-administered with IL-12 or cholera toxin. Several investigators have also shown that oral delivery of DNA vaccines in poly(lactide-co-glycolide) (PLG)

microcapsules generate mucosal and systemic immune responses (Shroff *et al.*, 1999:209).

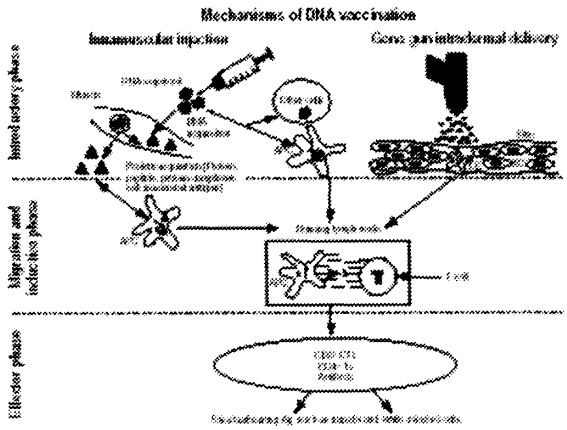


Figure 1.1: Mechanisms of DNA vaccination (Shroff *et al.*, 1999:209).

1.6.1.4 Advantages of DNA vaccines

Plasmid DNA vaccination is a highly versatile and safe procedure that has the potential to replace or supplement other vaccine approaches. Plasmids containing multiple cistrons have been tested and may be useful in simultaneously expressing two or more exogenous proteins from a single cell. In addition, individual plasmids, each encoding multiple antigens, may be mixed to further expand the antigenic diversity of a vaccine (Shroff *et al.*, 1999:210).

A further advantage of the pDNA vaccine approach is that immunogenic viral vectors are not used for delivery allowing for repeated immunisation with expression plasmids. It has been demonstrated that the immune response may be augmented or altered by co-administration of plasmids that encode cytokines or co-stimulatory molecules. Unlike retrovirus or adeno-associated virus vectors, pDNA has not been found to integrate into chromosomal DNA, an extremely important safety consideration (Shroff *et al.*, 1999:210).

Another important advantage of pDNA-mediated vaccination is the possibility of reducing the number of doses because of the prolonged antigen expression that DNA vaccination has to offer (Poland, 1999:1608).

1.6.1.5 Dangers of DNA vaccination

Although the immunogenicity of DNA vaccines is well established, concerns have been raised regarding their safety, more specifically their potential to induce harmful immune responses, such as autoimmunity, and the development of tolerance in immunised individuals. The potential of DNA vaccines to result in the formation of anti-DNA antibodies in healthy persons, as well as in individuals with autoimmune diseases (such as systemic lupus erythematosus) (SLE), is of special concern. An additional safety concern associated with the use of DNA vaccines is that myocytes could potentially become targets for antigen-specific T cells after taking up the injected plasmid and expressing the encoded antigen. Such a process could lead to the development of autoimmune myositis (Mor, 1998:1152).

1.6.2 Reverse vaccinology

Leclerc (2003:331) stated that in conventional vaccinology, the identification of protective antigens is based on purification of some of the molecules produced by a pathogen and analysis of their recognition by antibodies or immune cells. These biochemical, immunological and microbiological methods were successful in many cases but they require the pathogen to be grown in laboratory conditions, are time consuming and allow for the identification of only the most abundant antigens, which can be purified in quantities suitable for vaccine testing. Furthermore, when dealing with non-cultivatable microorganisms, there is no approach to vaccine development (Mora *et al.*, 2003:459).

The first complete genome sequence for any free-living organism *(H. influenza)* was published by Venter and co-workers in 1995, who employed a strategy of random whole-genome 'shotgun' sequencing. The possibility of determining the whole sequence of a bacterial genome led to the idea of using the genomic information to discover novel antigens that had been missed by conventional vaccinoloy (Mora *et al.*, 2003:460).

The availability of whole genome sequences and advances in bioinformatics has dramatically changed the way potential targets for vaccine development can be identified. Computer analysis can now be used to mine the genome sequences for potential surface targets. The only disadvantage of reverse vaccinology is that it is limited to proteins and thus cannot predict other pathogens such as polysaccharides or glycolipids (Leclerc, 2003:331).

1.6.3 Microspheres for vaccine delivery

Due to the advances in vaccinology, many future vaccines will be peptide or protein subunits made by chemical synthesis or recombinant DNA technology. Subunit vaccines are poorly immunogenic when compared to whole-cell vaccines, and therefore require several boosters with standard adjuvants (e.g., aluminium salts) in order to fully vaccinate an individual. As a result, these new vaccines will, in many cases, require improved adjuvants and delivery vehicles to improve antibody responses to levels that ensure protection against infectious disease (Hanes *et al.*, 1997:98).

Aluminium salts were among the first adjuvants discovered back in 1926. They are effective with many antigens, but repeated administration is necessary to achieve protection against infection. Their main method of adjuvanticity is due to their ability to provide a short-term depot effect for adsorbed proteins, slowly 'leaking' antigen to the body's immune system (Hanes *et al.*, 1997:98). Hanes *et al.* (1997:98) concluded that in contrast to aluminium salts, polymeric controlled-release systems could be designed to release entrapped antigens over a long period of time (weeks to months) following a

single immunisation, thereby eliminating the need for booster doses in many cases. This would benefit developing countries where the health conditions are poor and most individuals do not return for their booster doses, resulting in millions of deaths annually from immunisable diseases such as tetanus, pertussis and diphtheria.

1.6.3.1 Microsphere mechanism of adjuvanticity

Traditionally, the term adjuvant has been used to describe any molecule that improves the immune response to co-administered antigen. Many classical adjuvants, such as bacterial cell walls and their adjuvant-active extracts, work by stimulating a non-specific inflammatory response (consisting of various cells of the immune system) local to the site of antigen when given as a co-injection. However, well-designed antigen delivery systems significantly enhance immunity without invoking a vigorous inflammatory response (Hanes *et al.*, 1997:98).

It was initially proposed that controlled release delivery systems enhance immunity by providing a long-term suppository for the antigen, a phenomenon known as the depot theory for adjuvant action. In fact, controlled release systems can provide a release of antigens for weeks to months, a time far exceeding the depot effect of aluminium salts or water/oil emulsions such as Freud's adjuvants. In addition, microspheres can be made to deliver antigens in a continuous or pulsatile fashion over several months. Continuous release mimics the delivery of many small boosters given very close together and pulsatile release may mimic the administration of traditional bolus primary and booster immunisations (Hanes *et al.*, 1997:98).

It is now known that microspheres enhance the immune response to antigens by several mechanisms in addition to the depot effect. For example, microspheres are capable of providing enhanced antigen processing through their ability to target phagocytosis by professional antigen presenting cells (APCs). Microspheres of less than 10 μm in diameter are readily phagocytosed by macrophages, the primary antigen presenting cells (APCs) in the body, leading to direct intracellular delivery of the antigen for processing

by the major histocompatibility complex (MHC) class II pathway (exogenous antigen). Recently, it has also been shown that the encapsulation of antigens within particulates, or on their surface, can lead to antigen presentation by the major histocompatibility complex (MHC) class I pathway (endogenous antigen) as well. Presentation of antigens by major histocompatibility complex (MHC) class II molecules generally leads to enhanced antibody production (i.e. the induction of a humoral immune response), whereas antigen presentation by major histocompatibility complex (MHC) class I molecules primes cytotoxic T lymphocytes (CTL–mediated immune response). A humoral immune response is generally effective for protection from blood-borne pathogens and toxins, while a cellular immune response is thought necessary for the eradication of infected or altered cells of the body, as in the case of cancer cells or virus-infected cells (Hanes *et al.*, 1997:99).

Microspheres are also capable of protecting antigens from rapid destruction *in vivo*, allowing for presentation of the antigen in its native conformation to various cells of the immune system. Native antigen is of particular importance to antibody affinity maturation, the process by which the immune system selects the pool of B cells, which produce antibodies with the highest affinity for the antigen being delivered. If the delivered antigen is not in its native state, one may expect an affinity maturation that selects for antibodies with lower affinity for the native antigen (and therefore, lower toxin or pathogen neutralising capacity) (Hanes *et al.*, 1997:99).

Hanes *et al.*, (1997:99) also concluded that the protection provided to the antigen by encapsulation in polymer microspheres allows the antigen to be delivered via the oral route. The protective polymer coating of the microspheres is thought to at least partially protect the antigen from destruction by the low pH of the stomach, and the high levels of proteases and bile salts in the intestine. Furthermore, microspheres smaller than 10 μm in diameter are taken up from the intestine into the immune-inductive environment of the Peyer's patches where they can induce both mucosal and systemic immune responses.

Finally, microspheres can deliver adjuvants or be made of polymers that break down into adjuvant-active molecules, thereby providing long-term delivery of antigen associated with a vaccine adjuvant for further potentiation of the immune system (Hanes *et al.*, 1997:99).

1.6.3.2 Sterility and manufacturing considerations

Antigen-microspheres are too large to pass through a filter, thus leaving the manufacturer two options for producing a sterile product: aseptic processing throughout the micro-encapsulation process, or terminal sterilisation. Terminal sterilisation is usually achieved by gamma irradiation or electron beam irradiation. However, radiation can cause the formation of free radicals, leading to significant polymer degradation with lactide/glycolides. This polymer degradation can cause changes in the performance of antigen-microspheres, such as altered kinetics. The formation of free radicals because of irradiation can also have harmful effects on the antigen, such as oxidation, denaturation and aggregation, which can lower the potency of the antigen. As a result, the recommended method of attaining a sterile antigen-microsphere product is via aseptic processing procedures (Hanes *et al.*, 1997:101).

1.6.3.3 Challenges: Antigen stability during microsphere preparation and release

Although micro-encapsulation has been used extensively in the pharmaceutical and chemical industries, the technology remains far from being fully developed. The results obtained to date on the development of the controlled release of therapeutic or antigenic agents from biodegradable poly(lactic acid) (PLA) or poly(lactide-co-glycolide) (PGLA) have not been satisfactory. A crucial issue in the development of such formulations is the difficulty of controlling the manner and timing of protein delivery while preserving its bioactivity (Sánchez *et al.*, 1999:256). Future developments in this area greatly depend on the ability to overcome the instability of microencapsulated proteins. In this respect, the main hurdle is related to the complex structure inherent to protein molecules, which make them highly susceptible to physical and chemical instabilities. It is very important that the

protein (or subunit antigen) stays stable in the polymer microsphere because the native form is often required to invoke neutralising antibody responses, as well as to promote appropriate affinity maturation of antibodies. Antigens are often exposed to harsh conditions during the micro encapsulation process and *in vivo* prior to release (Table 1.3). Therefore, to maximise the probability of releasing intact antigen from PLGA microspheres, initial studies of antigen stability should be performed. Initial studies should include screening of stabilisers to prevent denaturation during encapsulation and incubation at physiological pH, ionic strength and temperature over the desired release time (Hanes *et al.,* 1997:102).

Table 1.3: Conditions during antigen encapsulation and release that may affect antigen stability (Hanes, *et al.,* 1997:102).

Conditions to which antigen may be exposed	Typical effect on antigen	Method(s) to minimize the effect on antigen stability
Organic solvent/water interface	Unfolding, aggregation	Stabilisers, surfactants
Shear	Unfolding, aggregation	Surfactants
Heat during encapsulation	Unfolding, aggregation	Operate at lower temperature
Freezing and drying	Unfolding, aggregation	Stabilisers
Hydrophobic polymer surfaces during drying	Unfolding, aggregation	Surfactants, stabilisers
Incubation in aqueous environment at 37 °C	Deamidation, oxidation	Chemical modification of protein
Low pH environment	Unfolding, iso-Asp formation	Make microspheres more porous, use partially insoluble buffering excipients
Silicone oil and heptane with coacervation method	Unfolding, aggregation	Stabilisers, surfactants, use solvent evaporation technique

1.6.4 Lipopeptide antigens encapsulated in novel liposomes prepared from the polar lipids of various *Archaeobacteria*

The facultative intracellular bacterial pathogen, *Listeria monocytogenes*, is capable of parasitising both host phagocytes and parenchymal cells such as enterocytes and hepatocytes. Systemically initiated infection of mice with *L. monocytogenes* has long served as a model for studying adaptive immunity to intracellular pathogens in general. Mice that recover from a primary sub-lethal infection with *L. monocytogenes* acquire an enhanced resistance to re-infection, which is considered to be a classical example of antigen-specific, $CD8^+$ T cell-mediated, macrophage expressed immunity. Likewise, specific immunity against other intracellular bacterial pathogens appears to require the participation of this defence mechanism. Naturally, this immunity is acquired following exposure to sub-lethal doses of the specific virulent organism. Artificially, it can be generated by vaccination. Generally, live attenuated vaccines have proven most effective against this class of pathogen. A major goal of modern vaccinology is to emulate the efficacy of such live vaccines with suitably adjuvanted, defined acellular vaccines (Conlan *et al.*, 2001:3509).

In the case of *L. monocytogenes*, several experimental viable and non-viable vaccines have been reported. Many of these are based on an immunodominant epitope of the virulence factor, listeriolysin. This epitope has been formulated entrapped and co-entrapped with Quill-A in conventional liposomes, expressed with anthrax toxin as a fusion protein, or encoded in plasmid DNA. It has also been expressed in recombinant vaccinia virus or recombinant *Salmonella typhimurium*. All of these vaccines have been tested in the murine model of systemic listeriosis. In all cases, vaccination elicited varying degrees of protection against subsequent exposure to *L. monocytogenes*. However, regulatory concerns, including safety concerns, still surround many of these vaccination strategies (Conlan *et al.*, 2001:3510).

Conlan *et al.* (2001:3510) investigated the utility of archaeosomes, which they defined as liposomes prepared from the polar lipids of various *Archaeobacteria*, developed as

vaccine and drug delivery systems. Compared to the natural and synthetic ester phospholipids used to make conventional liposomes, archaeobacterial polar lipids possess distinct chemical features. Consequently, archaeosomes display several unique properties, including enhanced stability against the extremes of pH, oxidation, elevated temperatures and the actions of lipases.

It was recently demonstrated that humoral and cell mediated immune responses to model protein entrapped alone in various archaeosomes were superior to those generated by the same antigens entrapped in conventional liposomes, or adsorbed to alum, and were equal to those achieved with Freud's adjuvant. It was also found that the soluble antigens entrapped in archaeosomes induced antigen specific $CD8^+$ T cell responses. To further explore the latter ability in a more biologically relevant system, Conlan *et al.* (2001:3510) undertook a study that used a mouse infection model to examine the ability of synthetic antigens encompassing an immunodominant epitope of listeriolysin encapsulated in various archaeosomes to elicit protective immunity against systemic infection with *L monocytogenes*. The results showed that immunisation with archaeosome-based vaccines rapidly generated protective immunity, which would persist for several months.

1.6.5 Virosomes

The immunopotentiating reconstituted influenza virosome (IRIV) delivery system is comprised of spherical unilamellar vesicles with a diameter of approximately 150 nm. Particular attention was paid to the components in the virosomal formulation to ensure that the components are suitable for human use, lack toxicity and possess adjuvant activity (Mischler & Metcalfe, 2002:B17).

The main constituents of IRIVs consist of naturally occurring phospholipids (PL) and phosphatidylcholine (PC). Previous uses of phosphatidylcholine (PC) have included numerous pharmaceutical preparations, specifically for malnutrition treatment through oral and intravenous solutions. Phosphatidylcholine (PC) has been shown to be non-

immunogenic even when combined with potent adjuvants, and forms approximately 70 % of the virosomal structure. The remaining 30 % of membrane components are composed of envelope phospholipids originating from the influenza virus used to provide neuraminidase (NA) and haemagglutinin (HA) glycoproteins (Mischler & Metcalfe, 2002:B17).

The purified haemagglutinin (HA) and neuraminidase (NA) antigens entercalated within the phospholipid bilayer provide a natural presentation of antigens (figure.1.2) Virosomes are biologically degradable, contain no preservatives or detergents and present fewer localised effects when compared to conventional parenteral vaccines. The virosomes bind to antigen presenting cells (APCs) at the influenza virus surface lycoprotein haemagglutinin (HA) and enter the cells by receptor-mediated endocytosis, the virosomes then fuse with the endosomal cell membrane (figure 1.3). This process provides optimal processing and presentation of antigens to immunocompetent cells (Glück & Metcalfe 2002:B11).

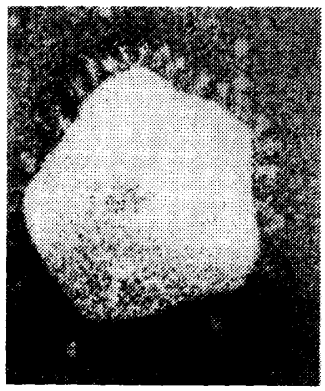


Figure 1.2: Electronmicrograph of an IRIV vesicle carrying two hepatitis A virion particles. The influenza glycoproteins (on the top of the picture) haemagglutinin and neuraminidase form spikes that protrude from the IRIV-membrane. The HAs have not yet been activated by the low endosomal pH (Glück & Metcalfe, 2002:B11).

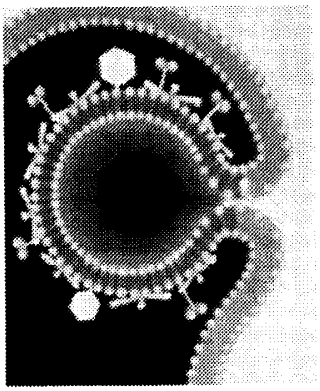


Figure 1.3: Computergraph of the fusion between the IRIV carrying two hepatitis A particles and the endosomal membrane at pH 5.0 (Glück & Metcalfe, 2002:B11).

The neuraminidase (NA) can readily intercalate into the phospholipid membrane and is a tetramer composed of four equal, spherical subunits hydrophobically embedded in the IRIV membrane by a central stalk. The influenza haemagglutinin (HA) intercalated into the phospholipid bilayer acts to stabilise the liposome base by preventing fusion with other liposomes. It is also the major antigen of the influenza virus, containing epitopes of both HA1 and HA2 polypeptides. Furthermore, haemagglutinin (HA) is responsible for the fusion of the virus with the endosomal membrane (Mischler & Metcalfe, 2002:B17).

The neuraminidase (NA), present on the IRIV's surface, aids its action by the same mechanism through which it enhances influenza virus pathogenicity. Neuraminidase (NA) catalyzes the cleavage of *N*-acetylneuraminic acid (sialic acid) from bound sugar residues, resulting in a decreased viscosity of the host's mucus and allowing the influenza virus easier access to epithelial cells. The same process leads to destruction of the haemagglutinin receptors within the cell membrane to which viruses and IRIVs bind. This allows the virus particles to avoid aggregation, as newly formed virus particles do not adhere to the infected host cell membrane after budding, allowing the influenza virus to retain its mobility. The actions of neuraminidase greatly enhance the infectivity of the virus and therefore the action of the virosomes. With IRIVs, the actions of neuraminidase (NA) may be useful, as, after coupling with haemagglutinin (HA), IRIVs not absorbed

into the cell by endocytosis can be cleaved off to potentially react with alternative cells. As an additional benefit, the reduction of viscosity of the host's mucus may prove useful in the development of an intranasal vaccine (Mischler & Metcalfe, 2002:B18).

The influenza haemagglutinin (HA), intercalated into the liposomal bilayer, plays an essential role in the mode of action of the IRIVs. Two polypeptides, haemagglutinin, HA1 and HA2, forming the haemagglutinin (HA) membrane protein are responsible for the fusion of the virus with the endosomal membrane. The sialic acid site for haemagglutinin is contained in the HA1 globular head group. The interaction of haemagglutinin (HA) with its natural receptors, sialyted lipids, enables IRIVs to bind to sialic acid receptors of antigen presenting cells (e.g. macrophages, lymphocytes) initiating an immune response. The HA2 polypeptide mediates the fusion of viral and endosomal membranes initiating the "infection" of cells. The low pH of the host cell endosome (approximately pH 5.0) produces a conformational change in the haemagglutinin (HA) that is a prerequisite for fusion to occur. A second action of the haemagglutinin (HA) relies on an individual's immunological memory to haemagglutinin (HA) established by previous influenza immunisation or infection. The binding of IRIVs and associated antigens to primed antigen processing cells, such as macrophages, is facilitated through the haemagglutinin (HA) expression of highly conserved T cell epitopes. The rapid release of the transported antigen into the membranes of the target cells results from virosomes stimulating the activity of natural influenza virus (Glück & Metcalfe, 2002:B11).

The specific fusion mechanism of virosomes allows targeting of the major histocompatability complex (MHC) class I or class II pathways. Antigens linked to the surface of virosomes are degraded upon endosomal fusion and are presented to the immune system by major histocompatability complex (MHC) class II receptors. Antigens encapsulated in the virosomes are delivered to the cytosol during the fusion event, thus entering the major histocompatability complex (MHC) class I pathway. Therefore, virosomes are able to induce either a B cell or T cell response. Using IRIVs as a delivery vehicle, two vaccines are currently on the market (Glück & Metcalfe, 2002:B11).

1.6.5.1 Epaxal®

The first virosome based vaccine for human use was licensed in 1996, a virosomal hepatitis A vaccine (Glück & Metcalfe, 2002:B12). Epaxal® is an aluminium-free vaccine based on formalin-inactivated hepatitis A virus (HAV) particles, which are attached to the surface of special liposomes, so called immunopotentiating reconstituted influenza virosomes (IRIVs). In contrast to other commercially available hepatitis A virus vaccines, Epaxal® does not contain aluminium as adjuvant. The virosomes are safe, efficient and an easily prepared carrier system for small virions such as hepatitis A viruses. The surface of the virosomes contains the haemagglutinin (HA) antigen from the influenza virus, which enhances the immune response to the inactivated hepatitis A virus (HAV). Epaxal® has been shown to be safe, well tolerated and highly immunogenic (Usonis *et al.*, 2003:4588).

1.6.5.2 Infexal® V

The use of virosomes to deliver influenza antigens to the endosome and stimulate a strong immune response of immunocompetent cells forms the basis of Infexal® V. Infexal® V is a parenteral trivalent virosome influenza vaccine that consists of a mixture of three monovalent virosome pools, each formed with one influenza strain's specific haemagglutinin (HA) and neuraminidase (NA) glycoproteins (Mischler & Metcalfe, 2002:B20).

1.6.5.3 Alternative uses of virosomes as delivery systems

The potential of virosomes as delivery systems for peptide and nucleic acid based vaccines has been investigated for several diseases including malaria, melanoma, hepatitis C virus and Alzheimer's disease. Virosomes as antigen carriers protect the incorporated peptide and adjuvants and they provide additional immunogenicity (Glück & Metcalfe, 2002:B13).

1.6.5.3.1 Peptide vaccine delivery through virosomes

The antigenically unique life stages of *Plasmodium falciparum*, the causative agent of malaria, intensify the problem of the development of a vaccine against this parasite. The ability of virosomes to present several peptides may provide the base for the development of a suitable vaccine against *Plasmodium falciparum*. The use of virosomes as a delivery system for a multi-epitope malaria peptide vaccine has been studied. The results from this study indicated that IRIVs, incorporated in the design of molecularly defined combined vaccines, could be used to target multiple antigens and development stages of one parasite, as well as multiple pathogens (Glück & Metcalfe, 2002:B13).

