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

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Abstract

Vaccination plays a very important part in daily life. It is essential to get vaccinated at an early age. The conventional parenteral method used is not always effective and not cost efficient. It requires qualified personnel and sterile conditions for administration of the vaccines.

The aim of this study was to investigate the effect of chitosan, *N*-trimethyl chitosan chloride (TMC) and Emzaloid™ particles on the local and systemic immune response of mice after oral vaccination with Diphtheria toxoid (DT). The different formulations used were chitosan microparticles (± 10 µm), chitosan nanoparticles (± 400 nm), TMC microparticles (± 5 µm), Emzaloid microparticles (± 4 µm) and Emzaloid nanoparticles (± 500 nm). All of these formulations proved to be very good delivery systems and can entrap large amounts of the antigen.

Balb/c mice were used to determine the local and systemic immune response of these formulations. The mice were vaccinated orally on three consecutive days in week 1 and 3 with 40 Lf DT per week with a total volume of 300 µl. Blood samples were taken from the mice and analysed for a systemic immune response (IgG). The same mice were used to determine the local immune response (IgA). Faeces were collected from each mouse on day 1, 3, 4, 6, 14 and 20 for analysis. An enzyme-linked immunosorbent assay (ELISA) was used to determine IgG and IgA titers.

It can be concluded that chitosan nanoparticles was the only formulation with a higher response than that of the currently used vaccine. Emzaloid nanoparticles showed no significant difference in response when compared to the currently used vaccine. All the other formulations showed a much smaller response than that of the conventional method of vaccination.

Key words: Oral vaccination, Chitosan microparticles, Chitosan nanoparticles, *N*-trimethyl chitosan chloride (TMC) microparticles, Emzaloid microparticles, Emzaloid nanoparticles, Diphtheria toxoid, ELISA assay.

Uittreksel

Vaksinering speel 'n belangrike rol in elke mens se lewe. Dit is noodsaaklik dat 'n mens reeds sedert sy vroeë kinderjare vaksienes moet ontvang. Konvensionele parenterale vaksinering is nie altyd effektief of koste effektief nie. Parenterale vaksinering benodig opgeleide personeel en behels die gebruik van steriele preperate en tegnieke.

Die doel van hierdie studie was om ondersoek in te stel na die effektiwiteit van kitosaan, *N*-trimetiel kitosaan chloried (TMC) en Emzaloid mikrodeeltjies op die lokale en sistemiese immuun respons van muise na vaksinering met difterie toksoïed (DT). Kitosaan mikrodeeltjies (10 μm), kitosaan nanodeeltjies (50 – 450 nm), TMC mikrodeeltjies (5 μm), Emzaloid mikrodeeltjies (5 μm) en Emzaloid nanodeeltjies (400 nm) is die formules wat in hierdie studie getoets is. Al hierdie formules was in staat om genoegsame hoeveelhede van die antigeen te enkapsuleer.

Balb/c muise is gebruik om die lokale en sistemiese immuunrespons van die formules te bepaal. Die muise is oraal gevaksineer op 3 opeenvolgende dae in week 1 en 3 met DT (40 Lf DT totaal). Bloedmonsters is van die muise geneem en geanaliseer vir die sistemiese immuunrespons (IgG). Dieselfde muise is ook gebruik om die lokale immuunrespons (IgA) te bepaal deur feses monsters te neem op dag 1, 3, 4, 6, 14 en 20. 'n ELISA analise metode is gebruik om die IgG en IgA vlakke te bepaal.

Hierdie studie het aangetoon dat kitosaan nanodeeltjies die enigste formule was met 'n beter immuunrespons as die bestaande gebruikte vaksien. Die formule wat Emzaloid nanodeeltjies bevat het dieselfde effek getoon as die bestaande gebruikte vaksien. Al die ander formules het laer immuunrespons getoon as die bestaande gebruikte vaksien.

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Introduction and Aim of study

Immunisation is the most cost-effective strategy to prevent needless morbidity and mortality. The currently used vaccines are mostly intended for parenteral use and require qualified personnel for administration of the vaccines and it must also be kept sterile. The currently used vaccines give only cellular protection and to date there has not been a potent enough oral vaccine that also gives humoral protection.

Chitosan easily forms micro- and nanoparticles that can encapsulate large amounts of antigens such as ovalbumin, diphtheria toxoid or tetanus toxoid. In previous studies it was shown that the loaded particles are taken up by the Peyer's patches of the gut associated lymphoid tissue (GALT). The particles also have the ability to open the tight junctions and can therefore be absorbed in the lymphoid tissue. These result in the enhancement of systemic and mucosal immune responses after oral administration. Chitosan can also be formulated in different sizes and therefore can target different regions in the intestine. The microparticles must be smaller than 10 μm to be taken up by the M-cells of the Peyer's patches. Nanoparticles are smaller than 500 nm and this allows even greater absorption of the particles.

Emzaloid™ is also a very promising vaccine delivery system and can also entrap large amounts of antigen. The formulations of the Emzaloid™ are much more cost and time effective than that of particles of chitosan and chitosan derivatives.

The objectives of this study were to:

1. Conduct a literature study on oral vaccination.
2. Conduct a literature study on chitosan, *N*-trimethyl chitosan chloride (TMC) and Emzaloid™ as absorption enhancers and to determine their role in vaccination.
3. Prepare and characterise chitosan, TMC and Emzaloid™ particles for use in a vaccination study.

4. Conduct an *in vivo* vaccination study in mice with diphtheria toxoid to compare the effectiveness of the chitosan, TMC and Emzaloid™ particles in prolonging a local and humoral immune response.

Chapter one contains current information regarding vaccination whilst Chapter two describes the properties of chitosan, TMC and Emzaloid™ particles. Chapter three focuses on the preparation and characterisation of chitosan, TMC and Emzaloid™ particles and Chapter four describes vaccination studies in mice with the prepared particles.

CHAPTER 1
Vaccinology: History and development

1.1 Introduction

The National Immunization Program of the Centre for Disease Control and Prevention in Atlanta, USA, clearly stated that without vaccines, continuing immunisation and high levels of immunity, the epidemics of vaccine-preventable infections would return (Krustak, 2002:580).

Of all the branches of modern medicine, vaccinology can claim to be the one that has contributed most to the relief of human misery and the spectacular increase in life expectancy in the last two centuries. It is the only science that has eradicated the infectious disease, smallpox, responsible for 8-20 % of all deaths in several European countries in the 18th century (Andrè, 2003:593).

Mass vaccination has proven to be very significant in the decrease of disease incidence (Krustak, 2002:580). According to Andre *et al.* (2003:593) it is estimated that immunisation saves the lives of 3 million children a year but 2 million more lives could be saved by improving existing vaccines and the development thereof. However; the use of vaccines is no longer restricted to the prevention of infections and they are now considered as therapeutic tools (Audibert, 2003:1187).

Due to the exploding costs of research, development and manufacturing of new vaccines over the last 2 decades, vaccine usage has been hampered. Emphasis is still placed on therapy instead of prevention in medicine. This has led to the mistaken perception that vaccines are expensive, although they are, in most cases, more cost-effective than the popular wait-see-treat approach (Andrè, 2003:593).

It should also be noticed that until the last few years, vaccines were exclusively used to prevent infectious diseases. Little effort has been made towards the development of therapeutic vaccines capable of treating pre-existing infectious diseases or non-infectious pathologies. Considering the expanding number of technologies available for making vaccines, it becomes possible for the first time in the history of vaccinology to design vaccines based on a rational approach, leading to increased efficacy and safety (Leclerc, 2003:330).

The oral route for administration is particularly well suited for vaccines against infections entering through or afflicting the airways or the gastrointestinal tract, for instance where mucosal protection is needed. The traditional vaccines used parenterally normally induce a good systemic humoral response, while the mucosal response is less pronounced. However, the mucosal associated lymphoid tissues (MALT) have a high capacity giving rise to a diversified immune response, including both cellular and humoral components, as well as a local and systemic response. An oral vaccine has to penetrate the intestinal epithelial barrier to reach the immune competent cells located in the epithelium, in the lamina propria, or beneath the basal membrane. To be able to do so, the vaccine components have to be formulated with carriers, taking them through the barriers. In free form, the antigens will not survive in the gastrointestinal tract and are normally not taken up by the enterocytes. However, when bound to particulate carriers, it is generally accepted that the antigens can be transported over the barriers by the M-cells in the Peyer's patches (Wikingsson *et al.*, 2002:3355).

The striking advantage of mucosal vaccination is the production of local antibodies at the sites where pathogens enter the body. Because vaccines alone are not sufficiently taken up after mucosal administration, they need to be co-administered with penetration enhancers, adjuvants or encapsulated in particles. Chitosan easily forms microparticles and nanoparticles that can encapsulate large amounts of antigens such as ovalbumin, diphtheria toxoid or tetanus toxoid. It has been shown that ovalbumin loaded chitosan microparticles are taken up by the Peyer's patches of the gut associated lymphoid tissue

(GALT). This unique uptake demonstrates that chitosan particulate drug carrier systems are promising candidates for oral vaccination (Van der Lubben *et al.*, 2001:139).

"a Nurse in Botswana has injected about 170 schoolchildren with a single needle while immunising them against whooping cough, tetanus and polio, sparking an AIDS and HIV scare, as reported by a newspaper" (Anon, 2003). The obvious disadvantages of invasive injections compared to non-invasive mucosal vaccination are the low patient compliance and high cost due to the need of a sterile manufacturing process and for qualified personnel to administer the vaccine. However, the best advantage of mucosal vaccine delivery is that it facilitates the neutralisation of pathogens at the moment that they enter the body across the mucosae (Van der Lubben *et al.*, 2001:140).

1.2 The history of vaccination

1.2.1 Origin of vaccination

The science of vaccinology took of on 14 May 1796 when Edward Jenner inoculated James Phipps, a 13-year-old boy, with the vaccinia virus obtained from a young women 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 variola virus "some months afterwards". Soon afterwards, in 1798, Jenner predicted that systematic 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 certified that one of the worst scourges of humanity had been wiped out by a vaccine developed nearly 200 years before (Andrè, 2003:593).

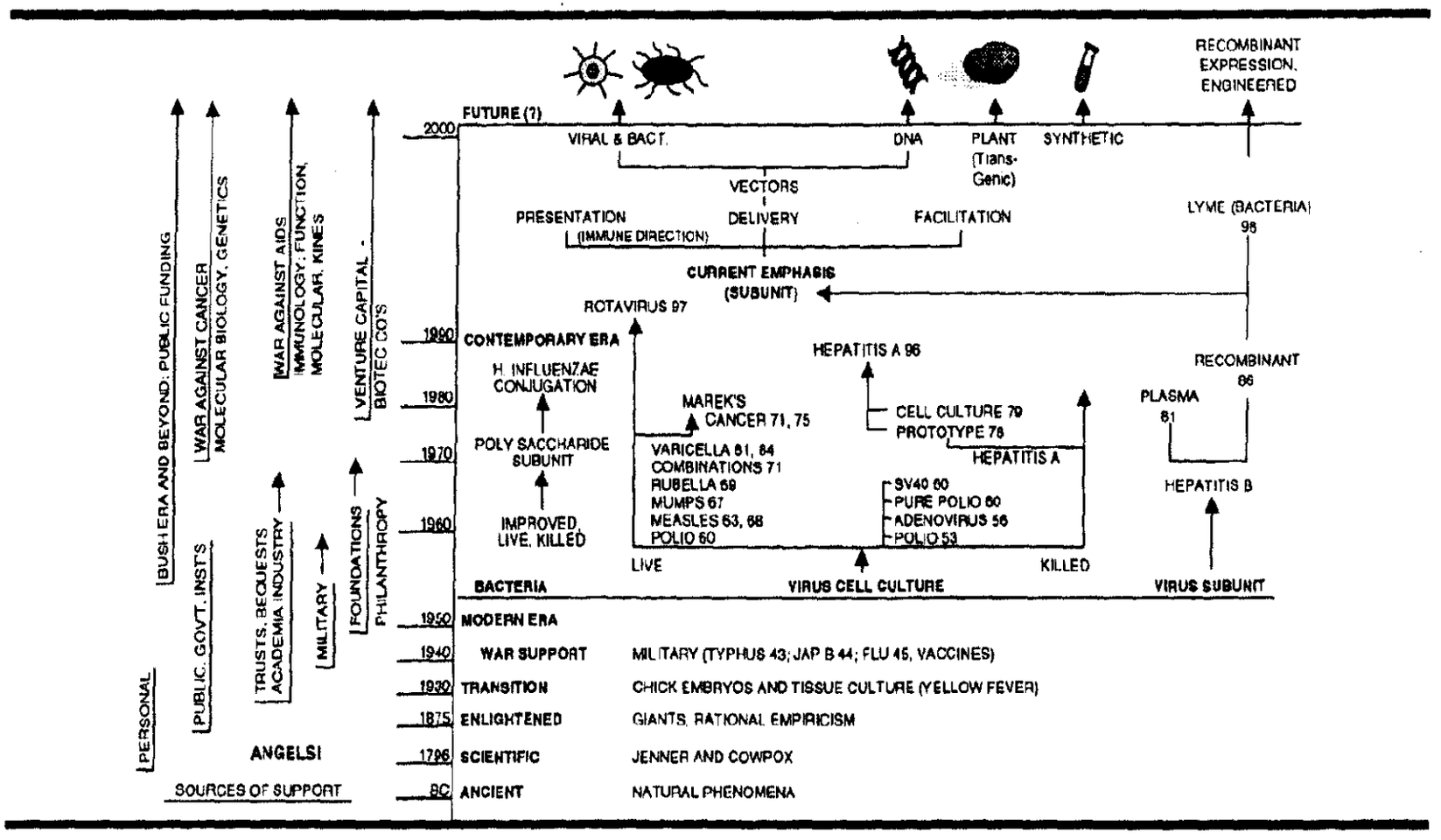


Figure 1.1: A diagrammatic outline of the history of vaccines (Hilleman, 2000:1437)

Twenty-six infectious diseases are now vaccine-preventable. Table 1.1 shows the dates of introduction of the most commonly used vaccines, as monovalent preparations, as well as in combination with other vaccines (Andrè, 2003:593).

Table 1.1: Dates of the introduction of commonly used vaccines (Andrè, 2003:594).

Vaccine	Date
Smallpox	1796
Rabies	1885
Cholera	1896
Typhoid	1896
Plague	1896
Diphtheria (D)	1923
Pertussis (Pw)	1926
Tetanus (T)	1927
Tuberculosis (BCG)	1927
Yellow fever	1935
Influenza	1936
Polio (IPV)	1955
DTPw	1957
Polio (OPV)	1958
DTIPV	1961
Measles (M)	1963
DTPIPV	1966
Mumps (M)	1967
Rubella (R)	1969
MMR	1971
Meningococcus	1972
Pneumococcus	1976

Acellular P (Pa)	1981
Hepatitis B (HB)	1981
Varicella (V)	1984
rDNA HB	1986
H. influenzae b (Hib)	1988
Hepatitis A (HA)	1991
DTPwIPVHib	1993
DTPa	1994
DTPwHB	1996
HBHA	1996
DTPaHib	1997
DTPaIPVHib	1997
Lyme	1998
Rotavirus	1998
Dtpa	1999
HATy	1999
DTPaHBIPV	2000
DTPaHBIPVHib	2000
MCCV[b]	2000
PCV[a]	2000

Despite the eradication of smallpox, there has been an explosion of interest in the vaccinia virus in the eighties. This interest has stemmed in part from the application of molecular genetics to clone and express foreign genes from recombinant vaccinia virus. These recombinant viruses have multiple applications in research and vaccinology and led to the development of various vaccines. The use of the recombinant vaccinia viruses as efficacious *in vitro* expression system and live vaccine has raised concerns about its safety. The work of the scientific community of the last 20 years has contributed drastically to improve the safety of the poxvirus-derived vectors. Firstly, the safety of vaccinia virus has been enhanced by the production of genetically attenuated strains. Secondly, alternative poxvirus vectors, such as avipoxviruses, were proved to be

extremely safe and efficacious non-replicating vectors when used in non-avian species (Pastoret *et al.*, 2003:343).