1.6.5.3.2 Targeting of virosomes for cytosolic drug delivery

IRIVs have also attracted attention as delivery vesicles for cytosolic drug delivery through their membrane fusion activity. It has been shown that virosomes can be targeted towards ovarian carcinoma cells with preservation of this fusion activity. It has also been shown that the use of a virosome with polyethylene glycol (PEG) derived lipids, incorporated into the membrane prevents haemagglutinin (HA) from interacting with sialic residues. This, in combination with the coupling of Fab´ fragments of an anti-epithelial glyocoprotein to the distal ends of the PEG lipids, enabled the specific binding of virosomes to ovarian carcinoma cells. In addition, it has been suggested that cationic virosomes (virosomes with a single positively charged membrane) may provide an efficient delivery system for antisense oligonucleotides in cancer therapy. The interaction between the positively charged lipids on the virosomes and the negatively charged oligonucleotides aids the specific targeting and binding of the virosomes. Reconstituted fusion-active viral envelopes conjugated with an antibody against a tumour antigen have potential as new selective drug delivery systems for the treatment of tumours expressing a selective tumour antigen (Glück & Metcalfe, 2002:B13).

1.6.5.3.3 Virosomal gene delivery

DNA vaccines are comprised of plasmid DNA which, when delivered into cells, encodes antigen molecules directly in the target organism. However, it has been noted that the outcome of DNA vaccination is dependant upon species and strain of the vaccinated host, vaccine formulation, host and route of delivery. The need for high quantities of DNA and numerous doses for optimal vaccination has led to the use of delivery systems able to target cells and improve immunogenicity. Viral vectors can improve the long-term expression of the therapeutic gene by utilising the natural integration of the viral genome into that of the host (Glück & Metcalfe, 2002:B13).

The administration of a DNA vaccine delivered by influenza virosomes via the intranasal route against respiratory pathogens has shown to be efficient for inducing an immune response both mucosally and systemically (Cusi & Glück, 2000:1435). These results suggest that cationic virosomes may have great potential as an efficient delivery system for intranasal immunisation with the advantage of providing an immunological barrier at the point of virus entry (Glück & Metcalfe, 2002:B13).

1.6.6 Polysaccharide conjugate vaccines

The causative agent of many bacterial infections contains surface structures made of polysaccharides, against which protective antibodies are directed (Glück & Metcalfe, 20002:B15). Although the antibodies directed at the polysaccharide are protective, the polysaccharides are thymus-independent antigens. These antigens cannot be processed and presented on the major histocompatibility complex (MHC) and, thus, do not induce T cell 'help' to the polysaccharide-specific B cell. At a gross level, the lack of help results in antibody responses dominated by IgM, with very little class switching to IgG. Of perhaps even greater importance to successful immunoprophylaxis is the inability of infants and young children (the main target group of vaccines) to respond in even this limited way to these antigens (Heath, 2001:428).

Early work by Goebel and Avery indicated that a potential way around this problem could be to conjugate the polysaccharide to a T-dependant protein antigen prior to immunisation. The theory behind this approach is that the polysaccharide-specific B cell takes in the conjugate and, although it is unable to process the polysaccharide moiety, it can process and present peptides derived from protein. T cells specific for these peptides will then lend help to the B cell and the response to the polysaccharide will now look more like a thymus-dependant response, therefore, high levels of IgG antibodies will be produced, and this should also happen in normally refractive infants. The conjugation approach has proved to be extremely successful, and the H. influenza type B conjugate is widely used (Heath, 2001:428).

1.6.7 Identification of T cell epitopes using bioinformatics

In the past few years, the understanding of the antigenic structure recognised by CD4 and CD8 T cell receptors has evolved rapidly. The demonstration that T cells recognise short peptidic sequences bound to major histocompatibility complex (MHC) class I or class II molecules has opened new perspectives in the field of therapeutic and prophylactic vaccines. The sequencing of peptides eluted from major histocompatability complex (MHC) molecules showed that peptides that can bind to major histocompatability complex (MHC) molecules share common patterns. These 'MHC binding motifs' could be used to identify all the peptides inside a protein that could bind MHC molecules and thus stimulate a T cell response. Many MHC-binding algorithms are available, and other algorithms have been developed to identify highly conserved regions or sequences, which share homology with other known proteins. Using these approaches it has been possible to identify hundreds of novel, conserved, T cell epitopes from HIV and *Mycobacterium tuberculosis* (Leclerc, 2003:332).

1.6.8 Conferring immunogenicity to vaccines based on purified proteins and synthetic peptides

The identification of new antigenic or epitopic targets is the first step in the design and development of new vaccines. Most antigens are poorly immunogenic and require the co-administration of an adjuvant to induce an optimal immune response. Historically, most vaccines were based on live attenuated pathogens or on whole killed bacteria. The stimulation provided by these microorganisms is usually enough to stimulate the immune system. However, the new approaches under development are based on purified recombinant proteins, synthetic peptides or plasmid DNA that are usually poorly immunogenic when injected alone. Moreover, depending on the pathogen, the selective activation of immune effector mechanisms, such as antibodies, cytotoxic T cell responses (CTL) or Th1/ Th2 CD4 T cell responses is in most cases strictly required to induce protective immunity. Adjuvants could also be required to increase the duration of the memory responses or to promote mucosal immunity (Leclerc, 2003:333).

Dendritic cells (DCs) have been described as "nature's adjuvant" because of their unique capacity to turn on naïve cells. They are involved in the initiation of both innate and adaptive immunity (Audibert, 2003:1189). Dendrtic cells (DCs) originate from bone marrow and are located in most peripheral tissues, in particular at the surfaces of pathogen entry sites, such as the skin and mucosal surfaces. As antigen presenting cells (APCs), dendritic cells (DCs) have the ability to capture antigens, process them into peptides and present these peptides in association with major histocompatability complex (MHC) class I or class II molecules to T cells. Immature dendritic cells (DCs) can capture antigens but they must differentiate or mature to become capable of stimulating naïve T cells. This maturation process is associated with the loss of endocytic capacity and the upregulation of co-stimulatory molecules such as CD40, CD80 and CD86 as well as a change in major histocompatability complex (MHC) class II molecule expression (Leclerc, 2003:333).

1.6.8.1 Targeting antigens to dendritic cells

A variety of molecules expressed at the surface of dendritic cells (DCs) can be used to increase the capture and endocytosis of antigens by these cells. Complement, scavenger and Fc receptors, as well as C-type lectins, DC-sign, mannose receptors and CD205 are some examples of molecules that can be used to target antigens to dendritic cells (DCs) for efficient uptake and endocytosis. The first possibility is to use antibodies specific for such molecules linked to the antigen to increase its uptake by binding to the dendritic cell surface. However, this strategy is difficult or even impossible to use for vaccine development. Another alternative is based on the binding of the vaccine to ligands for dendritic cell surface molecules (for instance mannose). However, this possibility remains limited since many of the natural ligands of dendritic cell surface receptors are still unknown. The capacity of some bacterial molecules to use dendritic cell surface molecules as receptors is also an attractive possibility. For instance, the specific binding of heat-shock proteins to dendritic cells (DCs) may certainly explain their strong capacity to trigger T cell immune responses. A bacterial toxin produced by *B. pertussis*, the adenylate cyclase, was also recently shown to selectively target the major histocompatability complex (MHC) class I pathway of dendritic cells through its capacity to bind the CD11b integrin, and subsequently to translocate to the cytosol of these cells. When used as a vaccine vector, this approach was shown to be highly efficient in stimulating cytotoxic T lymphocytes (CTL) and Th1 responses against a variety of viral and tumoural epitopes (Leclerc, 2003:333).

It has become very clear that the initial reaction between dendritic cells (DCs) and microbial products, using appropriate delivery systems, will dictate the outcome of the effector functions triggered by the dendritic cells (DCs). The dendritic cell targeting and the dendritic cell-based therapy requires the generation and validation of appropriate dendritic cell delivery systems, as well as the generation and validation of modified dendritic cells (DCs). This is achieved by developing protocols to obtain homogenous, highly effective populations of modified dendritic cells (DCs), by developing vectors and vehicles to deliver efficient antigens to dendritic cells (DCs) (Del Giudice, 2003:S2/84).

1.6.8.2 Exploiting the cross-presentation pathway

Emerging evidence indicates that dendritic cells (DCs) have the capacity to present exogenous antigens into the major histocompatibility complex (MHC) class I pathway. The phenomena, termed cross-presentation, could open new perspectives in the development of vaccines capable of inducing strong cytotoxic T lymphocytes (CTL) responses. Dendritic cells (DCs) possess the unique ability to present exogenous or cell-associated antigens such as heat-shock proteins, antibody complexes, exosomes, apoptotic cells, necrotic cells and viral pseudo-particles to CD8 T cells in association with major histocompatibility complex (MHC) class I molecules. This could explain the capacity of some exogenous antigens to induce CTL responses (Leclerc, 2003:334).

Leclerc (2003:334) recently showed that an antigen-delivery system based on nonreplicative, recombinant parvovirus-virus-like particles (PVV-VLPs) formed by self-assembly of the VP2 capsid protein of porcine parvovirus (PPV) induced strong CTL responses in the absence of an adjuvant. The strong immunogenicity of these inert particles is due to the very high capacity of dendritic cells (DC) to capture parvovirus-virus-like particles (PVV-VLPs) *in vivo* and to deliver them to major histocompatability complex (MHC) class I pathway after cytosolic processing. Such virus-like particles (VLPs) could represent a new and promising strategy to deliver epitopes or antigens to the immune system for prophylactic and therapeutic vaccines (Leclerc, 2003:334).

1.6.9 Adjuvants

Vaccines can be classified as either 'live' or 'inactivated'. Live vaccines stimulate immunity via a transient infection caused by a replicating live organism, for example, the smallpox vaccine pioneered by Jenner in 1796. Although cost effective and easy to produce, they carry the intrinsic risk associated with live pathogens. Inactivated vaccines that include killed organisms and isolated non-replicated sub-cellular components stimulate a lower level and shorter duration of immunity than that elicited by live

vaccines. In 1916, Le Moignie and Pinoy reported that mineral oil emulsions increased the immune response against an antigen (Marciani, 2003:934).

Immunological adjuvants can be defined as substances that enhance the ability of antigens to elicit an immune response (Seferian & Martinez, 2001:661). Because inactivated vaccines stimulate a lower level and shorter duration of immunity, the adjuvant component often determines whether the vaccine is effective or ineffective.

Aluminium salts have been used for over 70 years as adjuvants in vaccines. The adjuvant effect of aluminium was first reported in 1926 based on the observation that alum-precipitated diphtheria toxoid induced a better immune response than a soluble diphtheria toxoid (HogenEsch, 2002:S34).

Aluminium adjuvants are the only adjuvants allowed for use in human vaccines and are present in many veterinary vaccines. They have been administered to millions of people with only rare reports of local reactions (HogenEsch, 2002:S34). These local reactions include erythema, subcutaneous nodules, contact hypersensitivity and granulomatous inflammation (Gupta, 1998:164). From an immunological standpoint, the main drawbacks of aluminium adjuvants are their weak or absent adjuvant effect with certain vaccine antigens, the inability to induce cell-mediated and cytotoxic T cell responses and the tendency to induce IgE-mediated immune responses. Aluminium adjuvants augment the type 2 immune response without enhancing the type 1 immune response. This makes aluminium adjuvants less suitable for vaccines against certain pathogens, for which antibodies alone provide insufficient protection. The induction of antigen-specific IgE responses may predispose susceptible individuals to allergic reactions against vaccine components (HogenEsch, 2002:S34).

1.6.9.1 Required properties of an adjuvant

Adjuvants have to comply with a number of properties to render them safe and effective:

1) Adjuvants have to be non-toxic or must have a negligible toxicity at the dose range needed for effective adjuvanticity.
2) Adjuvants must stimulate a strong humoral and/or T cell immune response.
3) Adjuvants must provide good immunological memory or long-term immunity.
4) Adjuvants may not induce autoimmunity.
5) Adjuvants have to be non-mutagenic, carcinogenic and teratogenic.
6) Parental adjuvants must be non-pyrogenic.
7) Adjuvants have to be stable under broad ranges of storage time, temperature and pH (Marciani, 2003:935).

The requirements mentioned above are rarely met, thus, the availability of suitable adjuvants are limited.

1.6.9.2 Mechanisms of immune stimulation

So far, although adjuvants are widely used in vaccination, their mechanisms of action remain poorly understood. The immune response to pathogens relies both on innate and adaptive immune responses. The innate immune system is able to discriminate between a large number of pathogens. This recognition strategy is based on the detection of conserved molecular patterns that are essential products of microbial physiology. These invariant structures are referred to as pathogen-associated molecular patterns (PAMPs). PAMPs are not produced by the host and are invariant among microorganisms and thus are perceived as molecular signatures of infection and trigger an innate immune response. PAMPs include various components such as lipopolysaccharides (LPS), lipoproteins, peptidoglycans, unmethylated cytosine-phosphate-guanosine-oligonucleotides (CpG-DNA) and double strand RNA that interact with pattern-recognition receptors (PRRs)

known as Toll-like receptors (TLRs) in mammals. The expression of Toll-like receptors (TLRs) on the professional antigen presenting cells (APCs), and notably on dendritic cells (DCs), plays a major role in the initiation of immune responses. Triggering Toll-like receptors (TLRs) on these cells induces their maturation, as illustrated by the upregulation of co-stimulatory molecules and major histocompatibility (MHC) class II molecules, as well as by the induction of cytokine and chemokine production, leading to the activation of naïve T lymphocytes into effector and memory T cells. It has been proved that many adjuvants exert their effect on one of the ten distinct Toll-like receptors (TLRs). Moreover, triggering the Toll-like receptors by microbial adjuvants such as CpG-DNA would be required for Th1 activation, through the production by dendritic cells of cytokines such as IL-12 (Leclerc, 2003:336). The structural characterisation of several adjuvants and the identification of cellular receptors that are associated with their activities, such as Toll-like receptors and co-stimulatory ligand receptors, are eliciting a better understanding of their mechanisms of action on a molecular level (Marciani, 2003:935).

One mechanism is the depot formation that is observed with alum, emulsion-based and insoluble adjuvants (known as particulate adjuvants), where antigens and adjuvants are sequestered at the injection site and are released over a time to stimulate antigen-presenting cells (APCs), such as macrophages and dendritic cells (DCs). Adjuvants also promote an increase in the number of antigen-specific lymphocytes in lymph nodes draining the injection site (Marciani, 2003:935).

Targeting is the mechanism by which an adjuvant-antigen complex is delivered to antigen-presenting cells (APCs) for processing. Particulate adjuvants bind to antigens, forming aggregates that are engulfed by antigen-presenting cells (APCs) via endocytosis to form endosomes. More effective targeting is achieved by using adjuvants with residues that are recognised by receptors on antigen-presenting cells (APCs). For instance, the mannose receptor that belongs to the endocytic- Pattern Recognition Receptors (PRRs), binds compounds containing mannose (e.g. mannans), *N*-acetylglucosamine or fructose residues (e.g. some saponins) and sulfated oligosaccharides. Binding of these adjuvant-

antigen complexes to endocytic- Pattern Recognition Receptors (PRRs) initiates efficient receptor-mediated endocytosis and antigen processing (Marciani, 2003:935).

1.6.9 3 Classification of adjuvants

It is instructive to classify adjuvants according to the immunological events they evoke. Certain vehicles of administration such as ISCOMS, Quil A, $Al(OH)_3$ and liposomes can facilitate the activities of antigen presenting cells (APCs). Others allow a prolonged delivery of antigens because they help form a depot, this action is achieved by oil emulsions, $Al(OH)_3$, polymers, microspheres and gels. Some immunomodulators play an important role in activating the innate immune cells and they often represent compounds isolated from pathogenic microorganisms or mimicking certain structures of microorganisms. Unmethylated cytosine-phosphate-guanosine-oligonucleotides (CpG-DNA) motifs, lipopolysaccharide (LPS) and muramyl peptides belong to this category. Stress or stress-related structures such as heat-shock proteins (HSPs) can also activate the innate immune responses. Finally, mediators of the immune system such as selected cytokines or co-stimulatory molecules have been used with vaccines and act directly on T and B cells as well as on the maturation process of antigen presenting cells (APCs) (Audibert, 2003:1189).

1.6.9.4 The future of adjuvants in vaccine development

Despite many recombinant antigens available for use in vaccines, the only human viral recombinant sub-unit vaccine is hepatitis B. This lag in vaccine development reflects the use of adjuvants that elicit only Th2 immunity, such as alum, and a poor understanding of the adjuvant's crucial role in immunomodulation. However, discoveries of innate and adaptive immunity receptors and their mechanisms of action, enable the rational design of novel Th1 or Th2 immunity adjuvants (Marciani, 2003:941).

The effects of different adjuvants or modified adjuvants interacting with specific Toll-like receptors (TLRs) or co-stimulatory receptors can be either cumulative or synergistic, therefore, adjuvant combinations and/or modifications could yield improved immunity

with the induction of specific responses, for example cytotoxic T lymphocytes (CTL) against virally infected cells (Marciani, 2003:941).

As opposed to live vaccines, where the immune response depends on an individual's immunocompetence, new sub-unit vaccine adjuvants would be able to modulate the immune system to stimulate a safe and effective response even under immunodeficient conditions relating to aging, certain congenital defects, certain diseases or people undergoing immune suppressive therapy. For these populations, which are extremely susceptible to infections and not eligible for immunisation with live vaccines, the new adjuvants would provide safe and effective sub-unit vaccines (Marciani, 2003:941).

Availability of new adjuvants that are capable of stimulating both mucosal and systemic immune responses would facilitate the development of sub-unit vaccines against pathogens that infect via the mucosal membranes of the urogenital, respiratory and digestive tracts (Marciani, 2003:941).

1.7 CONCLUSION

It was reported in a newspaper that a nurse in Botswana injected approximately 170 schoolchildren with a single needle while immunising them against whooping cough, tetanus and polio, causing a HIV scare (Cape Times, March 2003). This example makes it clear that the development of new vaccines and delivery systems that will improve the effectiveness and safety of vaccines are essential. There are many delivery systems that show great potential to improve the effectiveness, safety, patient compliance and therapeutic outcome of immunisation.

CHAPTER 2

CHITOSAN, *N*-TRIMETHYL CHITOSAN CHLORIDE (TMC) AND EMZALOID™ TECHNOLOGY AS NOVEL DELIVERY SYSTEMS FOR NASAL VACCINATION

2.1 INTRODUCTION

Conventionally, the nasal route of delivery has been used for the delivery of drugs for treatment of local diseases such as nasal allergy, nasal congestion and nasal infections. Recent years have shown that the nasal route can be exploited for the systemic delivery of drugs such as small molecular weight polar drugs, peptides and proteins that are not easily administered via routes other than injection or where rapid onset of action is required (Illum, 2003:187).

The major factor, which impedes absorption of vaccines at mucosal sites, is the low and incomplete transport across the epithelial barrier. A transient and reversible opening of tight junctions between the epithelial cells by safe penetration enhancers such as chitosan and *N*-trimethyl chitosan chloride as well as novel carrier systems such as Emzaloid™ technology may allow for the permeation of antigens across the epithelial barrier and subsequently the production of an immune response.

In recent years, there has been an increasing interest in the development of novel vaccine systems for prophylactic and therapeutic purposes. Formulation strategies and adjuvants that can affect the immune response in both quantitative and qualitative terms have attracted the interest of those more familiar with problems of drug delivery (Illum & Davis, 2001:1).

2.2 NASAL DRUG DELIVERY

2.2.1 Physiology of the nasal cavity and mechanism of drug permeation

The nasal absorption of drugs is considered to take place mainly in the respiratory region comprising of the turbinates and part of the nasal septum. As is the case for all biological membranes, drugs can cross the nasal mucosal membrane using two different pathways: transcellularly (across the cell) and paracellularly (between the cells). This depends on the lipophilicity of the compound. Lipophilic drugs are transported transcellularly by an efficient concentration-dependent passive diffusion process, by receptor or carrier mediation and by the vesicular transport mechanisms. Polar drugs pass through the epithelium via the gaps or pores between the cells, namely tight junctions. Although, these tight junctions are dynamic structures that can open and close to a certain extent, the size of these cannels is less than 10 Å. Hence, the paracellular route will be less efficient for large molecules and is dependent upon the molecular weight of the drug (Illum, 2002:1185; Arora *et al.*, 2002:968).

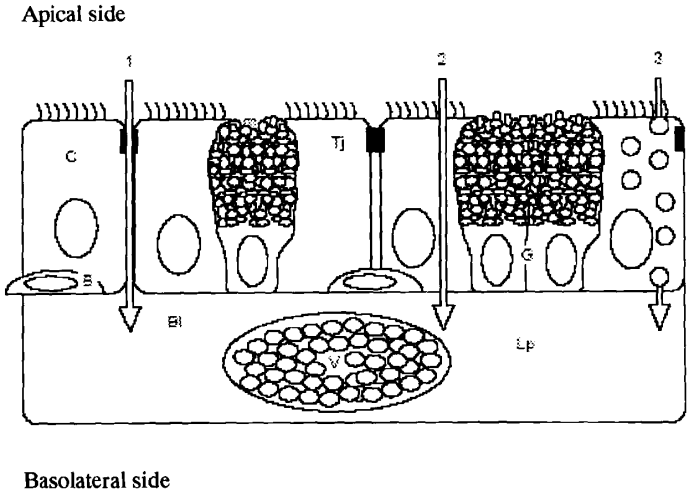


Figure 2.1: Schematic representation of transport routes across nasal mucosa.
(1) paracellular transport across tight junctions, (2) transcellular transport, (3) transcytotic transport. Mucus-secreting goblet cells (G), ciliated columnar cells (C) and tight junctions (Tj). Basal cells (B) are located on the basal lamina (Bl) adjacent to the lamina propria (Lp)
(Junginger & Verhoef, 1998:372).

2.2.2 Advantages and limitations of nasal drug delivery

2.2.2.1 Advantages of nasal drug delivery

Nasal drug delivery provides a viable alternative for the administration of many pharmaceutical agents. Some of the major advantages offered by the nasal route include:

1) Rapid absorption, higher bioavailability, therefore lower doses.
2) Fast onset of therapeutic action.
3) Avoids degradation of drug resulting from hepatic first pass metabolism.
4) Avoids degradation of drug in gastrointestinal tract resulting from acidic or enzymatic degradation.

5) Avoidance of irritation of the gastrointestinal membrane.
6) Reduced risk of overdose.
7) The nasal route of drug administration is a non-invasive route, therefore, there is a reduced risk of infection.
8) Self-medication is possible through this route.
9) Improved patient compliance.
10) Can be a beneficial adjunct product to an existing product.
11) Reduced risk of infectious disease transmission (Behl *et al.,* 1998:96).