In the last century, vaccines have been one of the most powerful tools for preventing infectious diseases. Smallpox has been eradicated and other disease such as poliomyelitis or measles have been reduced to very low levels in many regions of the world. However, infectious diseases remain the leading cause of death worldwide (Leclerc, 2003:329).

1.2.2 Mechanism of action of vaccines

Vaccines primarily use a harmless form of a pathogen, or some component of it, to induce a protective immune response involving one or both arms of the immune system: humoral and or cell-mediated immunity. Humoral immunity is based on antibodies and the B cells that produce them. Antibodies are proteins that recognise a specific target, usually part of the surface of a protein. Neutralising antibodies, which normally bind to the outside of a virus, can play an important role in fighting viral infections.

The acquired cellular immune response comprises $CD4^+$ and $CD8^+$ T cells. Antigens (generally proteins or peptides) activate CD4+ T-cells, after their processing by antigen-presenting cells (APC). These cells may be dendritic cells, macrophages or B cells. $CD4^+$ T cells who recognise antigens, processed through the exogenous pathway by APC, expresses major histocompatibility complex (MHC) class II molecules. This recognition leads to the differentiation of $CD4^+$ T cells into the functional subset T helper 1 (TH_1) and TH_2. The signature cytokines is interferon (IFN-γ) for TH_1 and interleukin (IL)-4 for TH_2 cells. The TH_2 cell subset mediates the production of specific antibodies by sensitised B-cells. TH_1 cells, mediates the killing of organisms responsible for a variety of intracellular infections through the production of IFN-γ. The induction of a functional TH_1 response is crucially dependent upon another cytokine IL-12, which is produced by APC, especially dendritic cells. Thus, IL-12 can be considered as the cytokine inducer of TH_1 cells while IFN-γ is the effector cytokine mediating their efficacy. $CD8^+$ T cells

recognise antigens that are processed through the endogenous pathway and are presented by APC cells expressing MHC class I molecules (Audibert, 2003:1188).

1.2.3 Infection route

There is conflicting evidence concerning the role played by the route of infection on the risk of disease development (Johnson, 1994:279).

1.2.3.1 Age

In a study amongst all risk groups it was found that the relative risk of disease progression increased one and half fold for every ten years after adolescence. With increasing age, it becomes more difficult to replace both humoral and cellular immune cells, due to the loss of thymus capacity to generate new naïve T-lymphocytes. It is also suggested that older people may have lower levels of chemokines that play a role in progression, whilst babies have not yet developed an immune response.

It is possible that age-related differences may exist in response to vaccination and therapy, although the extent to which age influences the efficacy of vaccines and its relationship to viral set point is unknown. It has been suggested that teenagers have a better immune response. Thus, one would expect to see a significant trend towards increased vaccine efficacy and slower disease progression. However, such data is not generally available for most of the viral pathogens and longitudinal studies of vaccination and viral burden have to be carried out (Polo *et al.*, 1999:447)

1.2.3.2 Ethnicity

There appears to be significant differences in the way families within different race/ ethnicities approach the issue of immunisation. These approaches may also affect the utilisation of preventative healthcare initiatives in general (Middleman, 2004:415).

Findings from the National Immunisation Survey strongly suggest that the estimated vaccination coverage among children of Hispanic ancestry vary by group. Improved monitoring of vaccination coverage among Hispanics by the community is necessary, and where under-vaccination is identified, interventions should be matched to community needs (Guillermo *et al.*, 2001:69).

Most of the evidence available to date suggests no association between measles and pertussis vaccination and the subsequent development of asthma and atopy. This appears to hold true also for BCG vaccination among subjects originating from western countries. However, immigrants from the tropics living in clean environments might be genetically susceptible to the protective effect of BCG vaccination against atopic disease. Whether a certain cumulative vaccine dose is needed to confer protection against atopy among children from Western countries and whether this effect is persistent remains to be verified (Von Hertzen *et al.*, 2004:401).

Race and gender clearly play separate and distinct roles in healthcare and utilisation, unrelated to the traditional variables of socio-economic status often associated with access to care (Middleman *et al.*, 2004:414).

1.2.4 Vaccine safety

At the time of Jenner, the anti-vaccine movement was still very ineffective. Imaginary or real concerns about vaccine safety were of secondary relevance compared to the obvious benefits of disease control. More recently, with the disappearance of many vaccine-

preventable diseases as a result of widespread vaccination, the movement has regained its initial popularity. One major blow was dealt with in Scotland in 1974, when a Glasgow University professor became convinced, inaccurately as it was later established, that whooping cough vaccine was responsible for permanent neurological damage in infants. His campaigning for his believe, including television appearances, caused a dramatic fall from 81 – 30 %, in usage of the vaccine in the UK. This, predictably, led to a reappearance of the disease, with several deaths as a result. Similar scenarios, although for different reasons, were later played out in other countries like Japan, Sweden, Germany and Italy. Fortunately, in all these countries, universal vaccination, with less reactogenic acellular vaccines than the classic whole-cell vaccine, has returned and pertussis has again been brought under control. The lesson that has been learned from the pertussis saga is that unjustified scare mongering is damaging to public health. In the last 15 years, many scientifically unsubstantiated hypotheses have imperilled vaccination programmes in many countries. The origin of these hypotheses, usually propounded by one enthusiastic champion, is often country-specific. However, with the ease of global communication, they are rapidly spread, mainly through the internet, to a surfacing anti-vaccine diaspora. A non-exhaustive list of such beliefs, with their country of origin or originator, is shown in Table 1.2 (Andre, 2003:594-595).

Table 1.2: Hypothetical, unproven associations between vaccines and health conditions with country of origin or originator (Andre, 2003:595).

Health condition	Vaccine incriminated	Origin
Neurological damage	DTPw	Scotland
Unexplained death	DTPw	Japan
Chronic fatigue syndrome	Hepatitis B	Canada
Sudden infant death	DTPw	France
Multiple sclerosis	Hepatitis B	France
Crohn's disease	MMR	UK
Autism	MMR	UK
Diabetes mellitus	Hib	US

AIDS	OPV	Hooper (reporter)
Mental retardation	Thiomersal	US
Arthritis	Lyme	US
Vcjd	Bovine serum	UK
Immune overload	Combinations	US

It is worthy to note that the health conditions apparently "associated" with vaccines are all of unknown or poorly known aetiology. It must be recognised that vaccines can indeed cause adverse effects or reactions. Most of the frequent ones are benign, such as temporary pain, redness and swelling at the site of injection. Systemic reactions such as fever (sometimes leading to febrile convulsions), malaise or headache can also be attributed to vaccination. Serious reactions are very rare and it is not easy to establish scientifically that an observed temporal association with vaccination is causal. The few scientifically proven reactions, with their frequency of occurrence are shown in Table 1.3. Adverse events attributable to vaccination can be "programmatic errors" such as the use of wrong diluents or the transmission of pathogens due to poor aseptic technique. Errors of manufacture, such as the Cutter incident, where one lot of polio vaccines were not properly inactivated and this caused many cases of paralytic poliomyelitis. In the case of the Lubeck disaster, the use of virulent mycobacteria for the production of BCG vaccine was responsible for cases of tuberculosis. However, with modern methods of manufacture such preventable accidents are very unlikely to occur again (Andre, 2003:595).

Table 1.3: Frequencies of some scientifically proven serious reactions to vaccines (Andre, 2003:595).

Vaccine	Reaction	Frequency
All	Anaphylaxis	1:50000-1000000
OPV	Paralytic polio	1:750000(first doses)
Measles	Thrombocytopenic purpura	1:22300
Rotavirus (RotaSchild)	Intussusception	1:11000
Mumps (Urabe Am 9)	Meningoencephalitis	1:10000

1.2.5 Vaccine Effectiveness

It has been shown that vaccination of a large proportion of a population can lead to the protection of the entire population due to a "herd effect", that slows down circulation of a pathogen in the immunised population. The greatest achievement of vaccination remains the eradication of smallpox, a disease responsible for 8-20 % of all deaths in Europe before the introduction of vaccination. Other momentous achievements are the virtual disappearance of previous disabling and lethal diseases such as diphtheria, paralytic poliomyelitis, pertussis, measles, mumps, rubella and invasive H. influenzae b. Hepatitis B and A are also being brought under control in an increasing number of countries. The spectacular increases of life expectancy in the last two centuries are in great part due to vaccination. In 1974, when the Expanded Program on Immunisation (EPI) was launched, only 5 % of newborns, almost exclusively in developed countries, were being properly vaccinated against six diseases: tuberculosis, poliomyelitis, diphtheria, tetanus, pertussis and measles. In 1990, the global vaccination rate had reached 80 %. Unfortunately, this rate has been decreasing since then. Nevertheless, it is estimated that the EPI is currently saving the lives of 3 million children a year. Two million more lives could be saved if existing vaccines were more systematically used (Andre, 2003:593-594).

It was well documented that vaccines are one of the major beneficial players in medicine, preventing suffering, disability and deaths. For example, a new, very effective conjugated meningcoccal serogroup C vaccine, was produced and introduced into the United Kingdom Childhood Immunisation Program at the end of 1999, with the objective to immunise 15 million children and adolescents below 18 years of age, over a period of 12 months. The rapid disappearance of confirmed cases of serogroup C meningcoccal infection was noted, proving the high efficacy of this immunogenic and safe vaccine. As meningcoccal infection is the foremost cause of death, such an effective vaccine should be introduced without delay in all countries. For example, the Spanish health authorities included this vaccine in the routine immunisation schedule at 2, 4 and 6 months of age. Children and adolescents between 6 and 19 years of age were also vaccinated. The results of mass vaccination have been spectacular, as a very significant decrease of

disease incidence has been noted. In Catalonia, a short-term effectiveness of 100 % for meningcoccal C conjugated vaccination in children less than 6 years of age were observed (Kurstak, 2002:581).

1.2.6 Different types and general classification of vaccines

A number of strategies to produce protective immune responses have been explored globally and on this basis vaccines can be classified as follows:

- Live attenuated vaccines

  This is a defective pathogen that would be harmless to subjects. These types of vaccines are in some cases unsafe for human use.

- Inactivated or killed vaccines

  These types of vaccines have still not yet been fully evaluated for their ability to protect against pathogens.

- Recombinant sub-unit vaccines

  This vaccine seeks to stimulate antibodies to the pathogen by mimicking proteins on its surface. Subunit vaccines researched to date have been strain-specific and have produced poor antibody responses. Recent research into adjuvants has opened new areas of envelope vaccine research, with some vaccines capable of inducing neutralising antibodies effective against a range of pathogen strains.

- Recombinant vectored vaccines

  These vaccines consist of genes or fragments of genes of the pathogen incorporated into established or new delivery systems. Delivery systems may include live but harmless viruses. Vector vaccines have been shown to produce pathogen-specific cytotoxic T cell responses in subjects. These can be enhanced with DNA vaccine priming.

- DNA vaccines and replicons

  These vaccines involve genetic sequences injected into subjects to induce the expression of antigens by cells. In the case of replicons, these sequences are wrapped

in the outer coat of an unrelated virus. Such a strategy has been proposed for a vaccine against some of the malignant viruses, such as human papilloma virus.

- Combination vaccines or "prime and boost" vaccines

  These strategies combine two or more different vaccines to broaden or intensify immune responses. It is possible that two different vaccines could be given at the same time, where one acts more rapidly than the other. This would result in a "prime-boost" effect from a single dose (O'Hagan, 1998:273-280).

1.3 Delivery systems for vaccines

1.3.1 General delivery systems

1.3.1.1 Sorbitan Monostearate Organogels and Amphiphilogels

Unlike their cousin's hydrogels and organogels have been less widely studied and the literature is sparse, especially in the drug delivery field. Simply dissolving or dispersing the gelator in the hot solvent and cooling the resulting sol phase that sets to a semi-solid gel typically prepare the gel. Cooling the sol results in reduced solubility of the gelator in the solvent, and hence reduced affinity between solvent and gelator molecules. Consequently the gelator molecules self-assemble into aggregates such as rods, tubules, fibers, rope-like chains, ribbons and fan-like structures which interact with one another and form a 3-dimensional network that immobilises the solvent. Potential applications of these gels include: media for reactions and for the purification of organic solvents, separation membranes, sensors, carriers for drugs, vaccines and thermotropic liquid crystals and tools to study the behavior of membrane-bound proteins (Murdan, 2003:16).

1.3.1.2 Microemulsions

The microemulsion technique is favorable when working with substances unstable to the high mechanical stress produced by high-pressure homogenisation. By using this method huge amounts of surfactants and co-surfactants are used. Cationic nanoparticles are potential carriers for genes and have shown to be less rapidly cleared from the circulation than negatively charged particles. The preparation of aqueous cationic solid lipid nanopheres dispersions consisted of two steps: firstly, formulation of an oil-in-water microemulsion and secondly, preparation of the solid lipid nanopheres by dispersing the warm microemulsion into cold water. The lipid phase including the co-surfactant and the water phase were heated separately, mixed and subsequently titrated with the surfactant until a microemulsion was obtained. The microemulsion was dispersed 1:10 in ice-cold water at a constant speed (2 ml/min) using a syringe (Heydenreich *et al.*, 2003:83-84).

1.3.1.3 Proteinoid micropheres

In an interesting report, a novel microcapsule is described, made from thermally condensed amino acids, for the delivery of influenza virus antigens. Coacervation and entrapment of antigen is achieved by adjustment of the pH. Using these microcapsules for oral challenge, they were able to show anti-haemaglutinin and neuramidase responses several times more than seen with the non-encapsulated antigens. The proteinoid coating is acid resistant but degrades rapidly as pH exceeds 5 (Po *et al.*, 1995:104).

1.3.1.4 Ethylene-vinyl acetate polymers

In a study by Po *et al.* (1995:104) albumin was used as the model antigen and ethyl-vinyl acetate as the capsule polymer. They showed that the IgG antibody response over a six-

month period with an injection of this microcapsule was equivalent to two intramuscular injections of the encapsulated antigen.

1.3.2 M-cells for vaccine delivery and the importance of gastrointestinal uptake of particles

The administration of drugs and vaccines via mucosal routes offers important advantages over parenteral delivery. Firstly, mucosally delivered drugs and vaccines are easy to administer, requiring neither sterile needles nor trained personnel. Secondly, mucosal delivery may enhance efficacy if, for example, the drug to be administered exerts its effects at mucosal surfaces or, as in the case of vaccines, a strong mucosal immune response is required. The induction of mucosal immunity is a highly desirable feature for vaccines, since it provides a first line of defence against the many pathogens that invade via the mucosal surfaces (Clark *et al.*, 2001:82).

However, mucosal sites also include the organised mucosa-associated lymphoid tissues (O-MALT) that are the specialised antigen sampling sites of the mucosal immune system. The antigen sampling function of the O-MALT is performed predominantly by the membranous epithelial M-cells. While these cells are specialised for antigen sampling, they are also exploited as a route of host invasion by many pathogens. In addition, M-cells represent a potential portal for mucosal drug and vaccine delivery since they possess a high transcytotic capacity and are able to transport a broad range of materials including particulates (Clark *et al.*, 2001:83).