2.2.2.2 Limitations of nasal drug delivery

Some of the disadvantages of nasal drug delivery include:

1) Volume that can be delivered into nasal cavity is restricted to 25-200 µl.
2) Higher molecular weight compounds cannot be delivered through this route (mass cut off = 1 kDa).
3) Nasal drug delivery is adversely affected by pathological conditions.
4) Normal defence mechanisms like mucocillary clearance and cillary beating affect the permeability of the drug.
5) Enzymatic barrier to permeability of drugs (Arora *et al.,* 2002:968).

2.2.3 Factors affecting the nasal permeability of drugs

2.2.3.1 Structural features of the nasal cavity

The respiratory region is richly supplied with blood, has a large surface area and receives the maximum amount of nasal secretions rendering it most suitable for the permeation of compounds. These factors and the type of cells, density and number of cells present in that region, influences the permeability. Different types of cells constitute nasal epithelium, as shown in figure 2.2.

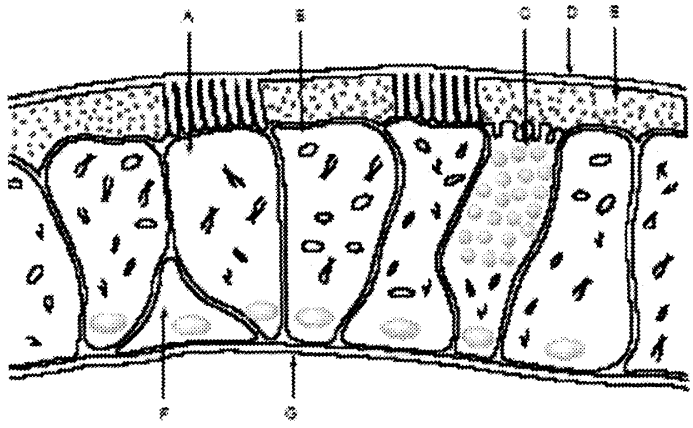


Figure 2.2: Cell types of the nasal epithelium showing ciliated cells (A), non-ciliated cells (B), goblet cells (C), gel mucus layer (D), sol layer (E), basal cell (F) and basement membrane (G) (Arora *et al.*, 2002:970).

According to Arora *et al.*, (2002:969), a large number of absorption enhancers, used in combination with drugs, increase the permeation of compounds by either increasing the membrane fluidity, decreasing viscosity of the mucosal layer, inhibiting proteolytic enzymes, disrupting the tight junctions, increasing the paracellular or transcellular transport, increasing the blood flow or dissociating protein aggregation.

2.2.3.2 Biochemical changes

One of the biggest challenges of nasal drug delivery is to overcome barriers posed by the nasal mucosal lining and the enzymes present in the nasal cavity (Mitra & Krishnamoorthy, 1998:136). Nasal mucus acts as an enzymatic barrier to the delivery of drugs because of the presence of a large number of enzymes, which include oxidative and conjugative enzymes, peptidases and proteases. These enzymes are responsible for the degradation of drugs in the nasal mucosa and result in the creation of a pseudo-first-pass effect, which hampers the absorption of drugs (Arora *et al.*, 2002:969).

2.2.3.3 Physiological factors

The presence of venous sinusoids and arteriovenous anastomosis gives the nasal mucosa the distinction of being a highly permeable site. Nasal cycles of congestion (increased blood supply resulting from parasympathetic stimulation) and relaxation (decreased blood supply resulting from sympathetic stimulation) regulate the rise and fall in the amounts of drug permeated (Arora *et al.,* 2002:971).

2.2.3.4 Nasal secretions

The viscosity of nasal secretions affects the permeability of drugs through the nasal mucosa. It has been reported that if the sol layer of the mucus is too thin, the viscous surface layer will inhibit the ciliary beating, and if the sol layer is too thick, mucociliary clearance is impaired because contact with cilia is lost. Impairment or modification of mucociliary clearance affects permeation of the drug by altering the time of contact of the drug and the mucosa (Arora *et al.,* 2002:971).

The pH of the nasal surface is 7.39 and the pH of nasal secretions varies between 5.5-6.5 in adults and 5.0-7.0 in infants (Behl *et al.,* 1998:106). Greater drug permeation is usually achieved at a nasal pH that is lower than the drug's pK_a because under such conditions the penetrant molecules exist as unionised species. A change in the pH of the nasal mucus can affect the ionisation and thus increase or decrease the permeation of the drug, depending on the nature of the drug. Because the pH of the nasal cavity can alter the pH of the formulation and vice-versa, the ideal pH of a formulation should be between 4.5 and 6.5 (Arora *et al.,* 2002:971).

2.2.3.5 Mucociliary clearance and ciliary beating

Cilia, the fine contractile projections located on the apical surface of nasal epithelial cells, and mucus, the thin layer of clear nasal fluid forming a continuous cover over the

epithelial surfaces, facilitate the removal of deposited materials from the nasal cavity, which is known as mucociliary clearance (Hinchcliffe & Illum, 1999:202).

The absorption of drugs from the nasal mucosa is influenced by the contact time between the drug and the epithelial tissue. Intranasally delivered drugs show a rapid rise to peak blood concentrations, and studies in animals and humans have shown that peptide and protein drugs are absorbed by the nasal epithelium within 5-15 minutes. Two factors contribute to the fast nasal absorption of drugs, the permeability of the nasal epithelium and the nasal mucociliary clearance. The permeability of the nasal epithelium is relatively high for large molecules, while the diffusion path length through the nasal mucosal epithelium is short; consisting of only two cell layers. Nasal mucociliary clearance limits the residence time of drugs administered into the nasal cavity, decreasing the time available for the drug to be absorbed. The normal half-time of clearance in humans is approximately 20 minutes (Marttin *et al.,* 1998:28). Reduced mucociliary clearance increases the time of contact between a drug and the mucus membrane and subsequently enhances drug permeation, whereas, increased mucociliary clearance decreases drug permeation. Some drugs, hormonal changes of the body, pathological conditions and formulation factors are reported to affect the mucociliary clearance and in turn exert significant influence on drug permeability (Arora *et al.,* 2002:971).

2.3 NASAL VACCINATION

2.3.1 Lymphoid structures associated with the mucosal immune system

The upper and lower respiratory tract has an immune system that can be divided into three parts:

1) An epithelial compartment at the surface of the epithelium and the underlying connective tissue that contains immunocompetent cells.

2) Lymphoid structures of the nose and bronchus-associated epithelium [nasal-associated lymphoid tissue (NALT), larynx-associated lymphoid tissue (LALT), bronchus-associated lymphoid tissue (BALT)].
3) Lymph nodes draining the respiratory system (Davis, 2001:22).

In man, the target site for a nasally administered vaccine formulation is believed to be the nasal-associated lymphoid tissue (NALT) situated mainly in the pharynx as a ring of lymphoid tissue, the Waldeyer's ring. The Waldeyer's ring comprises of the nasopharyngeal tonsil (adenoid), attached to the roof of the pharynx, the paired tubal tonsils, located at the Eustachain tube openings, the paired palatine tonsils, located at the oropharynx and the lingual tonsil (Illum *et al.,* 2001:82).

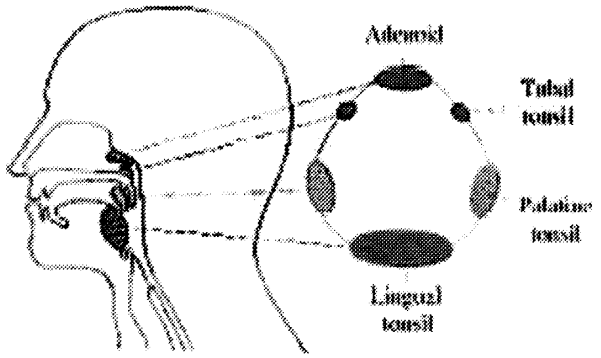


Figure 2.3: Schematic representation of the pharyngeal lymphoid tissue of the Waldeyer's ring (Davis, 2001:24).

2.3.2 Primary reasons for exploiting the nasal route for vaccine delivery

In recent years, there has been an increasing interest in the development of novel vaccine systems for prophylactic and therapeutic purposes. Formulation strategies and the use of adjuvants that can affect the immune response in both quantitative and qualitative terms

have attracted the interest of those familiar with problems in drug delivery (Illum & Davis, 2001:1).

Early efforts focused on injectable (parenteral) vaccines and the role of controlled release technologies. Mucosal routes of vaccination are attractive alternatives to parenteral immunisation since, with the appropriate system, it is possible to stimulate both arms of the immune system and provide both humoral (antibody) and cell-mediated responses (cytotoxic lymphocytes). Moreover, it is now recognised that the majority of the invading pathogens enter the body via some form of mucosal surface (Illum & Davis, 2001:1).

At first glance, oral vaccination would appear to be an attractive strategy but unfortunately, it is difficult to obtain good immune responses in larger animal models and humans. This may be due to poor access of the antigen to antigen presenting cells (APCs), degradation of the antigen in the harsh environment of the gastrointestinal tract and dilution of the delivery system in the gut contents. Nasal administration offers an attractive alternative since it is possible to use smaller doses and to deliver the formulation to the appropriate site (nasal associated lymphoid tissues – Waldeyer's ring in humans). In addition, because of the properties of the common mucosal immune system, the nose can act as an inducer and effector site and good secretory immune responses can be obtained at distant mucosal sites such as the intestines, lung and vagina (Illum & Davis, 2001:1).

Davis (2001:21) stated that the nasal mucosa is an important arm of the mucosal immune system since it is often the first point of contact for inhaled antigens and consequently, intranasal immunisation has emerged as possibly the most effective route for vaccination for both peripheral and mucosal immunity.

Partidos (2000:274) listed several reasons why the nose is an attractive route for immunisation:

1) The nasal route is easily accessible.
2) The nasal cavity is highly vascularised.
3) The presence of numerous microvilli covering the nasal epithelium generates a large absorption surface.
4) After intranasal immunisation, both mucosal and systemic immune responses can be induced.
5) An immune response can be induced at distant mucosal sites owing to the dissemination of effector immune cells in the common immune system.
6) The nose can be used for the easy immunisation of large population groups.
7) Nasal immunisation does not require needles and syringes, which are a potential source of infection.

2.3.3 Antigen uptake via the nose

During the evolution of the mammalian mucosal immune system, the nasal-associated lymphoid tissue (NALT) has adapted to fulfil several functions. For the protection of the nasal epithelium from colonisation by invading pathogens and entry by inhaled antigens, barriers to macromolecular absorption, mechanisms of antigen sampling, production of secretory IgA (s-IgA) antibody responses, as well as means of distributing effector T- and B-cells to local and distant mucosal sites have been developed. In addition, mechanisms of tolerance have also evolved to prevent harmful allergic immune responses to inhaled antigens (Partidos, 200:273).

The mucosal-associated lymphoid tissue (MALT) can be divided into two functionally distinct compartments or sites:

1) The inductive site, which is a defined lymphoid microcompartment.

2) The effector site, which contains diffuse accumulations of large numbers of lymphoid cells that do not associate into organised structures.

The antigen is first encountered in the inductive sites and initial responses are induced. By contrast, in the effector sites, IgA plasma cells are found, and the production of s-IgA antibodies result in local protection. In the upper respiratory tract of rodents and humans, the major inductive sites are the nasal-associated lymphoid tissue (NALT) and the tonsils, respectively. These sites possess M-cells, which are responsible for the sampling of and transporting of antigens to the underlying lymphoid tissue, germinal centers containing B- and T-cells, plasma cells and antigen presenting cells (APCs). These cells are involved in the regulation and induction of antigen-specific effector cells, which will ultimately mediate the protective humoral and cellular immune responses (Partidos, 200:274).

The balance between active immunity and tolerance greatly depends on the nature of the antigen and its interaction with the mucosal inductive sites, as well as on the dose, adjuvant, and the frequency of antigen administration and genetic background of the host. Particulate antigens can be removed either by the mucociliary clearance system or can be sampled by specialised cells that are similar in appearance to M-cells, which overlay the NALT and are transported to the posterior cervical lymph nodes. Soluble antigens can penetrate the mucosal epithelium and reach the superficial cervical lymph nodes. High doses of antigen administered intranasally are likely to reach the intestinal tract or are drained directly by the posterior cervical lymph nodes. The superficial cervical lymph nodes, which drain the nasal mucosa, appear to be instrumental in the induction of mucosa tolerance. Posterior cervical lymph nodes are involved in the generation of s-IgA responses. This suggests that lymphocyte composition differs in different lymph nodes because of their 'homing' behaviour, which underlies the observed divergence in the induction of either tolerance or immunity (Partidos, 2000:274).

2.3.4 The immune response following intranasal vaccination

Following intranasal immunisation, both cellular and humoral immune responses can occur. After the antigen is sampled and passed through endocytic vesicles to the underlying lymphoid cells in the submucosa, antigen processing and presentation occurs. The result is the activation of T cells, which in turn provide help to B cells to develop into IgA plasma cells. Isolated nasal associated lymphoid tissue (NALT) cells contain antigen-specific antibody-secreting cells that are predominately of the IgA isotype. In humans, there are two subclasses: IgA1 and IgA2. The nasal mucosa and lacrima glands contain mainly IgA1 plasma cells, whereas a greater portion of IgA2 plasma cells are found in the lamina propria of the lower intestinal tract. In mucosal defences, s-IgA plays an important role because of its high-affinity binding to mucosal surfaces in the body. Intranasal immunisation induces s-IgA responses in a wider range of mucosal tissues than oral immunisation. This is likely to be caused by the more dissolute profile of homing receptors possessed by the circulating IgA-secreting cells induced after intranasal immunisation. (Partidos, 2000:274).

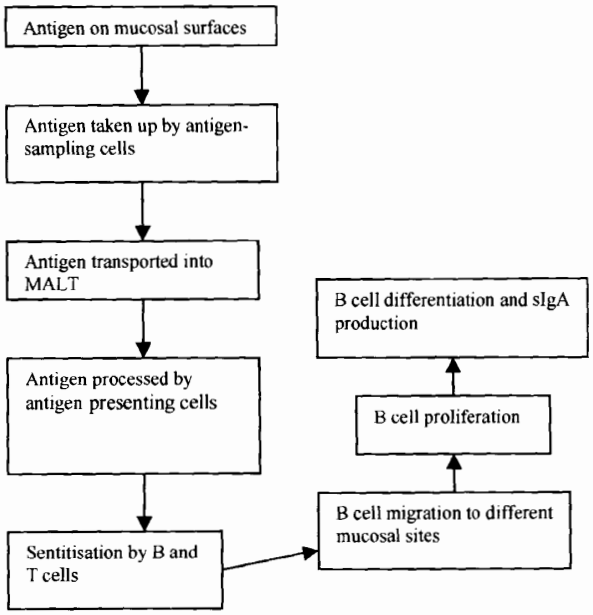


Figure 2.4: Induction of s-IgA following mucosal exposure to antigens (Chen, 2000:118).

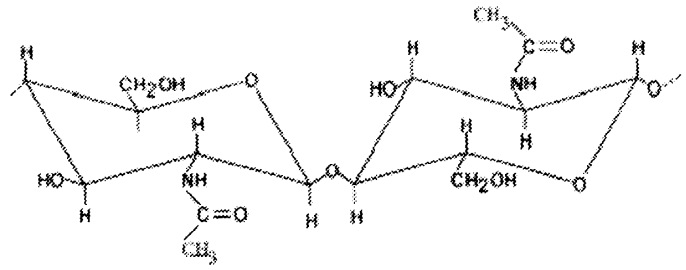
Small soluble antigens are able to penetrate the nasal epithelium (if assisted by formulation additives such as penetration enhancers) and interact with dendritic cells, macrophages and lymphocytes (intraepithelial and subnasal). Drainage then takes place to the posterior lymph nodes. In contrast to this, antigens incorporated into particles are largely taken up by M-cells in the nasal associated lymphoid tissue (NALT), and drained to the cervical lymph nodes. Antigens taken up in this manner can elicit a local mucosal immune response as well as distant mucosal immune response (Davis, 2001:27).

2.4 CHITOSAN AS DELIVERY SYSTEM FOR NASAL VACCINES

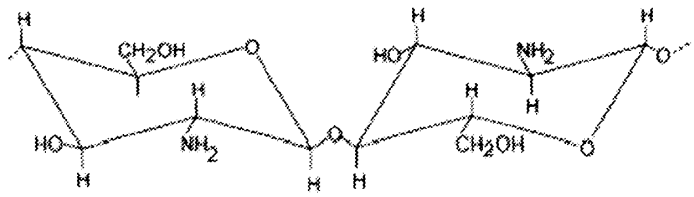
2.4.1 Origin and chemical structure of chitosan

The history of chitosan dates back to 1859 when Rouget discussed the deacetylated form of chitosan. He found that when chitin was boiled in a concentrated potassium hydroxide solution, a product was obtained that dissolved in dilute iodine and acids, unlike chitin that only stained brown (Paul & Sharma, 2000:5). Recently, chitosan has been considered for pharmaceutical formulation and drug delivery applications in which attention has been focused on its absorption enhancing, controlled release and bioadhesive properties. Synthesised from a naturally occurring source, this polymer has shown to be both biocompatible and biodegradable (Dodane & Vilivalam, 1998:246).

Chitosan is obtained by the deacetylation of chitin, the second most abundant polysaccharide in nature, cellulose being the most abundant. Chitin is found in the exoskeleton of crustaceans, insects and some fungi such as *aspergillus* and *mucor*. The main commercial sources of chitin are the shell wastes of shrimp, lobster, krill and crab (Hejazi & Amiji, 2003:151). Chitosan [α (1→4) 2-amino 2-deoxy β-D-glucan] is obtained by the alkaline deacetylation of chitin. The chitosan molecule is a copolymer of *N*-acetyl-D-glucosamine and D-glucosamine. The sugar backbone of chitosan consists of β-1,4 linked D-glucosamine with a high degree of *N*-acetylation, a structure very similar to that of cellulose, except that the acetylamino group replaces the hydroxyl group on the C-2 position. Thus, chitosan is poly (*N*-acetyl-2-amino-2-deoxy-D-glucopyranose), where the *N*-acetyl-2-amino-2-deoxy-D-glucopyranose units are linked by (1→4)-β-glycosidic bonds (Hejazi & Amiji, 2003:151).



(A)



(B)

Figure 2.5: Chemical structures of chitin (A) and chitosan (B) (Hejazi &Amiji, 2003:152).

Chitosan is marketed under a variety of forms with different molecular weights and degrees of deacetylation, or as chitosan base or salt. It is produced in different parts of the world as a solution and in flake, powder, bead and fibre forms. High purity chitosan (biomedical grades) is also available for biomedical applications including drug delivery. Briefly, to prepare chitin, crab and shrimp shells are treated in boiling aqueous sodium hydroxide solution after decalcification in dilute hydrochloric acid and deproteination in a dilute sodium hydroxide solution. Chitin is then deacetylated in a concentrated sodium hydroxide solution to manufacture chitosan. Biomedical grade chitosan is prepared by repeating the deacetylation process. Pharmaceutical grade chitosan has a degree of deacetylation > 90 % and food grade chitosan has a degree of deacetylation of > 75 % (Paul & Sharma, 2000:5).

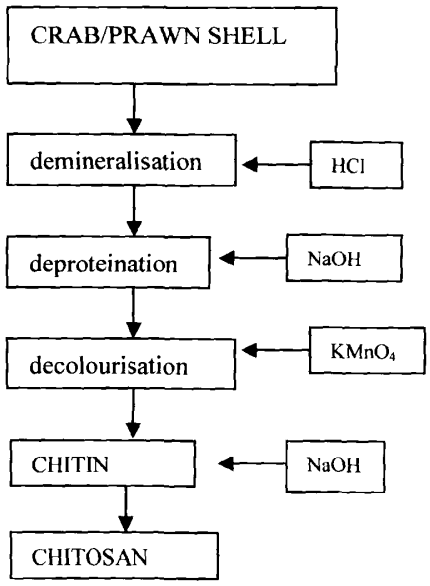


Figure 2.6: Production of chitosan from chitin (Paul & Sharma, 2000:5).

2.4.2 Physicochemical and biological properties of chitosan

Chitosan refers to a large number of polymers, which differ in their degree of *N*-deacetylation (40-98 %) and molecular weight (50 000 – 2 000 000 Da). These two characteristics influence the physico-chemical properties and thus have a major effect on the biological properties (Hejazi & Amiji, 2003:152).

Chitosan is a weak base with a pK_a value of 6.2 to 7.0 and, therefore, is insoluble at neutral and alkaline pH values. Chitosan does however form salts with inorganic and organic acids such as hydrochloric acid, acetic acid, glutamic acid and lactic acid. In acidic medium, the amine groups of the polymer are protonated resulting in a soluble, positively charged polysaccharide that has a high charge density. The solubility of chitosan salts depend on the degree of deacetylation and the pH of the solution. Chitosan salts with a low degree of deacetylation (< 40 %) are soluble up to a pH of 9, whereas

chitosan salts with a high degree of deacetylation (> 85 %) are soluble up to a pH of 6.5 (Hejazi & Amiji, 2003:152).

The viscosity of chitosan solutions is influenced by the degree of deacetylation. Chitosan solutions with a high degree of deacetylation have a higher viscosity than chitosan solutions with a low degree of deacetylation. This can be explained by the fact that chitosan with a high or low degree of deacetylation have different conformations in aqueous solution. Chitosan has an extended conformation with a more flexible chain when it is highly deacetylated because of the charge repulsion in the molecule. However, chitosan has a rod-like conformation or coiled conformation when it has a lower degree of deacetylation due to the low charge density in the polymer chain. Chitosan in solution exists in the form of quasiglobular conformation stabilised by intra- and intermolecular hydrogen bonding. The hydrogen bonding in chitosan chains due to the presence of amine and hydroxyl groups causes the high viscosity of chitosan solutions. Chitosan solutions with a lower degree of deacetylation have a lower degree of hydrogen bonding because of the lower number of amino groups in the polymer chain. The viscosity of chitosan solutions is also affected by factors such as concentration and temperature (Hejazi & Amiji, 2003:152).

2.4.3 Safety of chitosan

According to Dodane & Vilivalam (1998:246), chitosan lacks irritant or allergic effects and is biocompatible with both healthy and infected human skin. When chitosan was administered orally to mice, the LD_{50} was found to be in excess of 16 g/kg, which is higher than that of sucrose.

Numerous studies have demonstrated that the salt form, particle size, density, viscosity, molecular weight, degree of deacetylation as well as the pH at which chitosan is used influence the properties of this polymer in drug delivery systems. Therefore, these factors must be considered during formulation to ensure the safety of the drug delivery system (Dodane & Vilivalam, 1998:250; Sinha *et al.*, 2004:2).