1.3.2.1 M-cell structure and function

The intestinal epithelial barrier is composed of a single layer of epithelial cells that predominantly consists of enterocytes interspersed by mucus secreting goblet cells. The epithelial cells are sealed at their apical membranes by tight junctions, and while cells are

constantly extruded into the gut lumen, epithelial integrity is maintained by cell replacement from the crypts. O-MALT is located throughout the gastrointestinal tract and consists of lymphoid follicles arranged either singly or as clusters to form distinct structures such as Peyer's patches and the appendix. The epithelium overlaying the lymphoid follicles is termed the follicle-associated epithelium (FAE), and is distinguished from the intestinal epithelium at other sites mainly by the presence of the specialised antigen sampling M-cells (Figure 1.2). Together the FAE, lymphoid follicles and associated structures form the antigen sampling and inductive sites of the mucosal immune system. The M-cells are typically characterised by two features. Firstly, they have sparse, irregular microvilli on their apical surface. Secondly, they possess a basolateral cytoplasmic invagination that creates a pocket containing one or more lymphocytes and occasional macrophages. Both these features facilitate antigen sampling. The sparsity of the microvilli renders the M-cell apical membranes relatively accessible to reagents within the gut lumen. After M-cell adhesion, these agents need only be transported a short distance across the thin M-cell cytoplasmic rim before reaching the M-cell pocket and underlying lymphoid cells, a feature which permits rapid delivery of vaccine antigens directly to the inductive O-MALT sites (Clark *et al.*, 2001:83-84).

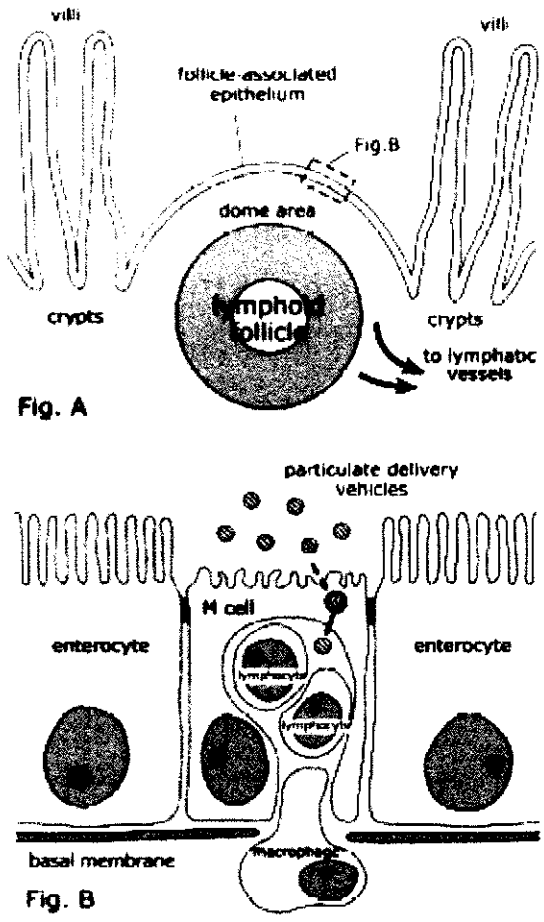


Figure 1.2: Schematic transverse sections of a Peyer's patch lymphoid follicle and overlying follicle-associated epithelium (FAE), depicting M-cell transport of particulate delivery vehicles. The general structure of intestinal organised mucosa-associated lymphoid tissues (O-MALT), are represented by the schematic transverse section of a Peyer's patch lymphoid follicle and associated structures in (A). The lymphoid follicle is situated beneath a dome area which protrudes into the gut lumen between villi, and which is covered by the follicle-associated epithelium (FAE). This epithelium is characterised by the presence of specialised antigen sampling M-cells (depicted in B). These cells typically possess a reduced number of irregular microvilli on their apical surface and a basolateral cytoplasmic invagination that creates a pocket harbouring lymphocytes and macrophages. Particulate delivery vehicles are largely prevented from passing between epithelial cells by tight junctions. However, since M cells possess a relatively high transcytotic capacity compared to that of enterocytes, the M-cell portal may represent an efficient route for the transport of drugs and vaccines carried by particulate delivery

vehicles across the intestinal epithelial barrier. Synthetic delivery vehicles may be targeted to M-cells by coating with appropriate ligands such as lectins of microbial adhesins, or the delivery vehicle may consist of a live attenuated micro-organism that innately targets to M-cells. After adherence to the M-cell apical membranes and transport across the thin apical cytoplasmic rim, reagents are delivered to the underlying inductive O-MALT sites, and may subsequently disseminate via the lymphatics (Clark *et al.*, 2001:84).

1.3.2.2 General principles of M-cell delivery

Many of the factors that determine the efficacy with which orally administered drugs and vaccines are delivered to intestinal epithelial cells are equally applicable to M-cell delivery. For example, administered reagents must survive the hostile gastric and intestinal intraluminal environments. They must then persist in the intestinal lumen for a sufficient length of time to make contact with and be transcytosed by the intestinal epithelial cells. The mucus gel layer, the closely packed microvilli and the cell surface glycocalyx inhibit access to the intestinal epithelial cell membranes. Together these structures entrap enzymes and create a highly degradative microenvironment at the apical cell surfaces. Various strategies have been devised to enhance drug and vaccine delivery by prolonging the intestinal residence time (Clark *et al.*, 2001:85).

Antigen sampling by M-cells is facilitated by the relative accessibility of the M-cell apical membranes. Secretory IgA and mucus are present in reduced quantities at the surface of the FAE compared to other intestinal epithelial sites, the M-cell microvilli are relatively sparse and irregular and the M-cell surface glycocalyx is relatively thin. To achieve effective delivery, the administered reagent should ideally target to and exhibit high levels of binding to the M-cell apical membranes, and subsequently be internalised and transported in an active form to the M-cell pocket. M-cells possess a high transcytotic capacity and are able to transport a variety of materials including macromolecules, inert particles and micro-organisms (Clark *et al.*, 2001:85).

Reagents may interact with M-cells via both non-specific and specific, receptor-mediated mechanisms. Non-specific mechanisms dependent on surface charge and hydrophobicity are thought to account for the observed efficacy with which synthetic particles selectively interact with M-cells in some experimental animal models. It is likely that surface positive charge and hydrophobicity favor the non-specific interaction of particulates with M-cells. Since negatively charged components of mucus may neutralise positively charged particles and surface hydrophobicity may be masked by the binding of gut luminal proteins, whereas delivery vehicles dependent on non-specific interactions with M-cell apical membranes and are likely to prove unreliable (Clark *et al.*, 2001:8).

The function of the epithelium is highly complex, being influenced by endocrine, paracrine, stromal and immune elements. Numerous studies have demonstrated that macromolecules can be absorbed through the gut in immunologically significant quantities, challenging previous suggestions that macromolecules were completely reduced to their component monomers in the gut, prior to uptake into the body. Molecules absorbed in such fashion in mammals have been found to interact with immunologically responsive cells in the gut that with lymphoid tissues in other mucosae, comprise the common mucosal immune system. However, oral delivery of peptide or protein drugs and antigens is frequently compromised by poor uptake of these molecules due to their size and hydrophilicity and by a reduction in the quantity of intact molecules reaching the circulation because of lumenal, brush border and intracellular degradation (Lavelle *et al.*, 1995:6).

There is now considerable evidence that microparticulate materials can be absorbed in small quantities from the mammalian gastrointestinal tract. The levels of uptake appear to be low, but may be sufficient for the effective induction of protective mucosal immune responses to orally administered entrapped antigen. Indeed, enhanced responses to orally delivered microencapsulated antigens have been reported on a number of occasions. Encapsulation of drugs or vaccine antigens in biodegradable microparticles may protect the molecules from enzymatic degradation, increase their uptake in intact form and potentially target the molecules to the desired sites in the body (Lavelle *et al.*, 1995:6).

1.3.2.3 Gut-associated lymphoid tissue

Characterisation of the cells and interactions involved in antigen uptake and immunity in the gastrointestinal tract is necessary if the oral route is exploited for therapeutic delivery. This may relate in part to a different antigen handling mechanism as a result of pre-processing of antigens by digestive enzymes. Mucosa-associated lymphoid tissue (MALT) is composed of scattered isolated lymphoid cells in the lamina propria and epithelium, scattered lymphoid follicles in the lamina propria, aggregations of lymphoid follicles in the Peyer's patches, the appendix and lymph nodes. The process of induction of immune B and T cells in MALT, followed by their migration to effector sites for the development of mucosal immune responses, is termed the common mucosal immune system. Gut-associated lymphoid tissue (GALT) is a major component of this interconnected network (Lavelle *et al.*, 1995:6).

1.3.2.4 Specialisation of the GALT associated with antigen uptake

M-cells possess short microvilli, small cytoplasmia vesicles and few lysosomes, and can endocytose and transport protein antigens, inert particles and micro-organisms, including bacteria, viruses and protosoans into the GALT. M-cells transport antigens from the surface luminal membrane to the pocket region of the Peyer's patch, with little degradation or chemical alternation (Lavelle *et al.*, 1995:7).

It has been suggested that since Peyer's patches have a reduced number of mucus-secreting goblet cells, compared with the surrounding epithelium, that these regions are more accessible for binding by micro-organisms. Particle binding to the apical membrane of M-cells led to rapid internalisation and transport to mucosal immune inductive regions. Distinct follicles are found under the dome region of the Peyer's patch that contains germinal centers where significant B-cell division occurs. B-cell conversion to IgA production and the process of affinity maturation occurs in these germinal centers (Clark *et al.*, 2001:85).

1.3.3 Delivery systems for oral vaccination

1.3.3.1 Introduction

A number of problems hamper the development and delivery of oral vaccines. Higher and more frequently administered antigen doses are generally required for oral compared to systemic immunisation. The poor response elicited to orally delivered antigens partly result from enzymatic degradation and low absorption levels, and consequently little immunogenic antigen reaches the gut-associated lymphoid tissue. Exploitation of a new generation of vaccine antigens and the delivery of peptides and proteins has been constrained by a lack of appropriate delivery systems; a situation that is particularly acute in the case of the oral delivery route. The literature on oral vaccination against enteric disease and experimental studies on mucosal responsiveness is overwhelmed by variable efficacy and is frequently difficult to explain. However, certain rules appear to apply:

1. Live micro-organisms provide much better antigens than killed bacterial or viral antigens, possibly as a result of their capacity to adhere to mucosal surfaces.
2. Most soluble antigens are less effective in inducing mucosal responses than particulate antigens. This is thought to result from different routes of entry and subsequent differences in the cell types involved in antigen processing. The uptake of particulates into Peyer's patches may lead to production by dendritic cells and macrophages and the induction of immunity. In contrast, the uptake of soluble antigens by Peyer's patches is less efficient, and antigen is taken up mainly across the villi and processed by macrophages in the lamina propria, which may have a suppressive effect on immune responses. Not all soluble proteins are poor mucosal immunogens; some proteins and glycoproteins such as cholera toxin, ricin and influenza virus haemaglutinin can effectively induce antibody responses in serum and secretions in orally immunised subjects.
3. It has been suggested that proteins with lectin/lectin-like binding activity are good mucosal immunogens, whereas those lacking such activity are ineffective or suppressive. Additionally, it was found that lectin-antigen conjugates, which bind

to the M-cell apical membranes, are more effectively transported than non-adherent conjugates.

All widely used vaccines, except the Sabin trivalent oral polio vaccine is presently administered by systemic routes. In many cases these vaccines are effective in inducing systemic cell mediated and antibody responses, but are poor at inducing mucosal immunity in humans who have not had a previous mucosal infection by the causative organism. A number of strategies are available to increase the efficacy of orally delivered molecules. Common approaches involve the avoidance or modification of gastrointestinal secretions by the use of gastric inhibitors, anti-proteases acid resistant films or encapsulation. An adjuvant activity has been demonstrated when muramyl dipeptide (MDP), liposomes of recombinant Gram-negative bacteria, are delivered orally. Cholera toxin is a potent enteric immunogen and exerts strong adjuvant effects on gut immune responses to unrelated antigens when presented concurrently. Immune stimulatory complexes (ISCOMS) confer immunogenicity on proteins delivered by the oral route, and very low amounts of antigen in such structures are immunogenic.
The incorporation of antigens in liposomes or microparticles protects them for harmful digestive secretions and thus allows the use of lower doses than is the case when soluble antigen is administered. An increased systemic and mucosal immune response to orally administered BSA, as a result of encapsulation in liposomes, has been reported. Oral live vaccines yield higher antibody titers in remote site secretions and in the serum than oral killed vaccines. Research is now focusing on the use of attenuated live organisms, both as oral vaccines and as carrier vehicles for enteric delivery of heterologous antigen (Lavelle *et al.*, 1995:9-10).

1.3.3.2 Chitosan

Chitosan formulations are used for ocular, oral, parenteral and nasal delivery, as well as for DNA transfection studies. Furthermore, chitosan can easily form microparticles. Advances in microparticulate drug delivery research have opened up the way to apply

these techniques for oral vaccination. Due to the high protein binding properties of some types of chitosan microparticles, they are also potential candidates for oral delivery of antigens. Mild preparation can protect the proteins when they are incorporated during preparation of the microparticles. In order to circumvent protein denaturation conditions, chitosan microparticles can be loaded passively. Besides antigens, DNA coding for antigens can also be taken up by the M-cells of the Peyer's patches. Transcription of this DNA leads to the production of the antigen (Van der Lubben *et al.*, 2001:688).

Recently, chitosan micro- and nanoparticles have been prepared according to several precipitation/co-acervation methods and some of these particles showed good antigen binding capacities. Chitosan micropheres were also designed for colonic drug delivery. For oral vaccination, microparticulate vaccine carrier systems not only need to associate a high amount of antigen, but also require specific release properties. After oral administration of such systems the vaccine should be well entrapped and protected from degradation in the GI-tract, and should only be released from the carrier system after uptake by the M-cells of the Peyer's patches. Nano- or microparticles should not exceed 10 μm in size. The hydrophobicity and the antigens presented on the surface of the carrier system are also important parameters. Microparticles smaller than 10 μm are taken up by the M-cells and transported to the dome of the Peyer's patches. Microparticles smaller than 5 μm are then transported to the spleen and lymph nodes, where specific IgM and IgG are produced. Since the cumulative size distribution showed that microparticles between 5 and 10 μm were formed, these microparticles might stay in the Peyer's patches. In this case additional antigen specific IgM is formed (Van der Lubben *et al.*, 2001:692).

Chitosan microparticles have a very porous structure and therefore have a high loading capacity and large quantities of antigen can be transported to the Peyer's patches. The release will only be after disintegration of the microparticles. Since chitosan is biodegradable, this might happen after M-cell uptake and chitinases are expected to play an important role in this degradation process. Uptake by the M-cells is the first step in

oral vaccination and chitosan microparticles are a promising method of efficient oral vaccine delivery (Van der Lubben *et al.*, 2001:692).

1.3.3.3 Non-replicating particulate systems for oral delivery

1.3.3.3.1 Polymeric particles

Biodegradable polymers have several advantages. First, they have demonstrated biocompatibility, and have been used in pharmaceutical and medical applications for many years. Second, biodegradation of the polymers results in release of encapsulated drugs over time, which enables the particles to serve as depots for controlled drug delivery. Examples of biodegradable polymers that have been examined for potential oral drug delivery include poly(lactide-co-glycolide) (PLG), poly-anhydrous, poly(methyl methacrylate) and poly-alkylcyanoacrylates. With the degradation of PLG, the polymer gives lactic and glycolic acids. In most cases, drugs are encapsulated in these particles using the solvent evaporation technique. Release of drugs from the particles is controlled by the particle degradation rate, which is in turn determined by the polymer composition and its molecular weight. Degradation of the particles in turn results in release of the encapsulated drugs (Chen *et al.*, 1998:343).