2.4.4 Mechanism of action of chitosan

In the field of mucosal drug delivery, chitosan has been shown to own strong mucoadhesive properties. In addition to this, the interaction of the positively charged amino group at the C-2 position of chitosan with the negatively charged sites on the cell surfaces and tight junctions allows paracellular transport of large hydrophilic compounds by opening the tight junctions of mucosal membrane barriers (Van der Lubben *et al.*, 2001:203). The interaction with the opening mechanism of the tight junctions has been demonstrated by a decrease in ZO-1 proteins and the change in the cytoskeletal protein F-actin from a filamentous to a globular structure. These characteristics reveal the potential of chitosan and chitosan salts as absorption enhancers for mucosal paracellular pathways (Junginger & Verhoef, 1998:374).

2.4.5 Chitosan microparticles and nanoparticles for mucosal vaccination

Most hydrophilic and macromolecular drugs like peptides, proteins and polysaccharides need to be injected due to low absorption at mucosal sites. The obvious disadvantages of parenteral drug delivery are low patient compliance and high costs due to high manufacturing costs of sterile products and the need for qualified personnel to administer the drug. The major factor, which impedes absorption at mucosal sites, is the low and incomplete transport across the epithelial barrier. A transient and reversible opening of tight junctions between the epithelial cells by safe penetration enhancers would allow for the permeation of non-absorbable drugs across the epithelial barrier and subsequent uptake of the drugs into the systemic blood circulation (Van der Lubben *et al.*, 2001:201).

A major advantage of mucosal vaccine delivery is that most pathogens enter the body via the mucosal surfaces and if the vaccine can be administered at the mucosal surfaces it can facilitate the neutralisation of the pathogens the moment they enter the body. Another

advantage of mucosal vaccination is that it stimulates both humoral and cell-mediated immune responses.

It has previously been demonstrated that nanoparticles and microparticles can easily be obtained from chitosan, and that it is a very efficient and non-toxic drug carrier for both orally and nasally administered peptide drugs (Van der Lubben *et al.,* 2001:140).

For vaccine delivery, the lymphoid tissue should be targeted. Access to mucosal associated lymphoid tissue (MALT) is provided by antigen sampling cells. These microfold cells (M-cells) are located between the epithelial cells and take up antigens and microparicles smaller than 10 μm. By incorporating the vaccine into microparticulate delivery systems, the vaccine is protected from degradation on its way to the mucosal tissue and efficiently targeted and taken up by the M-cells. Subsequent release in the Peyer's patches may result in the induction of immune responses (Van der Lubben *et al.,* 2001:201).

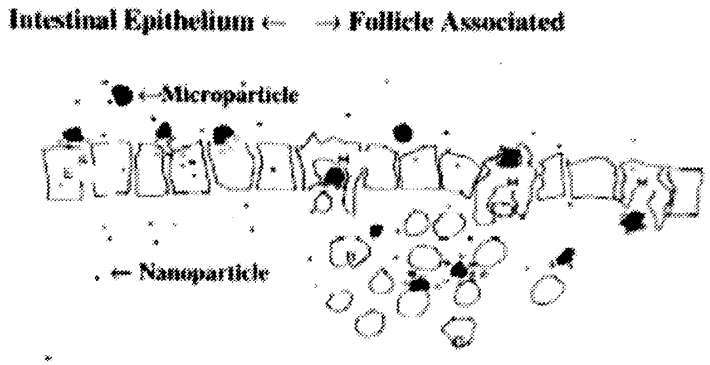


Figure 2.7: Schematic representation of the intestinal epithelium. M-cells (M) are located between the epithelial cells in the lymphoid associated epithelium. M-cells are the entrance port to the Peyer's patches and efficiently take up microparticles smaller than 10 μm. Epithelial cells (E) only take up nanoparticles (Van der Lubben *et al.,* 2001:202).

2.4.6 Chitosan microparticles and nanoparticles for nasal vaccination

Over the last decade, chitosan has been attracting attention as a biomaterial and pharmaceutical excipient for drug delivery because of its favourable biological properties. Besides its low toxicity and susceptibility to biodegradation, chitosan has shown mucoadhesive properties as well as an important drug penetration enhancement capacity across mucosal barriers. Specific reports on the nasal administration of high molecular weight chitosan preparations have shown that chitosan enhances the nasal absorption of peptide drugs as well as antigens (Vila *et al.,* 2004:123).

In the nasal associated lymphoid tissue (NALT), morphologically and functionally similar cells to the M-cells in the Peyer's patches have been described. Although nasal degradation in the nasal cavity is not as drastic as in the gut, a carrier system is still needed for the nasal route (Van der Lubben *et al.,* 2003:1401). In man, a simple powder or liquid formulation deposited in the nasal cavity will be cleared with a half-time of approximately 15-20 minutes. It can be argued that such a relatively rapid clearance time may not allow a formulated product enough time in the nasal cavity for a desired response, such as uptake of antigens by antigen presenting cells or tissues, to occur. Bio-adhesive systems (for e.g. chitosan) can slow down the process of mucociliary clearance and thereby allow a longer period of contact between the formulation and the nasal tissue (Illum *et al.,* 2001:83).

In contrast to oral administration, nasally administered vaccines are not exposed to low pH values and degrading enzymes. Therefore, in the case of nasal delivery, the vaccine does not necessarily have to be incorporated into micropariclcs or nanoparticles, but may also be co-administered with chitosan solution or powder formulations (Van der Lubben *et al.,* 2001:142).

2.4.7 Chitosan derivatives for mucosal vaccination

The absorption enhancing effects of chitosan as well as its effect on epithelial permeability is dependant on the pH of the solution. Chitosan is only soluble in an acidic pH range where the pH is less or equal to the pK_a value of chitosan (5.5-6.5). At higher pH values, the chitosan molecules exist in a coiled configuration, but as the pH decreases and the molecule becomes more ionised, the molecule uncoils and assumes a more elongated shape. Hence, at a lower pH range chitosan has a higher charge density and will therefore have a better possibility for contact with the epithelial membrane. This suggests that charge density may be important for enhancement of mucosal drug absorption (Van der Merwe *et al.,* 2004:227).

As previously mentioned, chitosan is a weak base and requires a certain amount of acid to transform the glucosamine units into the positively charged water-soluble form. Due to their loss of positive charge in an environment with a pH higher than 6.5, chitosan precipitates from the solution. This interferes with the biomedical application of chitosan, especially at the physiological pH of 7.4 where chitosan is insoluble and consequently ineffective as an absorption enhancer (Van der Merwe *et al.,* 2004:22; Polnok *et al.,* 2004:77).

Many efforts to prepare chitosan derivatives through chemical modifications in order to increase the solubility of chitosan in water have been reported. Removal of the one or two hydrogen atoms of the amino group of chitosan, and the introduction of some hydrophilic substituents through chemical modification resulted in an improvement in solubility of chitosan in aqueous media (Polnok *et al.,* 2004:77).

N-Trimethyl chitosan chloride (TMC) is a partially quaternised derivative of chitosan with improved solubility and easy preparation. This polymer has excellent absorption enhancing effects, especially in neutral environments where chitosan is ineffective as an absorption enhancer (Snyman *et al.,* 2003:62).

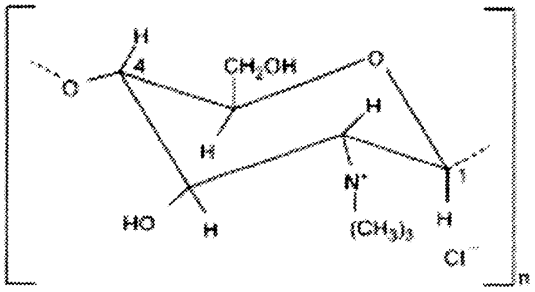


Figure 2.8: Chemical structure of TMC (Van der Merwe *et al.,* 2004:229).

It has been shown that the degree of quaternisation of TMC plays an important role in the absorption enhancing properties and mucoadhesive properties of this polymer, especially at neutral or basic pH environments (Jonker *et al.,* 2002:206). During the synthesis of TMC, the amount of fixed positive charges on the polymer chain is increased, causing an expansion of the polymer in solution. As the mucoadhesive properties of a polymer is a function of the chain flexibility, it is thus expected that the degree of quaternisation will have an effect on its mucoadhesive properties as an increase in the degree of quaternisation causes a decrease in the chain flexibility (Snyman *et al.,* 2003:62).

TMC with a degree quaternisation of 61.2 % and 12.3 % respectively were evaluated in Caco-2 cell monolayers with the hydrophilic model compound [^{14}C]-mannitol and it was confirmed that chitosan with a higher degree of quaternisation is a potent absorption enhancer at a pH of 7.4 whereas chitosan with a lower degree of quaternisation is ineffective as an absorption enhancer. The reason for this is that the chitosan with a higher degree of quaternisation has more quaternised amino groups that can interact with the cell membranes or negative sites within the tight junctions (Jonker *et al.,* 2002:206). In a study conducted by Hamman *et al.,* (2002:241), where [^{14}C]-mannitol was administered nasally to rats, it was discovered that the optimum degree of quaternisation is 48 % at a pH of 7.4 and that no further significant increases in absorption were observed even when the degree of quaternisation was increased to 59 %. The charge

density on the TMC molecule should reach a threshold value to induce significant interactions to open the tight junctions in order to increase paracellular transport in a neutral environment. It was concluded that TMC with a higher degree of quaternisation than the optimum value for absorption enhancement at physiological pH does not increase the absorption of compounds further due to possible steric effects of the attached methyl groups and the changes in the flexibility of the polymer chain.

2.4.8 Pharmaceutical and other application of chitosan

Chitosan has been used for its hypobilirubinaemic and hypocholesterolemic effects, antacid and anti-ulcer activities, wound and burn healing properties, immobilisation of enzymes and living cells and in ophthalmology. Since chitosan has the capacity to form a thin film, it is being investigated as a biopolymer for the development of contact lenses. Chitosan has been used for the manufacturing of ocular bandage lenses that are used as protective devices for acutely or chronically traumatised eyes. Chitosan membranes have also been found useful as artificial kidney membranes because of their suitable permeability and high tensile strength (Sinha *et al.,* 2004:2).

Chitosan has been used in the formulation and manufacturing of directly compressed tablets as a disintegrant, binder, granulating agent and as a drug carrier for sustained release preparations as well as a co-grinding diluent for the enhancement of the dissolution rate and bioavailability of water insoluble drugs (Sinha *et al.,* 2004:2).

Chitosan is also being investigated for its antitumour activity, as a drug delivery system in dental diseases as well as a carrier system for gene delivery (Dodane & Vilivalam, 1998:249; Hejazi & Amiji, 2003:154).

Table 2.1: Applications of chitosan (Paul & Sharma, 2000:6).

Field	Application
Health care	Contact lenses Eye bandages Wound healing ointments and dressings Anti-cholesterol (hypercholesterolemic agent) Fat-binding Surgical sutures Drug delivery Ophthalmology Dentistry Transportation of cells
Food and beverages	Food stabiliser Flavour and tastes Food packaging Nutritional additives Fruit preservation
Agriculture	Seed treatments (coating of seeds) Animal feeds Nematocides and insecticides
Cosmetics and toiletries	Hair treatment Skin care Oral care
Waste and water treatment (clarification)	Sewage effluents Drinking water Recovering metals Treating food wastes (food processor wastes)
Product separation and recovery (bioaplications)	Membrane separations Chromatographic matrix Immobilisation of enzymes/cells

2.5 EMZALOID™ AS A DRUG DELIVERY SYSTEM

2.5.1 The Emzaloid™ system

Emzaloid™ (further referred to as Emzaloid or Emzaloids) is a patented system comprised of a unique submicron emulsion formulation. An Emzaloid is a stable structure within a system that can be manipulated in terms of morphology, structure, size and function. Emzaloids consist mainly of plant and essential fatty acids namely ethyl esters of the essential fatty acids, linoleic acid and linolenic acid, as well as oleic acid, emulsified in water saturated with nitrous oxide. These Emzaloids can entrap, transport and deliver pharmacologically active compounds and other useful molecules (Saunders *et al.*, 1999:99).

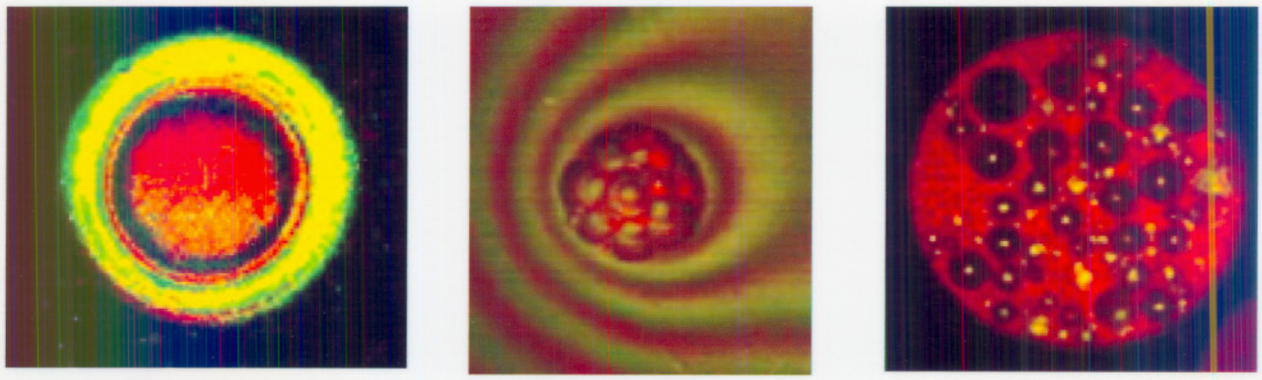
2.5.2 Emzaloid types, characteristics and functions

There are many barriers to drug delivery. Emzaloids entrap drugs and deliver them to target sites in the body. The Emzaloid penetrates keratinised tissue, skin, intestinal lining, nasal epithelium, vascular system, fungi, bacteria and parasites. Figure 2.9 shows confocal laser scanning micrographs of various formulations of the Emzaloid delivery system (Grobler, 2004:4).

While there are many existing delivery systems, the Emzaloid is unique in that its components are manipulated in a specific manner to ensure its high entrapment capabilities, fast rate of transport, delivery and stability. The absorption capabilities and drug release characteristics of the Emzaloid can thus be controlled. The entrapment of drugs within the Emzaloid generally creates a safer, more effective formulation than a formulation containing only the drug (Grobler, 2004:4).

Furthermore, the Emzaloid contains essential fatty acids that are necessary for many cell functions, but cannot be manufactured by human cells and therefore have to be ingested.

It has been shown that the western diet lacks these essential fatty acids. Some of the functions of the fatty acid components of the Emzaloid system are the maintenance of membrane integrity of cells, energy homeostasis and modulation of the immune system through leukotrins and prostaglandins and some regulatory aspects of programmed cell death. The Emzaloid system therefore has inherent therapeutic qualities that afford it significant advantages over some delivery systems (Grobler, 2004:4).



(A) (B) (C)

Figure 2.9: Confocal laser scanning micrographs of some of the basic Emzaloid types. (A), A bilayer membrane vesicle containing Rifampicin. (B), The formation of small pro-Emzaloids that are used in oral drug delivery. (C), A reservoir that contains multiple particles of coaltar (Grobler, 2004:5).

2.5.3 The Emzaloid versus other lipid based delivery systems

The fundamentals of the Emzaloid system show that the system differs substantially from conventional macromolecular carriers, such as liposomal delivery systems. The following table provides a comparison of the differences and key advantages of the Emzaloid and other lipid-based delivery systems.

Table 2.2: Differences and advantages of Emzaloid and other lipid-based delivery systems (Grobler, 2004:6).

Emzaloid	Lipid- based delivery systems
Consists mainly of essential fatty acids, a natural and essential ingredient of the human body.	Delivery systems generally contain a proportion of substances foreign to the human body.
Cytokine studies demonstrated that the Emzaloid elicits no immune response in man.	Some liposomal formulations have been shown to elicit immune responses in man.
By using different combinations of fatty acids and/or other substances, the Emzaloids can be targeted at subcellular level to some extent.	Since phospholipids are metabolised in the cell membrane, it is difficult to envisage how subcellular organelles can be targeted by this approach.
Since it is part of the natural biochemical pathways, the Emzaloid causes no cytotoxicity and assists with cell maintenance.	Cytotoxicity and impaired cell integrity are common problems with substances that enter the body. Liposomal systems may decrease the cytotoxicity of compounds or may cause cytotoxicity.
The Emzaloid is polyphonic and drugs that have different solubilities as well as insoluble drugs can be entrapped.	Most delivery systems are either lipophilic or hydrophilic.
The Emzaloid, due to its composition is sterically stabilised without the disadvantages of increased size or decreased elasticity.	Delivery systems generally need to be sterically stabilised. This generally leads to an increase in size and rigidity of the carrier.
Although the Emzaloid contains no cholesterol, the interior volume remains stable.	Most lipid-based delivery systems contain phospholipids and cholesterol to stabilise the interior of the vesicles.
The Emzaloid is designed to show a high degree of elasticity and fluidity, with a relatively high phase transition temperature.	Because of the general use of stabilising compounds and cholesterol, liposomal systems lose fluidity and elasticity. The phase transition temperatures of phospholipid groups are higher than that of the essential fatty acids, resulting in loss of elasticity.

Table 2.2 continued: Emzaloid	Lipid-based delivery systems
Due to the Emzaloid's composition, it is able to inhibit the drug efflux mechanism in the intestinal lumen and can thereby enhance the bioavailability of a compound.	Liposomal systems containing this feature have not been described. A separate compound (e.g. Cremophor is co-administered to achieve the same effect.
The entrapment efficiency of compounds inside the Emzaloid is high (between 85 % and 100 %).	Due to the charge and steric limitations of liposomal delivery systems, entrapment efficiencies may be problematic.
The type of Emzaloid formulated for a specific compound determines the loading capacity of the Emzaloid.	The loading capacity of most lipid-based delivery systems is dependant on the interior of the intramembrane volume and is therefore limited.
Emzaloid micro-sponges are ideal for combination therapies, as one drug can be entrapped in the interior volume and the other drug in the sponge spaces. Geographical separation of active compounds into different interior spaces minimizes drug interactions or interactions between drug compounds.	Combination therapies are problematic in most liposomal drug delivery systems.
Batch-to-batch reproducibility and stability has been proven with existing products containing Emzaloid, such as the registered product Exorex®.	Large scale manufacturing of other liposomal delivery systems sometimes show low batch-to-batch reproducibility as well as problems with size control.

2.5.4 Pharmaceutically applicable features of the Emzaloid system

2.5.4.1 Decreased time to onset of action

Research has indicated that the Emzaloid delivery system rapidly transverses most physiological barriers and delivers the active compound. An active compound delivered with the Emzaloid has been shown to act significantly faster than the same active compound delivered via a conventional approach, therefore suggesting a potentially faster relief from target symptoms (Grobler, 2004:9).

2.5.4.2 Increased delivery of active compounds

Both *in vitro* and *in vivo* studies have shown that by using the Emzaloid as a delivery system, the percentage active compound that is delivered to the target site can be dramatically increased by entrapment of the active compound in Emzaloids (Grobler, 2004:9).

2.5.4.3 Reduction of minimum drug concentration

Research conducted with the Emzaloid system has shown that, for certain active compounds, using as little as $1/40^{th}$ of the active compound may result in an effective drug plasma concentration. In practice, this characteristic will result in a reduction of patient side effects as well as major savings in treatment cost (Grobler, 2004:10).

2.5.4.4 Increased therapeutic efficacy

It has been shown that using Emzaloids as a delivery system increases the efficacy of the active compound incorporated in the Emzaloid system (Grobler, 2004:10).

2.5.4.5 Reduction in cytotoxicity

Side effects of drugs are in most instances caused by cellular damage. The Emzaloid system has the potential to minimise cellular damage that occurs as a result of membrane damage caused by active compounds. Therefore, by incorporating the drug in the Emzaloid system, the appearance of side effects can be dramatically reduced (Grobler, 2004:11).

2.5.4.6 Immunological responses

Some drugs, such as proteins and peptides, may induce an immunological response or adverse intolerance reactions. By masking these compounds using the Emzaloid system, this may prohibit the human immune system from recognising these compounds. By doing this, the dosage can be reduced without diminishing the potency or the dosage can be increased to enhance therapeutic effects (Grobler, 2004:12).

2.5.4.7 Transdermal delivery

Whilst the Emzaloid system is not limited to topical application, research has indicated that many medications can be administered topically instead of orally. The digestive system seems to be the origin of many side effects. Therefore, transdermal delivery will eradicate many of the unwanted side effects caused by oral medication (Grobler, 2004:12).

2.5.4.8 The ability to entrap and transfer genes to nuclei and expression of proteins

Experiments performed on the Emzaloid delivery system demonstrate its applicability in DNA vaccines and gene therapy. *In vitro* studies have shown entrapment of human and viral DNA of various lengths into Emzaloids. Reproducible expression of appropriate proteins was observed after transfection of cells by Emzaloid-entrapped genes (Grobler, 2004:13).

2.5.4.9 Reduction and elimination of drug resistance

In vitro studies have demonstrated that by incorporating a drug into the Emzaloid delivery system, drug resistance can be reduced or eliminated. Analysis of bacterial growth of multidrug resistant TB has shown that formulations containing the standard antimicrobial, rifampicin, entrapped in Emzaloids, obviated pre-existing drug resistance.

The ability to enhance the effectiveness of antibiotics such as penicillin can have widespread implications in the healthcare industry (Grobler, 2004:13).

2.5.5 Therapeutic and preventative uses of Emzaloid technology

2.5.5.1 Therapy of tuberculosis

"Tuberculosis kills 2 million people each year. Overall, one-third of the world's population is currently infected with the TB bacillus". World Health Organisation. Previous *in vitro* and animal studies suggested that the Emzaloid delivery system might have significant benefits when combined with TB medication. Therefore, a bio-equivalence study was performed to determine whether the Emzaloid delivery system would remain as effective in oral administration as it was proven to be in topical use (Grobler, 2004:14).

The purpose of the study was to prove that the use of the Emzaloid after administration in a pro-Emzaloid formulation could:

1) Increase the availability of the antimicrobials in human plasma.
2) Enhance the absorption of the formulated antimicrobials from the gastrointestinal tract without an increase in toxicity.
3) Increase the intracellular concentration of the antimicrobial in the target cells where the tuberculosis bacteria breed.
4) Extend the circulatory time of the active drugs.
5) Increase the bactericidal effect of the antimicrobials inside the target cells.
6) Decrease the side effects caused by the antimicrobials (Grobler, 2004:14).

Results of the bio-equivalence study, showed that:

1) The entrapment of the antimicrobials into Emzaloids led to an increase in absorption of the antimicrobials after oral administration, with a resulting increase in plasma levels of these antimicrobials.
2) The entrapment of the antimicrobials led to an increased rate of absorption and cellular response.
3) The therapeutic concentrations of the drugs were maintained for longer and the circulatory time of the drugs was extended, indicating that the exposure of the bacteria to the antimicrobials was increased.
4) The entrapment of the antimicrobials in the Emzaloid delivery system increased the delivery of the antimicrobials to the target cells, thereby decreasing the minimum inhibitory concentration.
5) A lower dosage can therefore be used to obtain similar effects.
6) A decrease in side effects was observed, with an increase in patient compliance, therefore suggesting that it could prevent the development of multi-drug resistance (Grobler, 2004:17).

2.5.5.2 Preventative therapies: Vaccines

Historically, vaccination is the only strategy that has led to the elimination of a viral disease, namely smallpox. An indirect relationship has been observed for vaccine immunogenicity and safety. Human immune responses to synthetic and recombinant peptide vaccines administered with standard adjuvants tend to be poor; hence there is an urgent need for effective vaccine adjuvants to enhance the immunogenicity and immunostimulatory properties of vaccines (Grobler, 2004:17).

2.5.5.2.1 A virus-based vaccine: Rabies

The efficacy of a commercially available rabies vaccine and a rabies vaccine incorporated in an Emzaloid delivery system was investigated and compared. An inactivated virus is used in the formulation of rabies vaccines. For the comparative animal studies, different formulations of the virus were used namely the inactivated virus, the inactivated virus with alum (aluminium hydroxide) as adjuvant and the inactivated virus incorporated in an Emzaloid delivery system. The inactivated virus incorporated in an Emzaloid delivery system showed a 9-fold increase in antibody response when compared to the other formulations (Grobler, 2004:18).

2.5.5.2.2. A peptide-based vaccine: Hepatitis B

The efficacy of a commercially available hepatitis B vaccine and a hepatitis B vaccine incorporated in an Emzaloid delivery system was investigated and compared. Non-recombinant hepatitis B vaccines are generally based on the use of the surface molecules of the virus as antigen. For the comparative animal studies, different formulations of this peptide-based vaccine were used namely the peptide, the peptide with alum as an adjuvant and the peptide incorporated in an Emzaloid delivery system. The use of Emzaloids as a drug delivery system led to more than a 10-fold increase in the efficacy of the peptide-based hepatitis B vaccine as measured by antibody response. The Emzaloid therefore has an obvious dual role in vaccinology, firstly as delivery system for disease specific antigens, and secondly as immuno-stimulatory adjuvant (Grobler, 2004:19).

2.5.5.2.3 Emzaloid technology for nasal vaccine delivery

The hypothesis for the nasal delivery of vaccines using the Emzaloid as delivery system is based on the same principle than that of microparticulate systems, such as chitosan and *N*-trimethyl chitosan chloride microparticles.

The antigen is loaded into the Emzaloids and administered where it is taken up by the microfold cells (M-cells) in the nasal epithelium which are responsible for the sampling of and transporting of antigens to the underlying nasal associated lymphoid tissue (NALT), germinal centers containing B cells and T cells, plasma cells and antigen presenting cells (APCs). These cells are involved in the regulation and induction of antigen-specific effector cells, which produce the protective humoral and cellular immune responses.

2.6 CONCLUSION

The two major physiological barriers that limit the mucosal delivery of protein and peptide drugs such as vaccines are the enzymatic and epithelial barriers. Due to the physico-chemical properties and high molecular weight, the absorption of peptide and protein drugs such as vaccines is limited to the paracellular pathway of transport. By the transient opening of the tight junctions between the epithelial cells of the nasal cavity, chitosan and *N*-trimethyl chitosan chloride (TMC) permit for the paracellular transport of protein and peptide drugs such as vaccines after nasal administration. Besides the opening of tight junctions, these above mentioned delivery systems as well as Emzaloids are able to associate large amounts of vaccines and enhance the uptake of antigens by the nasal associated lymphoid tissue.

Because of their relatively cheap production costs, biocompatibility and low toxicity, these systems may hold great potential for the future in the development of novel drug delivery systems.

CHAPTER 3

PREPARATION AND CHARACTERISATION OF CHITOSAN MICROPARTICLES AND NANOPARTICLES, TMC MICROPARTICLES AND EMZALOID™ DELIVERY SYSTEMS

3.1 INTRODUCTION

The pharmaceutical applications of chitosan, its more soluble derivative, *N*-trimethyl chitosan chloride (TMC) and Emzaloid™ technology have been discussed in chapter 2. The use of chitosan and TMC as microparticulate and nanoparticulate delivery systems as well as the potential use of Emzaloids for the entrapment and delivery of antigens via the nasal mucosa has also been discussed in chapter 2.

In this chapter, the preparation and characterisation of chitosan and TMC microparticles and chitosan nanoparticles are described. The nanoparticles and all the microparticles were prepared with the ionotropic gelation of chitosan and TMC with cationic salts. These formulations were characterised based on their size, morphology, diphtheria toxoid (DT) loading and diphtheria toxoid release profiles. The preparation and characterisation of micrometer and nanometer range Emzaloids will be described with respect to size and morphology.

3.2 PREPARATION AND CHARACTERISATION OF CHITOSAN MICROPARTICLES

3.2.1 Preparation of chitosan microparticles

3.2.1.1 Materials

ChitoClear® was obtained from Primex Ingredients ASA, Avaldsnes, Norway. The viscosity and the degree of deacetylation of the chitosan as determined by the supplier were 20 mPa.S, and 93 % respectively. Tween® 80, acetic acid and sodium sulphate was obtained from Sigma-Aldrich, Germany.

3.2.1.2 Method

A 0.25 % w/v chitosan solution was prepared in MilliQ water containing 2 % v/v acetic acid and 1 % v/v Tween® 80. Then 2 ml of a 10 % w/v sodium sulphate solution was added dropwise (approximately 1 ml/min) to 200 ml of the chitosan solution under magnetic stirring and continuous sonication using a Branson sonifier 250. After adding the sodium sulphate, stirring and sonication was continued for an additional 20 minutes. The microparticulate suspension was then centrifuged for 25 minutes at 2750 rpm. The pellet was resuspended in MilliQ water to wash the microparticles and centrifuged again. The washing process was repeated twice before the pellet was frozen in liquid nitrogen and freeze-dried overnight with a Christ freeze-dryer (Osterode Am Harz, Germany).

3.2.2 Characterisation of chitosan microparticles

3.2.2.1 Size of chitosan microparticles

Four different batches of washed and freeze-dried chitosan microparticles were dispersed in MilliQ water and the size of the microparticles was determined with a Malvern Mastersizer 2000 version 5.1 (Malvern, U.K.). Two measurements were taken for every batch of chitosan microparticles before and after stirring the suspension for 5 minutes in the sample bath.

3.2.2.2 Morphology of chitosan microparticles

Scanning Electron Microscopy (SEM) was performed to study the morphology of the chitosan microparticles. For Scanning Electron Microscopy (SEM) analysis, chitosan microparticles were adhered to carbon-containing double-sided tape. The microparticles were coated with gold/palladium (Aug/Pd) in a preparation chamber. The coated chitosan microparticles were then studied with a field emission SEM (Philips FEI Quanta 200 ESEM, Carl Zeiss, Germany). Digital images were made and stored.

3.2.3 Results and discussion

3.2.3.1 Size of chitosan microparticles

Table 3.1 gives the sizes (volume diameter) of the four batches of chitosan microparticles as well as the average particle size distribution at stirring time 0 min and stirring time 5 min. Figure 3.1 and 3.2 depict typical particle size distribution curves. After

characterisation, the four batches of chitosan microparticles were thoroughly mixed and used for further studies (chapter 4).

Table 3.1: Particle size analysis of chitosan microparticles at time 0 min and time 5 min.

Batch	Time (min)	Diameter d(0.1) (μm)	Diameter d(0.5) (μm)	Diameter d(0.9) (μm)
Chitosan 1	0	17.383	63.594	203.963
Chitosan 1	5	1.546	2.119	3.878
Chitosan 2	0	11.852	55.511	199.351
Chitosan 2	5	1.834	2.748	6.385
Chitosan 3	0	6.845	29.880	127.016
Chitosan 3	5	1.917	3.040	6.740
Chitosan 4	0	4.359	14.318	40.457
Chitosan 4	5	1.427	2.379	5.326
Average	0	10.11 ± 5.76	40.83 ± 22.78	142.70 ± 76.72
Average	5	1.68 ± 0.23	2.57 ± 0.41	5.58 ± 1.3

Time 0 : measurement with no stirring, Time 5: measurement after stirring for 5 min, d(0.1): 10 % of particles are smaller than the d(0.1) value, d(0.5): 50 % of the particles are smaller than the d(0.5) value, d(0.9): 90 % of particles are smaller than the d(0.9) value.

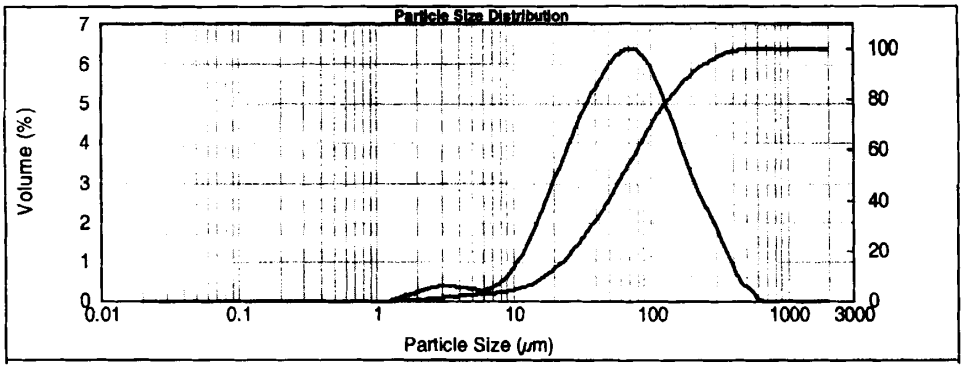


Figure 3.1: Particle size distribution of chitosan microparticles (chitosan 1) at stirring time 0 min.

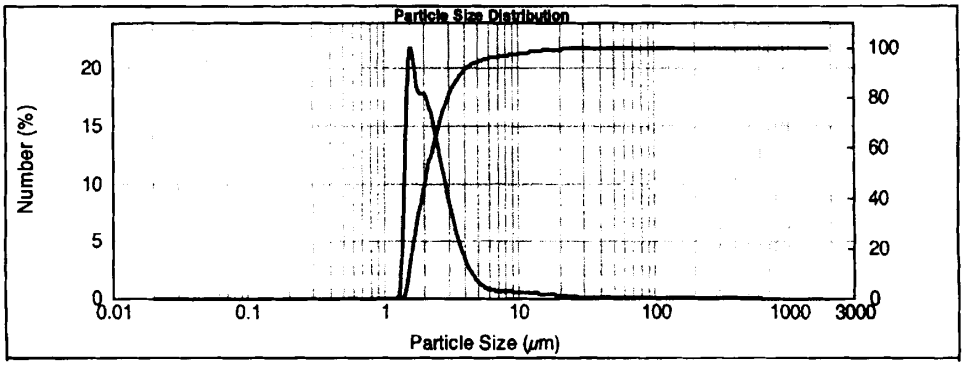


Figure 3.2: Particle size distribution of chitosan microparticles (chitosan 1) at stirring time 5 min.

It was found that by stirring the microparticulate suspension, the particle size of the chitosan microparticles could be reduced. The average particle size and the standard deviation (SD) were both dramatically reduced when the stirring time was prolonged to 5 min. This reduction in particle size is probably due to the fact that electrostatic forces exist between the individual particles. These electrostatic forces tend to cause aggregation of particles. However, stirring of the microparticles in water proves to be effective in reducing these electrostatic forces and subsequent aggregation. The SD after stirring for 5

min is indicative of the reproducibility of the preparation method as the SD is very small. This is, however not the case when the microparticulate suspension is not stirred, as the SD is bigger than 50% of the average value of the particle size.

3.2.3.2 Morphology of chitosan microparticles

Field emission Scanning Electron Microscopy (SEM) performed on freeze-dried chitosan microparticles showed that the rough surface of the chitosan microparticles is very porous. Figure 3.3(B) depicts a surface view of the chitosan microparticles. It could be seen that the microparticles appeared more porous on close inspection, which is a positive attribute, as the porosity could facilitate the entrapment of antigens into the particles.

(A) (B)

Figure 3.3: Scanning Electron Micrographs of (A), chitosan microparticles and (B), the surface of chitosan microparticles to illustrate the porous nature of the microparticles.

3.3 SYNTHESIS AND CHARACTERISATION OF TMC

N-trimethyl chitosan chloride (TMC) with a low degree of quaternisation was synthesised in a one-step reaction by a method based on the reductive methylation of chitosan with methyl iodide in the presence of sodium hydroxide at 60 °C as described by Sieval *et al.*(1998:158).

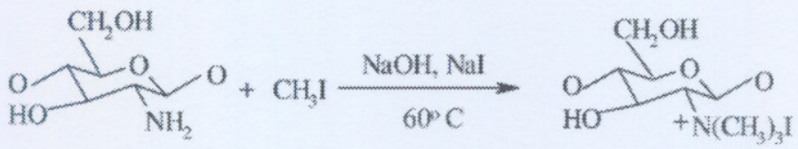


Figure 3.4: Synthesis of *N*- trimethyl chitosan chloride (TMC) (Thanou *et al.*, 2001:S96).

3.3.1. Materials

ChitoClear® was obtained from Primex Ingredients ASA, Avaldsnes, Norway. The viscosity and the degree of deacetylation of the chitosan as determined by the supplier were 15 mPa.S and 97 % respectively. Sodium iodide, sodium hydroxide, sodium chloride (Saarchem, R.S.A.), methyl iodide and *N*-methyl-2-pyrrolidinone (Sigma-Aldrich, Germany) were also used during the synthesis. Diethyl ether and ethanol used for precipitation were obtained from Merck, R.S.A.

3.3.2 Method

To prepare the TMC, a mixture of 2 g of chitosan, 4.8 g of sodium iodide, 11 ml of a 15 % w/v aqueous sodium hydroxide solution (NaOH) and 11.5 ml of methyl iodide in 80 ml of *N*-methyl-2-pyrrolidinone was stirred on a water bath at a temperature of 60 °C for 1 hour. A Liebig condenser was used to keep the methyl iodide in the mixture. The product was precipitated from solution with ethanol by slowly adding the ethanol to the mixture and subsequently isolated by centrifugation. After washing with ethanol and diethyl ether, the product was dissolved in 40 ml of a 5 % w/v aqueous NaCl solution to exchange the iodide ion with a chloride ion. The polymer was precipitated from solution with ethanol and isolated by centrifugation. The product was then dissolved in 40 ml water and precipitated with ethanol to remove the remaining NaCl from the material. This yielded a white water-soluble powder that was then dried overnight in a vacuum oven at 40 °C.

3.3.3 Characterisation of TMC with nuclear magnetic resonance (NMR) spectroscopy

^{1}H-NMR was used to characterise the synthesised TMC polymer. The ^{1}H-NMR spectrum was recorded in D_2O with a Bruker 600 MHz spectrometer (Bruker, Switzerland) at 80 °C. Minimum interference was observed from the water peak at this temperature and suppression of the water peak was not necessary. The following equation was used to calculate the degree of quaternisation of the TMC polymer from the ^{1}H-NMR spectrum:

$$DQ\ (\%) = [(TM/H) \times 1/9] \times 100 \qquad [1]$$

Where DQ (%) = degree of quaternisation expressed as a percentage

TM = integral of the trimethyl amino group peak

H = integral of the ^{1}H peaks

3.3.4 Results and discussion

The ^{1}H-NMR spectrum of the synthesised TMC polymer is depicted in figure 3.5. Sievel *et al.* (1998:158) assigned the peak at 3.3 ppm to the trimethyl amino group and the peaks between 4.7 and 5.7 ppm to ^{1}H. The integrals of the peaks were substituted into equation 1 to calculate the degree of quaternisation for the synthesised polymer. The degree of the quaternisation calculated for the polymer was 22.53 %.

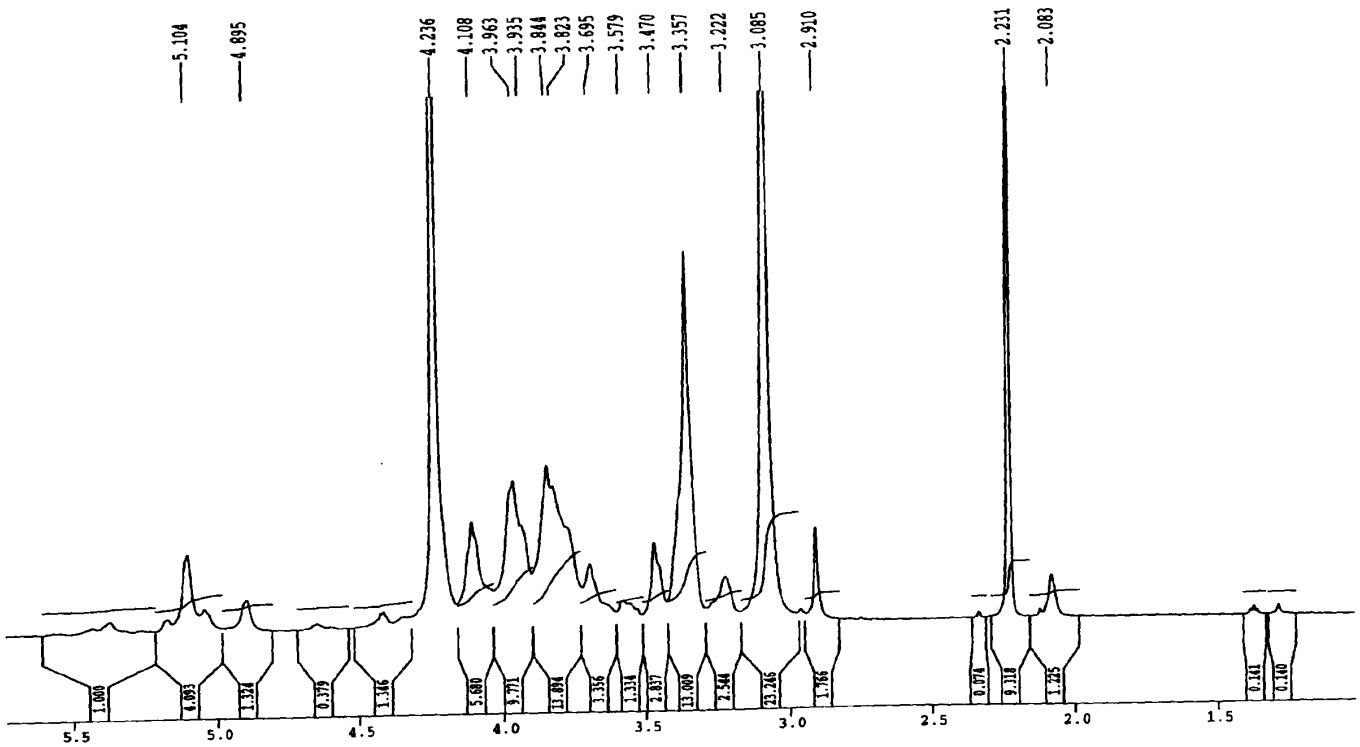


Figure 3.5: ^{1}H-NMR spectrum of TMC (degree of quaternisation = 22.53 %).

3.4 PREPARATION AND CHARACTERISATION OF TMC MICROPARTICLES

3.4.1 Preparation of TMC microparticles

3.4.1.1 Materials

TMC with a degree of quaternisation of 22.53 % was synthesised as described in section 3.3. Tween® 80 and tri-poly phosphate (TPP) were obtained from Sigma-Aldrich, Germany.

3.4.1.2 Method

A 0.25 % w/v TMC solution was prepared in MilliQ water containing 1 % w/v Tween® 80. Then 5.5 ml of a 5 % w/v tri-poly phosphate (TPP) solution was added dropwise (approximately 1 ml/min) to 250 ml of the TMC solution under magnetic stirring and continuous sonication using a Branson sonifier 250. After adding the TPP, stirring and sonication was continued for an additional 20 minutes. The microparticulate suspension was then centrifuged for 25 minutes at 2750 rpm. The pellet was resuspended in MilliQ water to wash the microparticles and centrifuged again. The washing process was repeated once before the pellet was frozen in liquid nitrogen and freeze-dried overnight with a Christ freeze-dryer (Osterode Am Harz, Germany).

3.4.2 Characterisation of TMC microparticles

3.4.2.1 Size of TMC microparticles

Two batches of washed and freeze-dried TMC microparticles were dispersed in MilliQ water before taking size measurements. The size of the microparticles was determined with a Malvern Mastersizer 2000, version 5.1 (Malvern, U.K.) Two measurements were taken for every batch of TMC microparticles, before and after stirring the suspension for 5 minutes in the sample bath.

3.4.2.2 Morphology of TMC microparticles

Scanning Electron Microscopy (SEM) was performed to study the morphology of the TMC microparticles. For Scanning Electron Microscopy (SEM) analysis, TMC microparticles were adhered to carbon-containing double-sided tape. The microparticles were coated with gold/palladium (Aug/Pd) in a preparation chamber. The coated TMC microparticles were then studied with a field emission SEM (Philips FEI Quanta 200 ESEM, Carl Zeiss, Germany). Digital images were made and stored.

3.4.3 Results and discussion

3.4.3.1 Size of TMC microparticles

Table 2 gives the sizes (volume diameter) of the two batches of TMC microparticles. Figure 3.6 and 3.7 depict typical particle size distribution curves. After characterisation, the two batches of TMC microparticles were thoroughly mixed and used for further studies (chapter 4).

Table 3.2: Particle size analysis of TMC microparticles at time 0 min and time 5 min.

Batch	Time (min)	Diameter d(0.1) (µm)	Diameter d(0.5) (µm)	Diameter d(0.9) (µm)
TMC 1	0	5.466	19.050	988.416
TMC 1	5	1.061	1.854	4.773
TMC 2	0	5.638	36.572	270.900
TMC 2	5	1.432	2.267	4.832
Average	0	5.55 ± 0.12	27.81 ± 12.39	629.6 ± 507.36
Average	5	1.25 ± 0.26	2.06 ± 0.29	4.8 ± 0.04

Time 0 : measurement with no stirring, Time 5: measurement after stirring for 5 min, d(0.1): 10 % of particles are smaller than the d(0.1) value, d(0.5): 50 % of the particles are smaller than the d(0.5) value, d(0.9): 90 % of particles are smaller than the d(0.9) value.

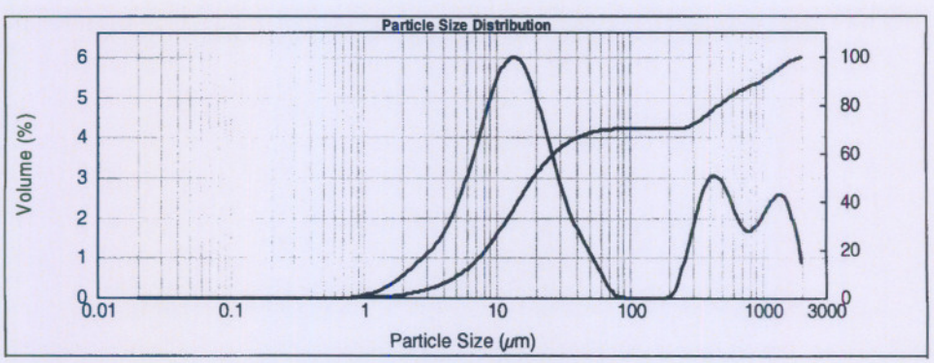


Figure 3.6: Particle size distribution of TMC microparticles (TMC 1) at stirring time 0 min.