1.3.3.3.2 Lipid particles

The most common form of lipid particles is liposomes. Liposomes are spherical vesicles made of concentric bilayers encasing an aqueous core. They can carry lipid-soluble drugs in their bilayers and at the same time water-soluble drugs in their aqueous cores. In addition, liposomes can be formed under mild conditions that minimise drug denaturation during encapsulation. Unfortunately, most liposome formulations cannot be used for oral delivery because they are susceptible to dissolution by intestinal detergents such as bile

salts and to degradation by intestinal phospholipases. Disruption of the liposomal membranes in the gastrointestinal tract leads to exposure of the encapsulated material and therefore the loss of their protective functions. To stabilise liposomes for application in oral delivery, polymerised liposomes have been developed. By creating a cross-linked network in the liposomal membranes, the liposome stability can be improved while they are inside the gastrointestinal track (Chen *et al.*, 1998:343-344).

1.3.3.3.3 Immune stimulating complexes and cochleates

Lipid molecules can form many other types of particles, such as immune stimulating complexes (ISCOM's) and cochleates. ISCOM's are three-dimensional cages of 30 nm to 70 nm in diameter and can be formed by mixing lipids, cholesterol and saponin (Quil A). Quil A is a potent immunoadjuvant and ISCOM's have therefore been used to deliver antigens orally. Hydrophobic antigens can be incorporated into the ISCOM's spontaneously. Incorporation of hydrophilic antigens, on the other hand, is more difficult and the antigens need to be modified before they can be inserted into the ISCOM's (Chen *et al.* 1998:344).

Cochlaetes are phospholipid-calcium precipitates with a unique structure consisting of a large continuous solid lipid bilayer sheet rolled up into a spiral. Cochlaetes are structurally distinct from liposomes and do not contain any aqueous space. The presence of the calcium ions maintains the cochleates in their rolled up forms. Removal of the calcium ions with chelating agents allows the cochleates to unroll and form large liposomes. It has been shown that hydrophobic drugs can be incorporated into the lipid bilayers of the cylindrical cochleates and delivered orally (Chen *et al.*, 1998:344).

1.3.3.4 Mucoadhesive delivery systems

Particles made of mucoadhesive polymers that can adhere to the mucus layer in the intestine have been widely studied to improve particle delivery efficiency. The transit of the polymeric carriers in the gastrointestinal tract is slowed down by the interaction between the particles and the mucus layer in the intestine. This result in a prolonged intestinal residence time for orally delivered particles. This in turn results in increased particle absorption efficiency (Chen *et al.*, 1998:346).

1.3.3.5 Delivery of DNA to mucosal surfaces

An alternative approach for mucosal vaccine delivery is the direct administration to mucosal surfaces of a DNA plasmid expression vector that encodes a protein antigen. Intramuscular injection of DNA expression vectors in mice or primates has been demonstrated to result in the uptake of DNA as well as the expression of the encoded proteins by the muscle cells. DNA plasmids have also been utilised for direct introduction of genes into other tissues. Plasmids were maintained episomally without replication, and the expression of the encoded proteins was observed to persist for extended time periods. DNA, encoding various genes, has been used to induce both humoral and cellular immune responses to the expressed proteins. Direct immunisation with DNA offers several advantages compared to protein subunit vaccines. Preparation of the DNA plasmids is simple and inexpensive. Furthermore, the expressed proteins have the ability to induce both humoral and cellular immune responses since they are introduced into the antigen-processing pathway that results in the generation of cytotoxic T-lymphocytes. The effect of mucosal administration of DNA has not been extensively investigated, and uptake of DNA from epithelial surfaces may not be as effective as direct injection of DNA into muscle cells. However, it should be possible to enhance the uptake of DNA by specific delivery mechanisms, such as incorporation into micropheres, liposomes, virosomes or cochleates, or administration of DNA with a mucoadhesive polymer (Mestecky *et al.*, 1997:252-253).

1.3.3.6 Nanoparticles

Recently, it has been shown that enhancement of the electrostatic interaction between the mucosal surfaces and drugs have a marked effect on their uptake and overall bioavailability. The epithelial cells in various tissues including the gastrointestinal tract, carry a negative charge on their surface due to the presence of negatively charged residues of proteins in the outer membrane of the cells and the selective active ion pumps of the membrane. Therefore, all epithelia are selective to positively charged solutes. Accordingly, it is anticipated that positively charged delivery systems that will have a strong interaction with the cells will result in better permeability and overall bioavailability of the drugs. One of the most successful approaches toward this aim has been the use of positively charged colloidal dispersions such as liposomes, submicron emulsions and self-emulsifying oily formulations. A common conclusion from these previous studies is that positively charged colloidal drug carriers increase the permeability and potential uptake of slightly soluble drugs when compared with neutral or negatively charged ones, thus improving their bioavailability and reducing their side effects. This behavior was attributed to the mucoadhesion mediated by electrostatic interaction between the positively charged colloidal particles and the negatively charged mucin on the mucosal surface. On the basis of the above-mentioned considerations, it was thought plausible to combine the advantages of nanopheres as oral delivery systems with the benefit of the presence of positive charges on their surfaces (El-Shabouri, 2002:102).

1.3.3.7 Biodegradable microparticles

The administration of bioactive molecules in microparticles is an expanding area of oral vaccine research and accumulated evidence indicates that biodegradable micro- and nanoparticles may act as efficient antigen delivery vehicles. The rationale behind the use of controlled release systems for vaccine delivery is to reduce the number of repeated administrations required to establish long-term protection, since the number of doses

required for a vaccine to be effective against an infectious agent is essential in achieving the appropriate level of immunity.

Two types of delivery may be possible:

1. Continuous antigen release, where antigen is progressively released over a period of time.
2. Pulsed antigen release, where a mixture of particles of different sizes and compositions are used to affect pulses of antigen release, somewhat akin to conventional booster immunisation with vaccines.

Microparticle technology has the potential advantages of reducing the number of inoculations and enhancing the immune response, after both parenteral and oral immunisation, and reducing the total antigen dose needed to achieve protection (Lavelle *et al.*, 1995:14).

1.3.3.8 Cellulose acetate phthalate

Mycoplasma pneumonia in swines can be vaccinated by using formalin-inactivated cells. However, the antigen is labile to low pH and intestinal enzymes and parenteral immunisation is required with standard formulations. An attempted was made to reduce the intestinal cleavage of the antigen by enteric-coating of the inactivated cells with cellulose acetate phthalate to produce micropheres. However, the products also included larger particles in the mm diameter range. They reported that oral administration of the particles as a booster dose provided protection against challenge by the micro-organism. Although their results are difficult to interpret because the priming dose was administered intramuscularly, enteric-coating appears to be a useful avenue to pursue further. Surprisingly, they reported that particles in the range 1-1.41 mm in diameter provided protection that was as good as particles of 350-650 μm in diameter (Po *et al.*, 1995:104).

1.3.3.9 Proteinoid micropheres

An interesting report describes a novel microcapsule, made from thermally condensed amino acids, for the delivery of influenza virus antigens. Coacervation and entrapment of the antigen is achieved by adjustment of the pH. By using these microcapsules for the oral challenge, they were able to show anti-haemaglutinin and neuramidase responses several times as were seen with the non-encapsulated antigens. The proteinoid coating is acid resistant but degrades rapidly as the pH exceeds 5 (Po *et al.*, 1995:104).

1.3.3.10 Virosomes

Virosomes are biologically degradable, contains no preservatives or detergents and present fewer localised adverse events when compared to conventional parenteral vaccinations. The virosomes bind to antigen-presenting cells on their surfaces and enter cells by receptor-mediated endocytosis. The virosomes then fuse with the endosomal cell membrane. This process provides optimal processing and presentation of the antigens to immunocompetent cells (Gluck *et al.*, 2002:B10-B11).

Two examples of virosome delivery systems are Epaxal® and Inflexal V®.

Epaxal®

The first virosome-based vaccine for human use was licensed in 1996, a virosomal hepatitis A vaccine, and is now registered in nearly all countries of the EU, America and Asia. The virosomal hepatitis A virus vaccine is able to induce a very fast immune response; 10 days after immunisation, 100 % of seroconversion has been measured. The use of virosomes as an antigen carrier system for Hepatitis A has also been shown to be considerably more tolerable than the conventional aluminium hydroxide adsorbed forms of vaccination (Gluck *et al.*, 2002:B11-B12).