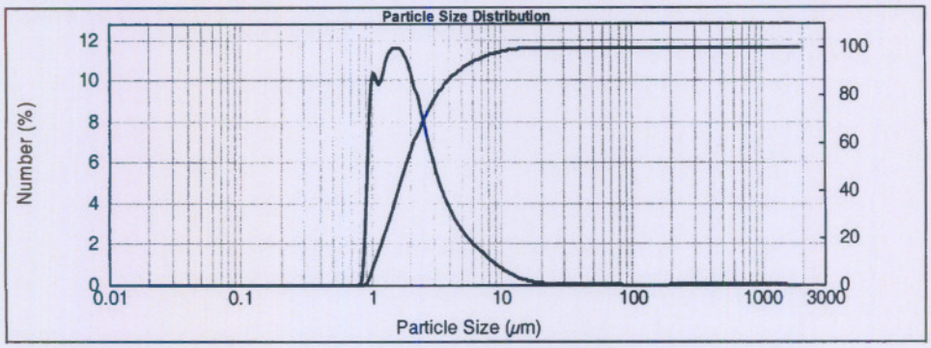
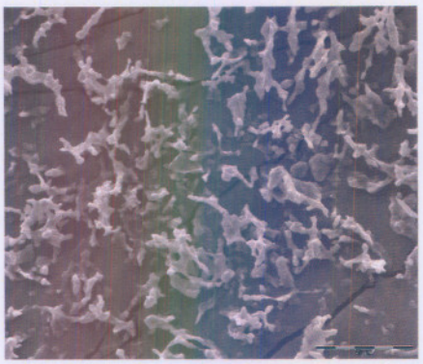


Figure 3.7: Particle size distribution of TMC microparticles (TMC 1) at stirring time 5 min.

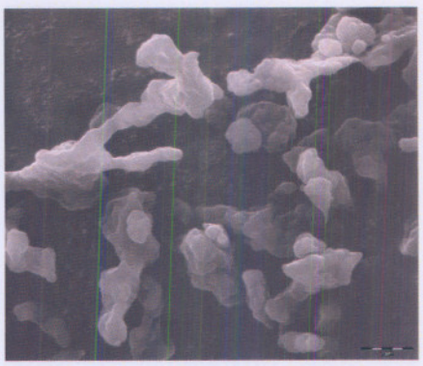
It was found that by stirring the microparticulate suspension the particle size of the TMC microparticles could be reduced. The average particle size and the standard deviation (SD) were both dramatically reduced when the stirring time was prolonged to 5 min. This reduction in particle size is probably due to the fact that electrostatic forces exist between the individual particles. These electrostatic forces tend to cause aggregation of particles. However, stirring of microparticles in water proves to be effective in reducing these electrostatic forces and subsequent aggregation. The SD after stirring for 5 min is indicative of the reproducibility of the method of preparation as the SD is very small. This is, however not the case when the microparticulate suspension is not stirred, as the SD is bigger than 50 % of the average value of the particle size.

3.4.3.2 Morphology of TMC microparticles

Field emission Scanning Electron Microscopy (SEM) performed on freeze-dried TMC microparticles showed that the surface of the TMC microparticles is much smoother compared to that of the chitosan microparticles. The TMC microparticles appeared to be less porous than the chitosan microparticles (fig. 3.8 B).



(A)



(B)

Figure 3.8: Scanning Electron Micrographs of (A), TMC microparticles and (B), the surface of TMC microparticles to illustrate the non-porous nature of the microparticles.

3.5 PREPARATION AND CHARACTERISATION OF CHITOSAN NANOPARTICLES

3.5.1 Preparation of chitosan nanoparticles

3.5.1.1 Materials

ChitoClear® was obtained from Primex Ingredients ASA, Avaldsnes, Norway. The viscosity and the degree of deacetylation of the chitosan as determined by the supplier were 15 mPa.S, and 97 % respectively. TPP was obtained from Sigma-Aldrich, Germany.

3.5.1.2 Method

A 0.25 % w/v chitosan solution was prepared in MilliQ water containing 0.5 % v/v acetic acid. Then 8 ml of a 0.1 % w/v TPP solution was added dropwise to 16 ml of the chitosan solution under magnetic stirring at a constant stirring speed of 1000 rpm. The nanoparaticulate suspension was then centrifuged for 10 min at 6500 rpm (Eppendorf 5415 C, Eppendorf, Germany). The pellet was then resuspended in MilliQ water and the washing step was repeated twice. Because the nanoparticles were small, strong electrostatic forces were observed between the particles and the particles were inclined to adhere to each other. To overcome this problem, the nanoparticles were placed in a sonication bath for 60 min and then air was forced through the nanoparticulate suspension by means of a Pasteur pipette before the suspension was frozen in liquid nitrogen and freeze-dried overnight with a Freezemobile 6 freeze-dryer (Virtis Gardiner, U.S.A).

3.5.2 Characterisation of chitosan nanoparticles

3.5.2.1 Size of chitosan nanoparticles

Due to the inability of the Malvern Mastersizer (Mastersizer X) to analyse particles smaller than 100 nm, the nanoparticles were analysed with a Transmission Electron Microscope (TEM), and therefore only an approximate particle size could be determined.

For Transmission Electron Microscopy (TEM) analysis, the freeze-dried nanoparticles were resuspended in phosphate buffered saline (PBS; pH 7.4) and placed in a sonication bath for 60 min, before air was forced through the nanoparticulate suspension by means of a Pasteur pipette. The samples were stained with uranyl acetate and placed on copper grids with Formvar films. The stained chitosan nanoparticles were then studied with a

Philips CM10 TEM (Carl Zeiss, Germany). Electronmicrographs were taken and analysed.

3.5.2.2 Morphology of chitosan nanoparticles

Transmission Electron Microscopy (TEM) was performed to study the morphology of the chitosan nanoparticles. The same process as described in 3.5.2.1 was followed to prepare the chitosan nanoparticles for viewing with a Philips CM10 TEM. Electronmicrographs were taken and analysed.

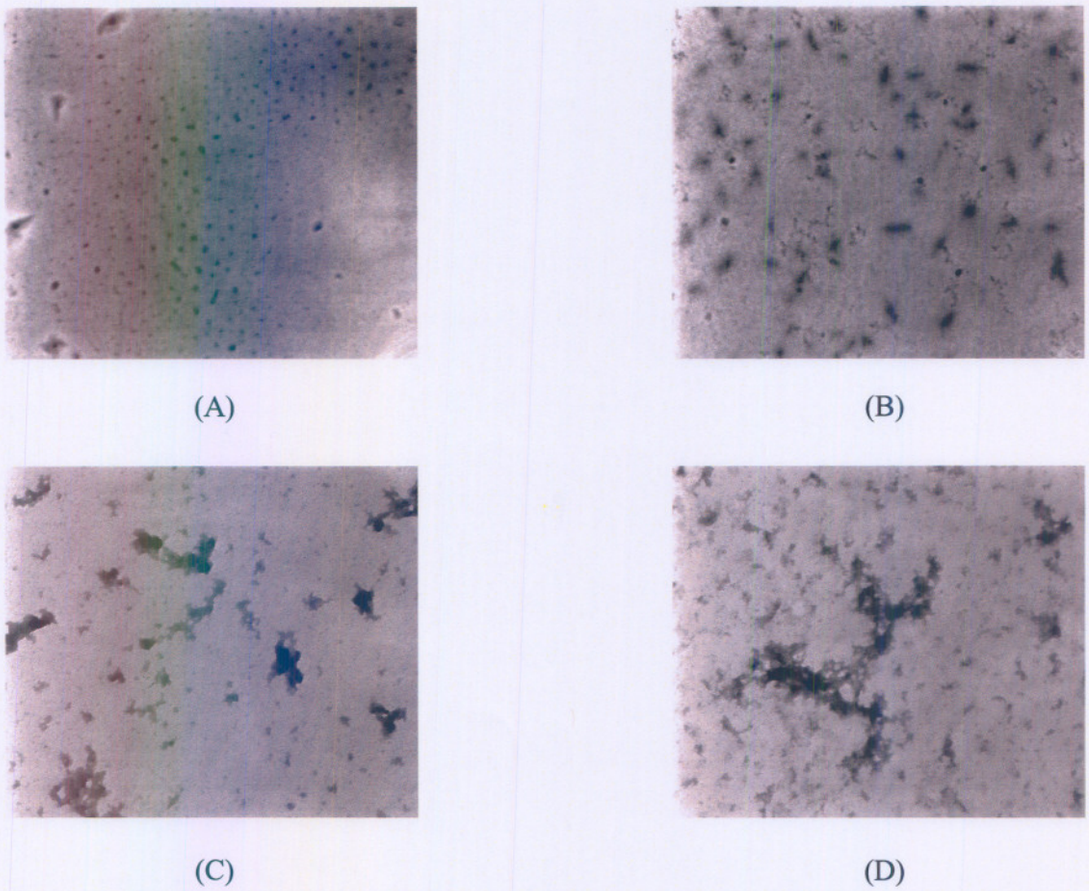
3.5.3 Results and discussion

3.5.3.1 Size of chitosan nanoparticles

The size of the chitosan nanoparticles was analysed by Transmission Electron microscopy (TEM) after washing and freeze-drying. It was established that the chitosan nanoparticles show an inconsistency in size, and the approximate size of the chitosan nanoparticles was found to be between 50 nm and 450 nm.

3.5.3.2 Morphology of chitosan nanoparticles

The morphology of the chitosan nanoparticles was analysed by Transmission Electron Microscopy (TEM) after washing and freeze-drying. As depicted in figure 3.9, chitosan nanoparticles appear to be amorphous in nature and show an inconsistency in size. It was also seen that chitosan nanoparticles do not form a gel when resuspended in PBS (pH 7.4), although the chitosan nanoparticles tend to form aggregates due to electrostatic forces present between the particles.



(A) (B)

(C) (D)

Figure 3.9: Transmission electronmicrographs of washed and freeze-dried chitosan nanoparticles resuspended in PBS (pH7.4). (A) and (B) indicate that the chitosan nanoparticles do not form a gel when resuspended in PBS. (B) and (C) indicate that the chitosan nanoparticles tend to form aggregates.

3.6 PREPARATION AND CHARACTERISATION OF EMZALOIDS

3.6.1 Preparation of micrometer range Emzaloids

3.6.1.1 Materials

Vitamin F ethyl ester was obtained from Kurt Richter Pharma, Germany. Cremaphor RH-40 was obtained from BASF, Germany. Schott bottles were obtained from Merck, RSA and 0.22 μm GD/X filters were obtained from Wattman, Germany.

3.6.1.2 Method

4.50 g of vitamin F ethyl ester and 1.36 g of cremaphor RH-40 was weighed in a closed glass container to create the oil phase of the preparation. The oil phase was then mixed and heated to 70 °C in a water bath. 300 ml of nitrous oxide (N_2O) saturated water was placed in a glass beaker and the opening of the beaker was closed before heating the N_2O saturated water to 70 °C on a hotplate. While the temperature of the N_2O saturated water was maintained at 70 °C, the oil phase was added to the N_2O saturated water to form an emulsion. This preparation was then mixed with a Braun mixer on its second speed setting until homogenous, before being transferred to Schott bottles. The Schott bottles were then shaken until room temperature was reached. This preparation was sterilised in a laminar flow hood with 0.22 μm GD/X filters and sealed in airtight containers.

3.6.2 Characterisation of micrometer range Emzaloids

3.6.2.1 Size of micrometer range Emzaloids

Confocal Laser Scanning Microscopy (CLSM) (PCM 2000 with Nikon digital camera DXM 1200, Nikon, Holland and He/Ne Spectra Physics laser with an excitation of 505 nm and emission of 568 nm as well as an ApoPlanar oil immersion objective was used to determine the approximate size of the Emzaloids. For CLSM, the Emzaloids were stained with Nile Red (Molecular Probes, Holland) at a concentration of 50 nM. The stained Emzaloids were placed on a glass slide and covered with a glass cover-slip. The glass slide and cover-slip were sealed together using adhesive to prevent the Emzaloids from drying out.

3.6.2.2 Morphology of micrometer range Emzaloids

Confocal laser scanning microscopy (CLSM) was used to study the morphology of the Emzaloids. The same process as described in 3.6.2.1 was used to prepare the Emzaloids for viewing. Digital images were taken and stored.

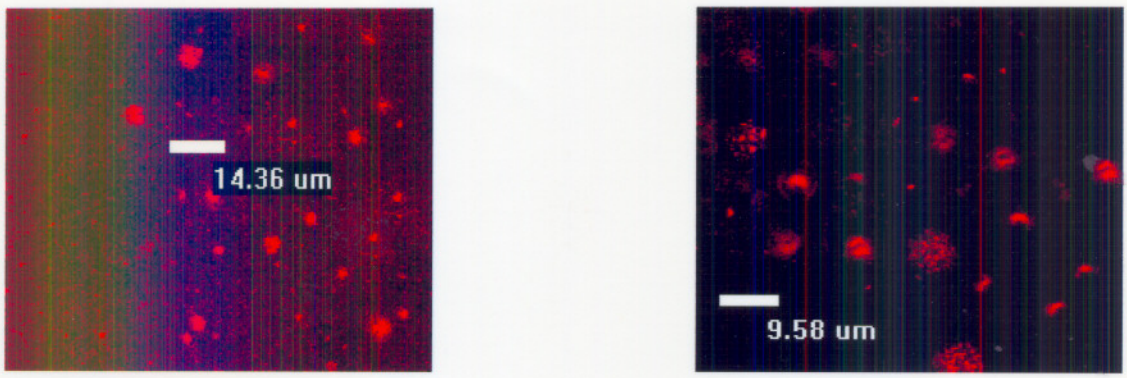
3.6.3 Results and discussion

3.6.3.1 Size of micrometer range Emzaloids

The size of the micrometer range Emzaloids was analysed by Confocal Laser Scanning Microscopy (CLSM). It was established that the Emzaloids are reasonably consistent in size, and the approximate size of the Emzaloids was found to be between 2 μm and 4 μm.

3.6.3.2 Morphology of micrometer range Emzaloids

The morphology of the micrometer range Emzaloids was analysed by Confocal Laser Scanning Microscopy (CLSM). As shown in figure 3.10 the Emzaloids are spherical in nature. It was seen that the diphtheria toxoid (DT) loading of Emzaloids caused a decrease in the quantity of Emzaloids in the preparation, as well as an increase in size of the Emzaloids in the preparation.



(A) (B)

Figure 3.10: Confocal Laser Scanning Micrographs of (A), unloaded micrometer range Emzaloids and (B), micrometer range Emzaloids loaded with diphtheria toxoid (DT).

3.6.4 Preparation of nanometer range Emzaloids

3.6.4.1 Materials

Vitamin F ethyl ester was obtained from Kurt Richter Pharma, Germany. Cremaphor RH-40 was obtained from BASF, Germany. Schott bottles were obtained from Merck, RSA and 0.22 μm GD/X filters were obtained from Wattman, Germany.

3.6.4.2 Method

4.50 g of vitamin F ethyl ester and 1.36 g of cremaphor RH-40 was weighed in a closed glass container to create the oil phase of the preparation. The oil phase was then mixed and heated to 70 °C in a water bath. 300 ml of nitrous oxide (N_2O) saturated water was placed in a glass beaker and the opening of the beaker was closed before heating the N_2O saturated water to 70 °C on a hotplate. While the temperature of the N_2O saturated water was maintained at 70 °C, the oil phase was added to the N_2O saturated water to form an emulsion. This preparation was then mixed with a Braun mixer on its second speed setting until homogenous. This preparation was then sonicated for 30 min at 37 °C before being transferred to Schott bottles. The Schott bottles were then shaken until room temperature was reached. The emulsion was sterilised in a laminar flow hood with 0.22 μm GD/X filters and sealed in airtight containers.

3.6.5 Characterisation of nanometer range Emzaloids

3.6.5.1 Size of nanometer range Emzaloids

Confocal Laser Scanning Microscopy (CLSM) (PCM 2000 with Nikon digital camera DXM 1200, Nikon, Holland and He/Ne Spectra Physics laser with an excitation of 505 nm and emission of 568 nm as well as an ApoPlanar oil immersion objective was used to determine the approximate size of the Emzaloids. For Confocal laser scanning microscopy, Emzaloids were stained with Nile Red (Molecular Probes, Holland) at a concentration of 50 nM. The stained Emzaloids were placed on a glass slide and covered with a glass cover-slip. The glass slide and cover-slip were sealed together using adhesive to prevent the Emzaloids from drying out.

3.6.5.2 Morphology of nanometer range Emzaloids

Confocal laser scanning microscopy (CLSM) was used to study the morphology of the Emzaloids. The same process as described in 3.6.5.1 was used to prepare the Emzaloids for viewing. Digital images were taken and stored.

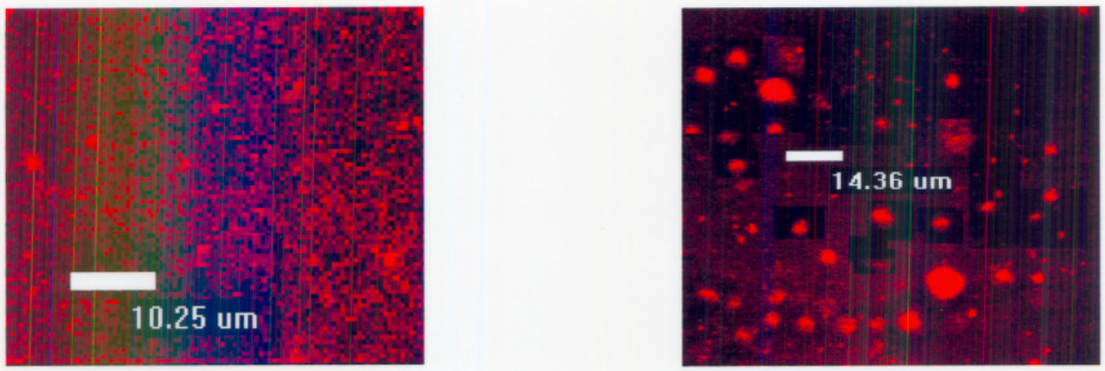
3.6.6 Results and discussion

3.6.6.1 Size of nanometer range Emzaloids

The size of the nanometer range Emzaloids was analysed by Confocal Laser Scanning Microscopy (CLSM). It was established that the Emzaloids are reasonably consistent in size, and the approximate size of the Emzaloids was found to be between 400 nm and 500 nm.

3.6.6.2 Morphology of nanometer range Emzaloids

The morphology of the nanometer range Emzaloids was analysed by Confocal Laser Scanning Microscopy (CLSM). As shown in figure 3.11 the Emzaloids are spherical in nature. It was seen that the diphtheria toxoid (DT) loading of Emzaloids caused a decrease in the quantity of Emzaloids in the preparation, as well as an increase in size of the Emzaloids in the preparation.



(A) (B)

Figure 3.11: Confocal Laser Scanning Micrographs of (A), unloaded nanometer range Emzaloids and (B), nanometer range Emzaloids loaded with diphtheria toxoid (DT).

3.7 LOADING, RELEASE AND STABILITY OF DIPHTHERIA TOXOID (DT) INTO CHITOSAN AND TMC MICROPARTICLES AND NANOPARTICLES

3.7.1 Materials

Diphtheria toxoid (DT) was a generous gift from Leiden University, The Netherlands. Chitosan microparticles, TMC microparticles and chitosan nanoparticles were prepared as described in section 3.2, 3.4 and 3.5. Phosphate buffered saline (PBS; pH 7.3) was freshly prepared for the experiment. The Lowry protein assay (BioRad Laboratries, Germany) was used to determine the loading capacity, loading efficacy and stability of loaded particles. It was not possible to determine the loading and release of micrometer and nanometer range Emzaloids with the Lowry protein assay.

3.7.2 Method

3.7.2.1 Diphtheria toxoid loading

Diphtheria toxoid (DT) loading of the particles was performed by incubating 1 % w/v of the particles and 0.25 % w/v (625 Lf) DT in phosphate buffered saline (PBS; pH 7.3) under shaking at 25 °C. After incubation for 180 min, the suspension was then centrifuged at 3000 rpm for 2 min to remove free, unloaded DT. The loading degree was determined by quantifying the non-bound DT in the supernatant with the Lowry protein assay method. Both loading capacity (LC) and loading efficacy (LE) were determined as follows:

LC = [(total amount DT)-(free DT)] / weight microparticles

LE = [(total amount DT)-(free DT)] / total amount DT

Diphtheria toxoid (DT) loading of micrometer and nanometer range Emzaloids was performed by incubating the Emzaloids and 0.25 % w/v (625 Lf) DT by shaking at 25 °C for 180 min. Diphtheria release from the Emzaloids could not be measured as the Emzaloids break when centrifuged.

3.7.2.2 Diphtheria toxoid release

DT release from the particles was determined in phosphate buffered saline (PBS; pH 7.3). After loading, the particles were resuspended in PBS (pH 7.3) to make a 1 % w/v particulate suspension. Samples of 1 ml were incubated at 37 °C under mild shaking. After 15, 30, 45, 60, 90, 120, 180, 240 min, the tubes were centrifuged at 3000 rpm for 2 min and samples of 250 µl were taken from the supernatant. These samples were replaced with PBS (pH 7.3). The non-bound DT was determined with the Lowry protein assay.

3.7.2.3 Stability studies

To determine the stability of the prepared particles over a period of 3 months, loaded particles were stored at 4 °C and ambient temperature (25 °C) as 1 % w/v suspensions in phosphate buffered saline (PBS; pH 7.3). Samples were taken from week 0 to week 13 after preparation. The degree of DT loading to particles and DT release from particles were determined as described in section 3.7.2.1 and 3.7.2.2.

3.7.3 Results and discussion

3.7.3.1 Loading, release and stability studies performed on chitosan microparticles

Table 3.3 gives an overview of the loading and release of diphtheria toxoid (DT) in chitosan microparticles. The loading capacity (LC) and loading efficacy (LE) of DT in chitosan microparticles were found to be 25.7 ± 0.44 % and 88.9 ± 1.52 % respectively. The release and stability studies showed that no DT was released from the chitosan microparticles over a period of 3 months, and that all the DT remained associated to the microparticles.

Table 3.3: Stability of DT-loaded chitosan microparticles at 4 °C and ambient temperature over a period of 13 weeks.

Temperature	Weeks	LE (%)	LC (%)	Release (%)
4 °C	0	88.9 ± 1.52	25.7 ± 0.44	0 ± 0
4 °C	13			0 ± 0
25 °C	0	88.9 ± 1.52	25.7 ± 0.44	0 ± 0
25 °C	13			0 ± 0

LE, loading efficacy; LC, loading capacity. Results are expressed as mean ± SD of 3 experiments

3.7.3.2 Loading, release and stability studies performed on TMC microparticles

Table 3.4 gives an overview of the loading and release of diphtheria toxoid (DT) in TMC microparticles. The loading capacity (LC) and loading efficacy (LE) of DT in TMC microparticles were found to be 18.3 ± 0.34 % and 63.1 ± 1.16 % respectively. The release and stability studies showed that no DT was released from the TMC microparticles over a period of 3 months, and that all the DT remained associated to the microparticles.

Table 3.4: Stability of DT-loaded TMC microparticles at 4 °C and ambient temperature over a period of 13 weeks.

Temperature	Weeks	LE (%)	LC (%)	Release (%)
4 °C	0	63.1 ± 1.16	18.3 ± 0.34	0 ± 0
4 °C	13			0 ± 0
25 °C	0	63.1 ± 1.16	18.3 ± 0.34	0 ± 0
25 °C	13			0 ± 0

LE, loading efficacy; LC, loading capacity. Results are expressed as mean ± SD of 3 experiments

3.7.3.3 Loading, release and stability studies performed on chitosan nanoparticles

Table 3.5 gives an overview of the loading and release of diphtheria toxoid (DT) in chitosan nanoparticles. The loading capacity (LC) and loading efficacy (LE) of DT in chitosan nanoparticles were found to be 8.03 ± 0.11 % and 27.74 ± 0.39 % respectively. The release and stability studies showed that no DT was released from the chitosan nanoparticles over a period of 3 months, and that all the DT remained associated to the nanoparticles.

Table 3.5: Stability of DT-loaded chitosan nanoparticles at 4 °C and ambient temperature over a period of 13 weeks.

Temperature	Weeks	LE (%)	LC (%)	Release (%)
4 °C	0	27.74 ± 0.39	8.03 ± 0.11	0 ± 0
4 °C	13			0 ± 0
25 °C	0	27.74 ± 0.39	8.03 ± 0.11	0 ± 0
25 °C	13			0 ± 0

LE, loading efficacy; LC, loading capacity. Results are expressed as mean ± SD of 3 experiments

3.8 CONCLUSION

In this chapter, chitosan and TMC microparticles, chitosan nanoparticles, micrometer range Emzaloids and nanometer range Emzaloids were prepared and characterised. These microparticles and nanoparticles were characterised based on their size, morphology, diphtheria toxoid (DT) loading and diphtheria toxoid release profiles. Only morphology and size determination studies were conducted on the prepared micrometer and nanometer range Emzaloids.

Chitosan microparticles were prepared by ionotropic gelation with sodium sulphate, which was found to be a reproducible method. Size analysis of the microparticles indicated that they have an average size distribution of d(0.1) 1.68 ± 0.23 μm, d(0.5) 2.57 ± 0.41 μm and d(0.9) 5.58 ± 1.3 μm. Morphology studies showed that chitosan microparticles are very porous in structure, which is a positive attribute, as the porosity could facilitate the entrapment of antigens into the particles.

A TMC polymer was synthesised by the reductive methylation of chitosan. The degree of quaternisation was calculated from the ^{1}H-NMR spectrum and was found to be 22.53 %. TMC microparticles were prepared by ionotropic gelation with TPP, which was found to be a reproducible method. Size analysis of the microparticles indicated that they have an average size distribution of d(0.1) 1.25 ± 0.26 μm , d(0.5) 2.06 ± 0.29 μm and d(0.9) 4.8 ± 0.04 μm. Morphology studies showed that TMC microparticles are less porous than chitosan microparticles.

Chitosan nanoparticles were prepared by ionotropic gelation with TPP, which was found to be a reproducible method. It was discovered that the chitosan nanoparticles show an inconsistency in size, and the approximate size of the nanoparticles was found to range from 50 nm to 450 nm. Morphology studies showed that chitosan nanoparticles appear to be amorphous in nature and are inclined to form aggregates due to electrostatic forces present between the particles.

Micrometer and nanometer range Emzaloids were also prepared for this study. CLSM was used to study the size and morphology of the Emzaloids. It was established that Emzaloids are consistent in size, and the average size was found to be 2 μm to 4 μm for micrometer range Emzaloids and 400 nm to 500 nm for nanometer range Emzaloids. Morphology studies showed that Emzaloids are spherical in nature, and it was seen that the DT loading of Emzaloids influences the quantity and size of the Emzaloids.

Loading, release and stability studies were carried out on chitosan and TMC microparticles as well as on chitosan nanoparticles to determine the loading capacity, loading efficacy and stability of particles. The loading capacity and efficacy of chitosan microparticles were notably higher than that of TMC microparticles due to the porosity of the chitosan microparticles. Moreover, the loading capacity and efficacy of both chitosan and TMC microparticles were higher than that of chitosan nanoparticles due to the small

size of the chitosan nanoparticles. Furthermore, the above preparations were very stable, as no DT release took place over a period of 3 months.

CHAPTER 4

IN VIVO EVALUATION OF CHITOSAN DERIVED FORMULATIONS AND EMZALOID™ TECHNOLOGY FOR NASAL VACCINATION AGAINST DIPHTHERIA

4.1 INTRODUCTION

The preparation and characterisation of chitosan microparticles, TMC microparticles, chitosan nanoparticles as well as micrometer and nanometer range Emzaloids for the entrapment and delivery of diphtheria toxoid (DT) via the nasal mucosa has been discussed in chapter 3.

In this chapter, the nasal efficacy of these formulations will be determined by conducting *in vivo* studies in mice. The mice will receive a total of 40 Lf DT associated to the different formulations. The mice will be vaccinated on three consecutive days in week 1 and week 3 receiving 6.67 Lf DT per day in a total volume of 10 µl/day (5 µl in each nostril). In weeks 4 and 6, 5 mice from each group will be bled and serum samples will be stored at -20 °C until analysis can be performed. After the mice have been bled in week 4 and week 6, nasal washing will be performed and the nasal lavages will be stored at -20 °C until analysis can be performed. The IgG titers (systemic immune response) and IgA titers (local immune response) will be determined using an enzyme linked immunosorbent assay (ELISA), which is a sensitive and specific assay widely used in the analysis of biological samples. The IgG titers as well as the IgA titers obtained with the ELISA assays will be statistically compared to the control groups by a Kruskal-Wallis ANOVA.

4.2 NASAL EFFICACY STUDIES IN MICE

4.2.1 Experimental procedure

4.2.1.1 Diphtheria toxoid loading

The diphtheria toxoid (DT) loading of the particulate formulations was performed as described in chapter 3 (section 3.7).

4.2.1.2 Experimental animals

Female SPF balb/c mice of 6 weeks old were used as experimental animals to study the nasal efficacy of the prepared particulate delivery systems against diphtheria. Balb/c mice are readily available, breed successfully and quickly in captivity and are easy to handle.

The female balb/c mice that were used in the study were kept in a closed, controlled environment at the Animal Research Centre of the North-West University. The animals were kept under controlled conditions to ensure an ideal environment for the good health of the animals. Exposure to pathogenic organisms were minimised by keeping the mice in a room with constant air circulation. They were fed Epol® mouse cubes (Epol, RSA).

The conditions to which the animals were exposed are controlled by the Animal Research Centre and are summarised in table 4.1.

Table 4.1: Environmental conditions at the Animal Research Centre at the North-West University.

Variable	Recommended value (international standard)	Value at Animal Research Centre, North-West University
Room temperature (°C)	18 - 24	21 ± 2
Relative humidity (%)	45 - 55	55 ± 15
Air changes per hour	15 - 20	18
Light cycle: hours on/ off	12/12	12/12
Acceptable background noise	Less than 50 db	Less than 50 db
Light intensity (lux one meter above ground)	350 - 400	350 – 400

4.2.1.3 Experimental animal groups

A total of 14 groups of mice were used for the nasal vaccination study. Each group consisted of 10 mice as 5 mice in every group had to be sacrificed in week 4 and week 6 to collect blood samples and nasal washings for the determination of IgG and IgA titers. DT associated to alum was injected subcutaneously as a positive control.

The following groups were vaccinated during the study:

I. Chitosan microparticles with 40 Lf DT
II. Negative control – non loaded chitosan microparticles
III. TMC microparticles with 40 Lf DT (TMC degree of quaternisation = 22.53 %)
IV. Negative control – non loaded TMC microparticles
V. Chitosan nanoparticles with 40 Lf DT
VI. Negative control – non loaded chitosan nanoparticles
VII. TMC solution with 40 Lf DT (TMC degree of quaternisation = 53 %)
VIII. Negative control – non loaded TMC solution
IX. Micrometer range Emzaloids with 40 Lf DT
X. Negative control – non loaded micrometer range Emzaloids
XI. Nanometer range Emzaloids with 40 Lf DT
XII. Negative control – non loaded nanometer range Emzaloids
XIII. Positive control – 40 Lf DT in phosphate buffered saline (PBS)
XIV. Positive control – 40 Lf DT associated to Alum (injected subcutaneously)

TMC with a high degree of quaternisation (53 %) used to prepare the TMC solution was a generous gift from the Department of Pharmaceutics at the North-West University, R.S.A, and was included in this study to investigate the effect of the degree of quaternisation of TMC on the immune response, as well as to determine which TMC formulation is the most effective formulation for nasal vaccination.

4.2.1.4 *In vivo* vaccination study in mice

For the nasal vaccination study, the mice were vaccinated on three consecutive days in week 1 and week 3 receiving 6.67 Lf DT per day in a total volume of 10 µl/day (5 µl in each nostril) with a micropipette. The mice were kept in a conscious state during vaccination.

4.2.1.4.1 Blood collection

In weeks 4 and 6, 5 mice from every group were decapitated and bled. Blood samples were taken from each mouse and centrifuged for 20 min at 14 000 rpm (Eppendorf 5415 C, Eppendorf, Germany). The obtained serum samples were stored a –20 °C until analysis for IgG titers was performed.

4.2.1.4.2 Nasal washing

The trachea of every decapitated mouse was canulated with a 0.5 × 1 mm PVC tube. Via this tube, 500 µl of PBS containing the protease inhibitor, PMSF (Sigma-Aldrich, Germany), was flushed through the nasal cavity and collected from the nostrils. This can be done when the head is placed in such a way that the nasal cavity is inferior to the oral cavity; so that most of the fluid passes through the nasal cavity. The nasal lavages were collected and stored at –20 °C until analysis for IgA titers was performed.

4.2.2 DT-specific IgG and IgA assays

4.2.2.1 Materials

Purified diphtheria toxoid (150 Lf/ml) was a generous gift from the University of Leiden, The Netherlands. The 96-well Microlon ELISA plates used for the assays were obtained from Greiner Bio One, Germany. The carbonate buffer was prepared from sodium hydrogen carbonate and sodium carbonate that were both obtained from Merck, Midrand, RSA. Bovine serum albumin (BSA), Tween® 20, peroxidase-conjugated anti-IgG and anti-IgA used during the assays were obtained from Sigma-Aldrich, Germany. 3,3', 5,5' tetramethylbenzidine (TMB) was obtained from Sigma-Aldrich, Germany. All other reagents used during the assays were of analytical grade.

4.2.2.2 Method

DT-specific IgG and IgA antibodies were measured by an adapted enzyme-linked immunosorbent assay (ELISA) method. DT (1 Lf/ml) in 0.04 M carbonate buffer (pH 9.6) was absorbed to 96-wells plates by overnight incubation at 20 °C. Plates were washed four times before use with a PBS and 0.1 % Tween® 20 mixture. Two-fold serial dilutions of samples were done, using a mixture of PBS, 0.5 % BSA and 0.1 % Tween® 20 as diluent. These samples were then incubated for 2 hours at ambient temperature. The plates were washed four times with a PBS and 0.1 % Tween® 20 mixture and peroxidase-conjugated anti-IgG was added for determination of IgG antibodies, while peroxidase-conjugated anti-IgA was added for determination of IgA antibodies. The plates were incubated for 2 hours at ambient temperature and subsequently washed four times with PBS and 0.1 % Tween® 20. Then 100 µl of a tetramethylbenzidine (TMB) solution (6 mg/ml) in 96 % ethanol was added to each well. After 15 min, the reaction was stopped by adding 50 µl of 2M H_2SO_4 to each well. The optical density (OD) of the samples was measured at 450 nm and the IgG and IgA titers of the samples were calculated from dilutions with an OD of between 0.25 and 0.3.

4.2.3 Results and discussion

4.2.3.1 Systemic immune responses (IgG)

Figures 4.1 and 4.2 as well as table 4.2 depict the IgG titers of chitosan-derived formulations and Emzaloid-derived formulations compared to PBS and alum in weeks 4 and 6.

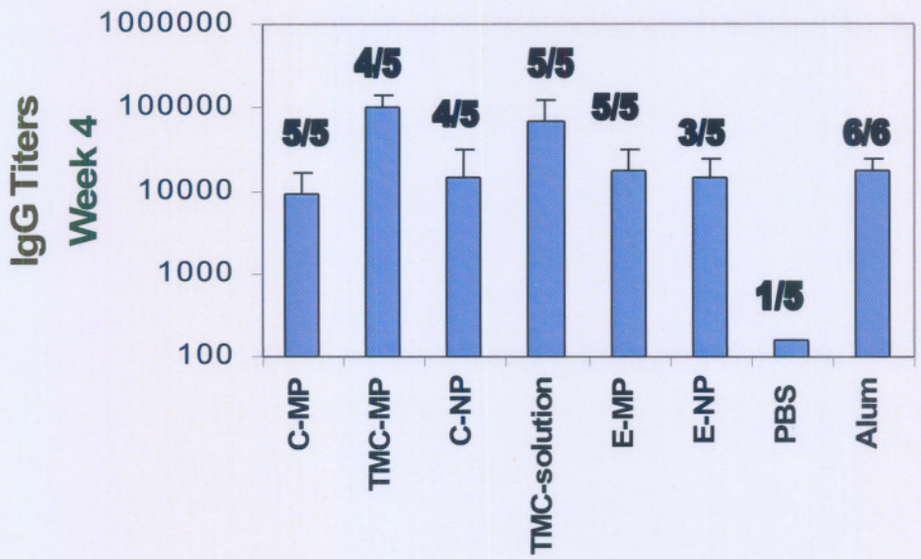


Figure 4.1: Serum IgG titers at 4 weeks after nasal vaccination with 40 Lf DT associated to chitosan derived formulations and Emzaloids compared to the positive controls of 40 Lf DT in PBS and 40 Lf DT associated to alum. Antibody titers are expressed as the mean ± SD. The numbers above the columns represent the number of sera giving a positive response per number of mice tested.

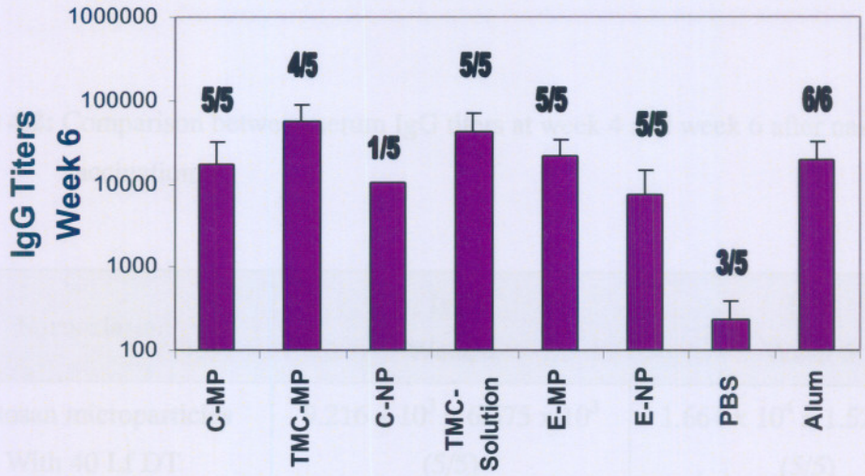


Figure 4.2: Serum IgG titers at 6 weeks after nasal vaccination with 40 Lf DT associated to chitosan derived formulations and Emzaloids compared to the positive controls of 40 Lf DT in PBS and 40 Lf DT associated to alum. Antibody titers are expressed as the mean ± SD. The numbers above the columns represent the number of sera giving a positive response per number of mice tested.

Table 4.2: Comparison between serum IgG titers at week 4 and week 6 after nasal vaccination.

Formulation	IgG Week 4	IgG Week 6
Chitosan microparticles With 40 Lf DT	$9.216 \times 10^3 \pm 6.675 \times 10^3$ (5/5)	$1.664 \times 10^4 \pm 1.520 \times 10^4$ (5/5)
TMC microparticles With 40 Lf DT	$1.024 \times 10^5 \pm 4.096 \times 10^4$ (4/5)	$5.632 \times 10^4 \pm 3.072 \times 10^4$ (4/5)
Chitosan nanoparticles With 40 Lf DT	$1.408 \times 10^4 \pm 1.828 \times 10^4$ (4/5)	1.024×10^4 (1/5)
TMC-solution with 40 Lf DT	$6.656 \times 10^4 \pm 6.079 \times 10^4$ (5/5)	$4.198 \times 10^4 \pm 2.719 \times 10^4$ (5/5)
Micrometer range Emzaloids with 40 Lf DT	$1.741 \times 10^4 \pm 1.429 \times 10^4$ (5/5)	$2.099 \times 10^4 \pm 1.359 \times 10^4$ (5/5)
Nanometer range Emzaloids With 40 Lf DT	$1.451 \times 10^4 \pm 1.034 \times 10^4$ (3/5)	$7.168 \times 10^3 \pm 7.550 \times 10^3$ (5/5)
PBS with 40 Lf DT	160 (1/5)	240 ± 138 (3/5)
Alum with 40 Lf DT	$1.792 \times 10^4 \pm 6.27 \times 10^3$ (6/6)	$1.963 \times 10^4 \pm 1.229 \times 10^4$ (6/6)

Data is expressed as mean ± SD and the numbers in brackets represent the fraction of positive responders

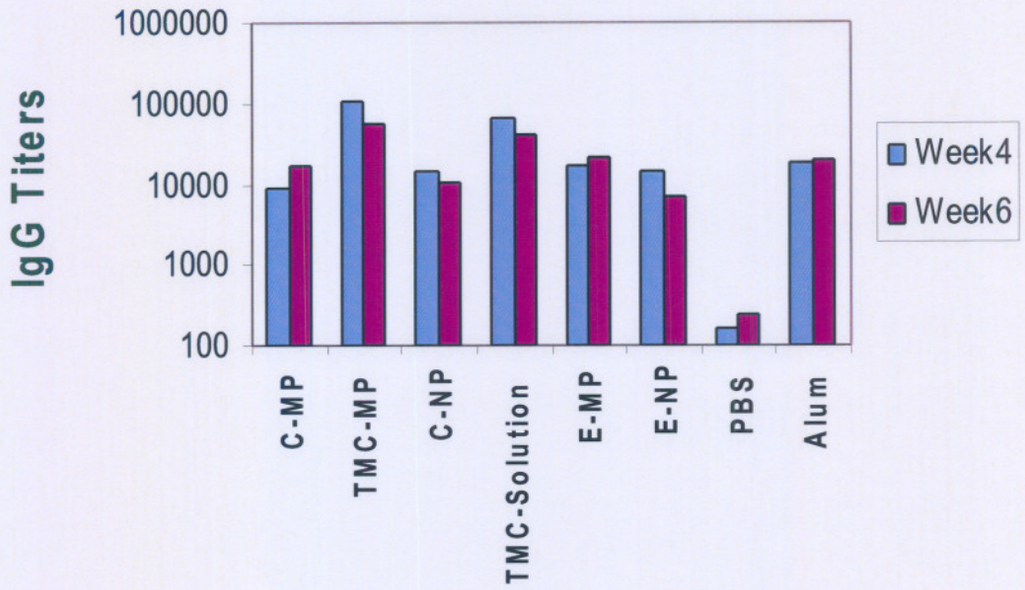


Figure 4.3: Comparison of serum IgG titers at weeks 4 and 6 after nasal vaccination with 40 Lf DT associated to chitosan derived formulations and Emzaloid derived formulations compared to the positive controls of 40 Lf DT in PBS and 40 Lf DT associated to alum.

The nasal vaccination study was performed to assess the efficacy of chitosan derived formulations, namely chitosan microparticles and nanoparticles, TMC microparticles and solution, as well as micrometer and nanometer Emzaloid formulations. It was clear that DT associated to all the above mentioned formulation produced a systemic immune response in mice. When comparing the formulations to the positive controls, it was apparent that the mice vaccinated with DT associated to these formulations produced significantly more systemic anti-DT IgG than mice vaccinated with DT in PBS. Furthermore, the mice vaccinated with DT associated to the TMC formulations showed a much higher immune response than the mice that were vaccinated subcutaneously with DT associated to alum, while the other formulations produced systemic immune responses that are similar to that of DT associated to alum.

The IgG titers of all the experimental formulations were statistically compared to each other as well as to the control groups by means of a Kruskal-Wallis ANOVA test. According to this test, only the TMC microparticles and TMC solution differed statistically from DT in PBS in week 4. In week 6, the TMC solution differed statistically from both DT in PBS and chitosan nanoparticles. These statistical results can however not be considered credible, as the titers indicate that all the experimental groups should differ statistically from DT in PBS. The inaccurate statistical validation of the titers can be attributed to the fact that the experimental animal groups were too small to produce credible results.

4.2.3.2 Local immune responses (IgA)

Figures 4.4 and 4.5 as well as table 4.3 depict the IgA titers of chitosan-derived formulations and Emzaloid derived formulations compared to PBS and alum in weeks 4 and 6.

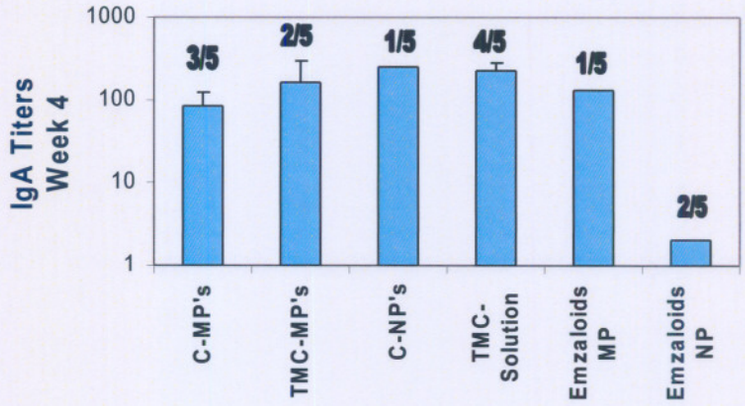


Figure 4.4: IgA titers at 4 weeks after nasal vaccination with 40 Lf DT associated to chitosan derived formulations and Emzaloid derived formulation compared to the positive controls of 40 Lf DT in PBS and 40 Lf DT associated to alum. Antibody titers are expressed as the mean ± SD. The numbers above the columns represent the number of sera giving a positive response per number of mice tested.

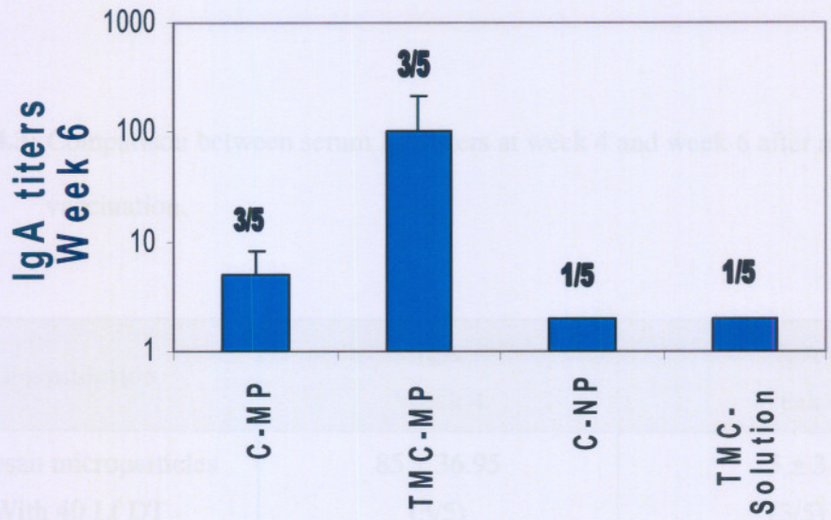


Figure 4.5: IgA titers at 6 weeks after nasal vaccination with 40 Lf DT associated to chitosan derived formulations and Emzaloid derived formulations compared to the positive controls of 40 Lf DT in PBS and 40 Lf DT associated to alum. Antibody titers are expressed as the mean ± SD. The numbers above the columns represent the number of sera giving a positive response per number of mice tested.

Table 4.3: Comparison between serum IgA titers at week 4 and week 6 after nasal vaccination.

Formulation	IgA Week 4	IgA Week 6
Chitosan microparticles With 40 Lf DT	85 ± 36.95 (3/5)	5 ± 3 (3/5)
TMC microparticles With 40 Lf DT	160 ± 135.76 (2/5)	102 ± 116.37 (4/5)
Chitosan nanoparticles With 40 Lf DT	256 (1/5)	2 (1/5)
TMC-solution with 40 Lf DT	224 ± 64 (4/5)	2 (1/5)
Micrometer range Emzaloids with 40 Lf DT	128 (1/5)	0
Nanometer range Emzaloids with 40 Lf DT	2 (2/5)	0
PBS with 40 Lf DT	0	0
Alum with 40 Lf DT	0	0

Data is expressed as mean ± SD and the numbers in brackets represent the fraction of positive responders

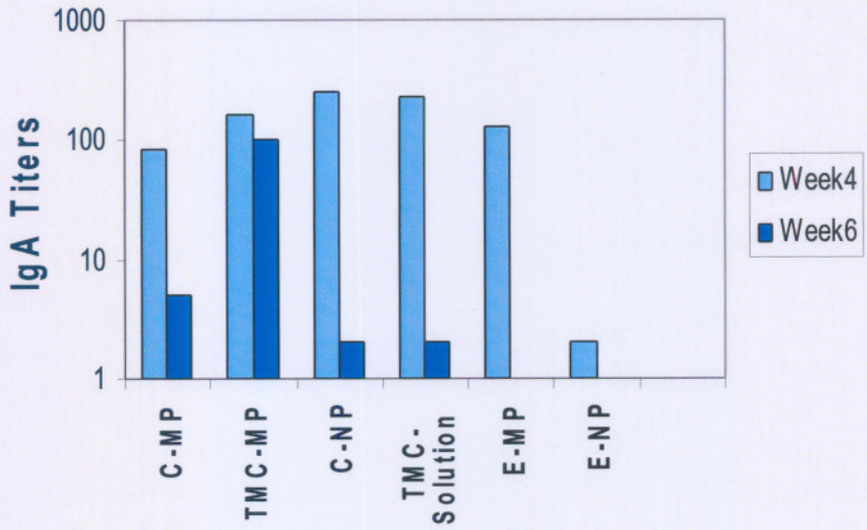


Figure 4.6: Comparison of serum IgA titers at weeks 4 and 6 after nasal vaccination with 40 Lf DT associated to chitosan derived formulations and Emzaloid derived formulations compared to the positive controls of 40 Lf DT in PBS and 40 Lf DT associated to alum.

In this study it was seen that DT associated to the chitosan derived formulations, namely chitosan microparticles and nanoparticles, TMC microparticles and solution, as well as micrometer and nanometer Emzaloid formulations produced a local immune response in mice in week 4, whereas only the chitosan derived formulations still produced a local immune response in mice in week 6. Moreover, when comparing the IgA titers of these formulations in weeks 4 and 6, it can be seen that only the DT associated to TMC microparticles produced a consistent local immune response in week 4 and 6 as all the IgA titers of the other formulations decreased in week 6. When comparing the experimental formulations to the positive controls, namely DT in PBS and DT associated to alum, it was seen that only the mice vaccinated with DT associated to the experimental formulations produced mucosal anti-DT IgA.

The IgA titers of all the experimental formulations were statistically compared to each other as well as to the control groups by means of a Kruskal-Wallis ANOVA test.

According to this test, there were no statistically significant differences between any of the groups in week 4 and week 6. These statistical results can however not be considered to be credible, as the titers indicate that there should be statistical differences between the groups. The inaccurate statistical validation of the titers can be attributed to the fact that the experimental animal groups were too small to produce credible results.

4.3 CONCLUSION

In this chapter, the nasal vaccination efficacy of chitosan microparticles and nanoparticles, TMC microparticles as well as micrometer and nanometer range Emzaloids were determined by conducting *in vivo* studies in mice. The mice were vaccinated on three consecutive days in week 1 and week 3, receiving 6.67 Lf DT per day in a total volume of 10 µl/day. In weeks 4 and 6, the mice were bled and serum samples were collected and analysed. After the mice were bled, nasal washing was performed and nasal lavages were collected and analysed. The IgG titers (systemic immune response) and IgA titers (local immune response) were determined using an enzyme linked immunosorbent assay (ELISA).

In the study conducted to determine the systemic immune response, it was seen that DT associated to all the above mentioned formulations produced a systemic immune response. When these formulations were compared to the positive controls, it was seen that the mice vaccinated with DT associated to these formulations produced a significantly higher immune response than the mice vaccinated with DT in PBS. Furthermore, the mice vaccinated with DT associated to the TMC formulations showed a much higher immune response than the mice that were vaccinated subcutaneously with DT associated to alum, whereas the other formulations produced systemic immune responses that were comparable to that of DT associated to alum.

In the study conducted to determine the local immune response, it was seen that DT associated to the above mentioned formulations produced a local immune response in week 4, whereas only the chitosan derived formulations still produced a local immune response in week 6. Furthermore, it was seen that only DT associated to TMC microparticles produced a consistent local immune response.

By studying these results, it can be concluded that the TMC formulations, moreover, the TMC microparticles seem to be the most effective formulation for nasal vaccination, although the Emzaloid studies should be repeated as the Emzaloids that were used for this study were not buffered and therefore may have had a low pH, which could have had an effect on the stability of the diphtheria toxoid, as it is unstable in acidic environments.

CHAPTER 5

SUMMARY AND FUTURE PROSPECTS

The two major physiological barriers that limit the mucosal delivery of protein and peptide drugs as well as vaccines are the enzymatic and epithelial barriers. Due to their physico-chemical properties and high molecular weight, the absorption of antigens across mucosal surfaces is limited. However, some polymers such as chitosan, *N*-trimethyl chitosan chloride (TMC) and Emzaloid systems permit for the transport of antigens after nasal administration. These delivery systems are able to associate large amounts of vaccines and enhance the uptake of antigens by the nasal associated lymphoid tissue (NALT). Because of their cheap production costs, biocompatibility and low toxicity, these delivery systems are very promising nasal vaccine carriers.

In this study, the ability of chitosan microparticles and nanoparticles, TMC microparticles as well as micrometer and nanometer range Emzaloids to enhance both the systemic and mucosal (local) immune response against diphtheria toxoid (DT), after nasal administration, in mice was investigated.

Chitosan microparticles were prepared by ionotropic gelation with sodium sulphate, which was found to be a reproducible method. Size analysis of the microparticles indicated that they have an average size distribution of d(0.1) 1.68 ± 0.23 μm , d(0.5) 2.57 ± 0.41 μm and d(0.9) 5.58 ± 1.3 μm. Morphology studies showed that chitosan microparticles are very porous in structure, which is a positive attribute, as the porosity could facilitate the entrapment of antigens into the particles.

A TMC polymer was synthesised by the reductive methylation of chitosan. The degree of quaternisation was calculated from the ^{1}H-NMR spectrum and found to be 22.53 %. TMC microparticles were prepared by ionotropic gelation with TPP, which was also found to be a reproducible method. Size analysis of the microparticles indicated that they have an average size distribution of d(0.1) 1.25 ± 0.26 μm , d(0.5) 2.06 ● 0.29 μm and d(0.9) 4.8 ± 0.04 μm. Morphology studies showed that TMC microparticles are less porous than chitosan microparticles.

Chitosan nanoparticles were prepared by ionotropic gelation with TPP. It was observed that the chitosan nanoparticles show an inconsistency in size, and the approximate size of the nanoparticles was found to range from 50 nm to 450 nm. Morphology studies showed that chitosan nanoparticles appear to be amorphous in nature and are inclined to form aggregates due to electrostatic forces present between the particles.

Micrometer and nanometer range Emzaloids were also prepared for this study. Confocal scanning laser microscopy (CLSM) was used to study the size and morphology of the Emzaloids. It was established that Emzaloids are consistent in size, and the average size was found to be 2 μm to 4 μm for micrometer range Emzaloids and 400 nm to 500 nm for nanometer range Emzaloids. Morphology studies showed that Emzaloids are spherical in nature, and it was seen that the DT loading of Emzaloids influences the quantity and size of the Emzaloids.

Loading, release and stability studies were carried out on chitosan and TMC microparticles as well as on chitosan nanoparticles to determine the loading capacity, loading efficacy and stability of particles. The loading capacity and efficacy of chitosan microparticles were notably higher than that of TMC microparticles due to the porosity of the chitosan microparticles. Moreover, the loading capacity and efficacy of both chitosan and TMC microparticles were higher than that of chitosan nanoparticles due to the small

size of the chitosan nanoparticles. Furthermore, the above preparations were very stable, as no DT release took place over a period of 3 months.

DT loaded and unloaded (empty) chitosan microparticles and nanoparticles, TMC microparticles, micrometer and nanometer range Emzaloids as well as DT in phosphate buffered saline (PBS) were administered nasally to mice. Mice were also vaccinated subcutaneously with DT associated to alum as a positive control. All mice were vaccinated in week 1 and boosted in week 3. Sera was analysed for anti-DT IgG and nasal lavages were analysed for anti-DT IgA using an enzyme linked immunosorbent assay (ELISA).

In the study conducted to determine the systemic (IgG) and local (IgA) immune responses it was seen that DT associated to all the experimental formulations produced a systemic immune response. The said formulations produced a significantly higher systemic immune response when compared to the formulation of DT in PBS. Furthermore, the mice vaccinated with DT associated to the TMC formulations showed a much higher systemic immune response than the mice that were vaccinated subcutaneously with DT associated to alum, whereas the other formulations produced systemic immune responses that were comparable to that of DT associated to alum. It was seen that DT associated to the experimental formulations produced a local immune response, however only DT associated to TMC microparticles produced a consistent local immune response.

It can be concluded from the *in vivo* experiments that the TMC formulations, moreover, the TMC microparticles is the most effective and promising formulation for the nasal delivery of vaccines, however the following recommendations should be considered when future studies are performed:

1) The Emzaloid study should be repeated, as the Emzaloids that were used for this study were not buffered and therefore may have had a low pH, which could have

had an effect on the stability of the diphtheria toxoid, as it is unstable in acidic environments.

2) Bigger experimental animals should be used as it is difficult to vaccinate mice nasally and the amount of formulation administered to the mice could not be controlled. Previous vaccination studies conducted by Suckow *et al.,* (2002:228) using rabbits as experimental animals have proven to be very successful as blood and nasal lavages can be collected and analysed more frequently and therefore a more accurate study can be conducted.
3) Bigger experimental animal groups should be used so that accurate statistical evaluation of *in vivo* results may be possible.
4) Further research needs to be done to determine whether these vaccine delivery systems can be used for other commonly used antigens.
5) Future studies should be performed to determine whether these delivery systems are also effective for oral vaccination in mice.

LIST OF FIGURES

LIST OF TABLES

REFERENCES

ANDRÉ, F.E. 2003. Vaccinology: past achievements, present roadblocks and future promises. **Vaccine,** 21:593-595.

ARORA, P., SHARMA, S., GARG, S. 2002. Permeability issues in nasal drug delivery. **Drug discovery today,** 7:967-975.

AUDIBERT, F. 2003. Adjuvants for vaccines, a quest. **International Immunopharmacology,** 3:1187-1193.

BEHL, C.R., PIMPLASKAR, H.K., SILENO, A.P., DEMEIRELES, J., ROMEO, V.D. 1998. Effects of physiochemical properties and other factors on systemic nasal drug delivery. **Advanced drug delivery reviews,** 29:89-116.

CHEN, H. 2000. Recent advances in mucosal vaccine development. **Journal of controlled release,** 67:117-128.

CONLAN, J.W., KRISHNAN, L., WILLICK, G.E., PATEL, G.B., SPROTT, G.D. 2001. Immunization of mice with lipopeptide antigens encapsulated in novel liposomes prepared from the polar lipids of various *Archaeobacteria* elicits rapid and prolonged specific protective immunity against infection with the facultative intracellular pathogen, *Listeria monocytogenes*. **Vaccine,** 19:3509-3517.

CUSI, M.G., GLÜCK, R. 2000. Potential of DNA vaccines delivered by influenza virosomes. **Vaccine,** 18:1435.

DAVIS, SS. 2001. Nasal vaccines. **Advanced drug delivery reviews**, 51:21-42.

DEL GIUDICE, G. 2003. Vaccination strategies, an overview. **Vaccine,** 21:S2/83-S2/88.

DODANE, V., VILIVALAM, V.D. 1998. Pharmaceutical applications of chitosan. **Pharmaceutical science and technology today,** 1:246-253.

GALAMBOS, L. 1999. A century of innovation in vaccines. **Vaccine,** 17:S7-S10.

GLÜCK, R., METCALFE, I.C. 2002. New technology platforms in the development of vaccines for the future. **Vaccine,** 20:B10-B16.

GROBLER, A.F. 2004. Emzaloid™ Technology. Potchefstroom: North-West University. 20p. **(Confidential: Concept document).**

GUPTA, R.K. 1998. Aluminum compounds as vaccine adjuvants. **Advanced drug delivery reviews,** 32:155-172.

HAMMAN, J.H., STANDER, M., KOTZÉ, A.F. 2002. Effect of the degree of quaternisation of *N*-Trimethyl chitosan chloride on absorption enhancement; *in vivo* evaluation in rat nasal epithelia. **International journal of pharmaceutics,** 2002:235-242.

HANES, J., CLELAND, J.L., LANGER, R. 1997. New advances in microsphere-based single-dose vaccines. **Advanced drug delivery reviews,** 28:97-119.

HEATH, A.W. 2001. Recent advances in vaccinology. **Current opinion in pharmacology,** 1:425-430.

HEJAZI, R., AMIJI, M. 2003. Chitosan based gastrointestinal delivery systems. **Journal of controlled release,** 89:151-165.

HILLEMAN, M.R. 2000. Vaccines in historic evolution and perspective: a narrative of vaccine discovery. **Vaccine,** 18:1436-1447.

HINCHCLIFFE, M., ILLUM, L. 1999. Intranasal insulin delivery and therapy. **Advanced drug delivery reviews,** 35:199-234.

HOGENESCH, H. 2002. Mechanisms of stimulation of the immune response by aluminum adjuvants. **Vaccine,** 20:S34-S39.

ILLUM, L. 2002. Nasal drug delivery: new developments and strategies. **Drug discovery today,** 7:1184-1189.

ILLUM, L. 2003. Nasal drug delivery – possibilities, problems and solutions. **Journal of controlled release,** 87:187-198.

ILLUM, L., DAVIS, S.S. 2001. Nasal vaccination: a non-invasive vaccine delivery method that holds great promise for the future. **Advanced drug delivery reviews,** 51:1-3.

ILLUM, L., FARRAJ, N.F., DAVIS, S.S. 1994. Chitosan as novel nasal delivery system for peptide drugs. **Pharmaceutical Research,** 11:1186-1189.

ILLUM, L., JABBAL-GILL, I., HINCHCLIFFE, M., FISHER, A.N., DAVIS, S.S. 2001. Chitosan as a novel nasal delivery system for vaccines. **Advanced drug delivery reviews,** 51:81-96.

JONKER, C., HAMMAN, J.H., KOTZÉ, A.F. 2002. Intestinal paracellular permeation enhancement with quaternised chitosan: *in situ* and *in vitro* evaluation. **International journal of pharmaceutics,** 238:205-213.

JUNGINGER, H.E., VERHOEF, J.C. 1998. Macromolecules as safe penetration enhancers for hydrophilic drugs- a fiction? **Pharmaceutical science and technology today,** 1: 370-376.

KOTZÉ, A.F., LUEßEN, H.L., DE LEEUW, B.J., DE BOER, A.G., VERHOEF, J.C., JUNGINGER, H.E. 1997b. Chitosans for enhanced delivery of therapeutic peptides across intestinal epithelia: *in vitro* evaluation of Caco-2 cell monolayers. **International journal of pharmaceutics,** 159:243-253.

LECLERC, C. 2003. New approaches in vaccine development. **Comparative immunology, microbiology & infectious diseases,** 26:329:341.

MARCIANI, D.J. 2003. Vaccine adjuvants: role and mechanisms of action in vaccine immunogenicity. **Drug discovery today,** 8:934-943.

MARTTIN, E., SCHIPPER, N.G.M., VERHOEF, J.C., MERKUS, F.W.H.M. 1998. Nasal mucociliary clearance as a factor in nasal drug delivery. **Advanced drug delivery reviews,** 29:13-38.

MISCHLER, R., METCALFE, I.C. 2002. Inflexal®V a trivalent virosome subunit influenza vaccine production. **Vaccine,** 20:B17-B23.

MITRA, A.K., KRISHNAMOORTHY, R. 1998. Prodrugs for nasal vaccination. **Advanced drug delivery reviews,** 29:135-146.

MORA, M., VEGGI, D., SANTINI, L., PIZZA, M., RAPPUOLI, R. 2003. Reverse vaccinology. **Drug discovery today,** 8:459-464.

MOR, G. 1998. Plasmid DNA: A new era in vaccinology. **Biochemical pharmacology,** 55:1151-1153.

PARTIDOS, C.D. 2000. Intranasal vaccines: forthcoming challenges. **Pharmaceutical science and technology today,** 3:273-281.

PAUL, W., SHARMA, C.P. 2000. Chitosan, a drug carrier for the 21st century: a review. **S.T.P. Pharma sciences,** 10:5-22.

POLAND, G.A. 1999. Current paradoxes and changing paradigms in vaccinology. **Vaccine,** 17:1605-1611.

POLNOK, A., BORCHARD, G., VERHOEF, J.C., SARISUTA, N., JUNGINGER, H.E. 2004. Influence of methylation process on the degree of quaternization of *N*-trimethyl chitosan chloride. **European journal of pharmaceutics an biopharmaceutics,** 57:77-83.

SÁNCHEZ, A., VILLAMAYOR, B., GUO, Y., MCIVER, J., ALONSO, MJ. 1999. Formulation strategies for the stabilization of tetanus toxoid in poly(lactide-co-glycolide) microspheres. **International journal of pharmaceutics,** 185:255-266.

SAUNDERS, J.C.J., DAVIS, H.J., COETZEE, L., BOTHA, S., KRUGER, A.E., GROBLER, A.F. 1999. A novel penetration enhancer: evaluation by membrane diffusion and confocal microscopy. **Journal of pharmaceutical sciences,** 2:99-107.

SEFERIAN, P.G., MARTINEZ, M.L. 2001. Immune stimulating activity of two new chitosan containing adjuvant formulations. **Vaccine,** 19:661-668.

SHROFF, K.E., SMITH, L.R., BAINE, Y., HIGGINS, T.J. 1999. Potential for plasmid DNAs as vaccines for the new millennium. **Pharmaceutical Science & Technology Today,** 2:205-212.

SIEVEL. A.B., THANOU, M., KOTZÉ, A.F., VERHOEF, J.C., BRUSSEE, J., JUNGINGER, H.E. 1998. Preparation and NMR characterisation of highly substituted *N*-trimethyl chitosan chloride. **Carbohydrate polymers,** 36:157-165.

SINHA, V.R., SINGLA, A.K., WADHAWAN, S., KAUSHIK, R., KUMRIA, R., BANSAL, K., DHAWAN, S. 2004. Chitosan microspheres as potential carriers for drugs. **International journal of pharmaceutics,** 274:1-33.

SNYMAN, D., HAMMAN, J.H., KOTZÉ, A.F. 2003. Evaluation of the mucoadhesive properties of N-Trimethyl chitosan chloride. **Drug development and industrial pharmacy,** 29:61-69.

SUCKOW, M.A., JARVINEN, L.Z., HOGENESH, H., PARK, K., BOWERSOCK. T.L. 2002. Immunization of rabbits against a bacterial pathogen with an alginate microparticle vaccine. **Journal of controlled release,** 85:227-235.

THANOU, M., VERHOEF, J.C., JUNGINGER, H.E. 2001. Chitosan and its derivatives as intestinal absorption enhancers. **Advanced drug delivery reviews,** 50:S91-S101.

USONIS, V., BAKASÉNAS, V., VALENTELIS, R., KATILIENE, G., VIDZENIENE, D., HERZOG, C. 2003. Antibody titers after primary and booster vaccination of infants and young children with a virosomal hepatitis A vaccine (Epaxal®). **Vaccine,** 21:4588-4592.

VAN DER LUBBEN, I.M., KERSTEN, G., FRETZ, M.M., BEUVERY, C., VERHOEF, J.C., JUNGINGER, H.E. 2003. Chitosan microparticles for mucosal vaccination against Diphtheria: oral and nasal efficacy studies in mice. **Vaccine,** 21:1400-1408.

VAN DER LUBBEN, I.M., VERHOEF, J.C., BORCHARD, G., JUNGINGER, H.E. 2001. Chitosan and its derivatives in mucosal drug and vaccine delivery. **European journal of pharmaceutical sciences,** 14:201-207.

VAN DER LUBBEN, I.M., VERHOEF, J.C., BORCHARD, G., JUNGINGER, H.E. 2001. Chitosan for mucosal vaccination. **Advanced drug delivery reviews,** 52:139-144.

VAN DER MERWE, S.M., VERHOEF, J.C., VERHEIJDEN, J.H.M., JUNGINGER, H.E. 2004. Trimethylated chitosan as polymeric absorption enhancer for improved peroral delivery of peptide drugs. **European journal of pharmaceutics and biopharmaceutics,** 58:225-235.

VILA, A., SÁNCHEZ, A., JANES, K., BEHRENS, I., KISSEL, T., JATO, J.L.V., ALONSO, M.J. 2004. Low molecular weight chitosan nanoparticles as new carriers for nasal vaccine delivery in mice. **European journal of pharmaceutics and biopharmaceutics,** 57:123-131.

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