Inflexal V®

Inflexal V® is a trivalent virosome-based influenza vaccine. Its trivalent properties stem from the creation of three virosome formulations. Inflexal V®, being based on virosome technology, accesses the immune system in a similar way to the natural pathogen. The cell surface glycoprotein haemaglutinin guides virosomes to antigen-presenting cells thereby enabling the introduction of antibody production by B-cells and T-cell mediated immunity and immunological memory. The superior immunogenicity of Inflexal V® verses conventional influenza vaccines has been shown by a statistically significant improvement over other vaccines when a larger than 4-fold increase in anti-hemaglutinin antibodies was found in elderly people. The superior immunogenicity and tolerability of Inflexal V® has also been shown by a comparison with Influvac®, a subunit vaccine. The use of a trivalent virosomal vaccine provides several advantages over current parenteral influenza vaccines. The natural antigen presentation produces improved immunogenicity and protective efficacy, whilst also showing better tolerability (Gluck *et al.*, 2002:B12).

1.3.4 Particle characteristics

Particles must have certain specific characteristics because of all the different types of particulate delivery systems that exist. Due to the unique physiological conditions in the GI-tract, the particulate systems are required to meet the following criteria before they can be used as effective oral delivery vehicles. Firstly, they need to be resistant to degradation in the GI-tract and in turn protect the encapsulated drugs from degradation. This is especially important for lipid-containing particulates such as liposomes, since the presence of bile salts in the small intestine is known to destroy most of the particles administered. In comparison, this is not as critical for polymeric particles such as biodegradable microspheres since their degradation typically takes place on a time scale of days, sometimes weeks. Secondly, the encapsulated drugs in the particles need to be absorbed with high efficiency in the GI-tract to be therapeutically effective. In other words, the particles themselves need to be absorbed at an efficient rate in the GI-tract to

deliver the encapsulated drugs to their *in vivo* targets. Currently, it is believed that less than one percent of the particles can be absorbed after oral administration. This extremely low absorption efficiency proves to be the largest obstacle toward the potential application of particulates in the oral delivery of complex molecules such as proteins and peptides (Chen *et al.*, 1998:340).

The extent and detail of particle uptake appears to vary considerably depending on the nature of the particle and possibly the animal model used. Particulate uptake has been reported to depend on the size, hydrophobicity, charge and polymeric composition of the particles. Particle size is a critical determinant of the fate of orally delivered microparticles and possibly of the elicited immune response to the antigen. Larger particles may be retained for longer periods in the Peyer's patches while smaller particles are progressively transported to other major organs. Particles of less than 10 μm are absorbed by M-cells and translocated to the Peyer's patch T and B-cell zones. Those larger than 5 μm in diameter were endocytosed and transported by MAC 1+ cells through efferent lymphatics to systemic lymphoid tissues, to stimulate a serum antibody response. Particles smaller than 5 μm in diameter remain in the Peyer's patches leading to sustained release of antigen in this S-IgA inductive area. The polymeric composition of particles also appears to be of importance in determining the extent of particle uptake. The extent of accumulation in Peyer's patches was also dependent on the hydrophobicity of the polymeric material (Lavelle *et al.*, 1995:11).

Particle charge is also an important factor that determines the extent of uptake from the gut. Specific charges may be imparted to the surface of the particulate antigen carrier system. For example, with liposomes, the zwitterionic liposomes produced from phosphtidylcholine and cholesterol can be made negatively charged by addition of diacetyl phosphate to the formulation. In previous studies it was reported that systemic immune response was activated only by negatively charged liposomes following nasal administration of bovine serum albumin-associated liposomes to mice. Activation of mucosal immune response was dependent on liposomal charge (Po *et al.*, 1995:106).

Species differences are also apparent in the uptake of particulate materials (Lavelle *et al.*, 1995:11).

1.4 Future trends in vaccinology

1.4.1 Reverse vaccinology

Conventional vaccines consist of live-attenuated microbes, killed inactivated micro-organisms, purified microbial components, polysaccharide-carrier protein conjugates or recombinant proteins. Two basic types of vaccines, based on the micro-organism in an attenuated but live form, or on the killed, inactivated micro-organism, were among the first vaccines generated. A third type of vaccine, made from the diphtheria and tetanus toxoids, were developed in the 1920's and represent a much more sophisticated product. The two toxins, which were previously shown to be essential for causing the disease, were chemically detoxified to yield the non-toxic toxoids. Although other purified single-component vaccines are immunogenic, they might not be able to induce immunological memory. The ability of a protein antigen to induce a T-cell immune response can be improved by conjugation to a polysaccharide. These glyoconjugate vaccines were first prepared and studied with *Haemophilus influenza* type b and have proved to be a great success (Mora *et al.*, 2003:469).

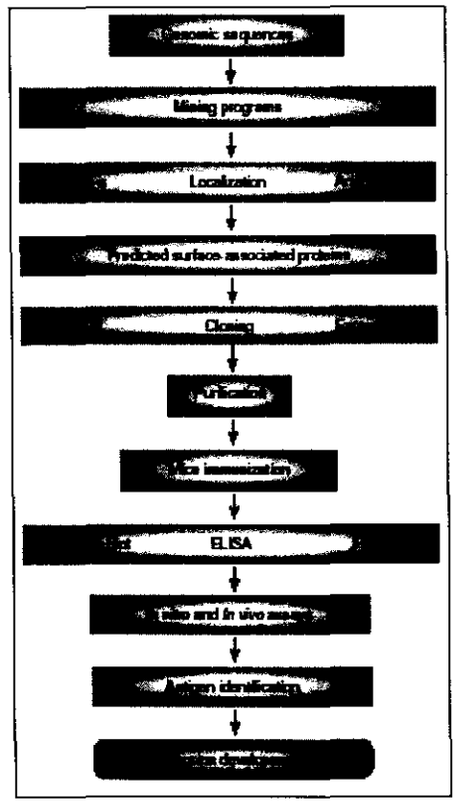


Figure 1.3: Flow chart of the genome-based approach to vaccine development. This approach involves the in silico analysis of microbial genome sequences followed by the high-throughput expression of the genes of interest. The recombinant proteins are then used to immunise mice and the post-immunisation sera are analysed to assess the ability of the polypeptide to elicit a quantitative and qualitative immune response (Mora *et al.*, 2003:460).

The late 1990's marked the beginning of the era of genomics. Genomic sequencing and analysis are now in a period of exponential growth. More than 90 eukaryotic and prokaryotic genomes have been completely sequenced, with analysis of more than 200 genome sequences currently under way. The availability of the entire genetic content of several important human pathogens has opened new innovative and efficient research avenues to identify a wide array of new antigens for vaccines against any infectious disease. However, genome data alone cannot be used to accurately predict the *in vivo* efficacy of candidate antigens. Therefore, vaccine candidates selected on the basis of in silico criteria need to be validated by using genomic, proteomic, genetic biochemical and

bioinformatic approaches, in addition to appropriate animal models (Mora *et al.*, 2003:463).

The advantage of the reverse vaccinology approach is that growth conditions or detection methods for gene expression do not limit it. This strategy identifies a small number of the most promising new vaccine candidates from a large number of antigens, and enables more rapid development of these candidates at a reasonable cost compared with traditional strategies (Mora *et al.*, 2003:463).

1.4.2 Recombinant measles virus vaccines

The aim of recombinant measles virus vaccines is to induce a long-lasting immunity against a particular pathogen. The measles virus has great potential as a vaccine, due to its ability to produce both humoral and cellular immune responses. The rescue of measles virus from cDNA allows the generation of a variety of recombinant and chimeric measles viruses for a variety of purposes, including recombinant vaccines. Studies in mice have proved that measles virus can be engineered to selectively eliminate cells expressing a targeted receptor. The potential for measles virus as a well functioning vector stems from its ability to tolerate additional "foreign" genes from other pathogens such as hepatitis B virus antigens and SIV and HIV genes. In addition, the strength of expression of these genes can be controlled (Gluck *et al.*, 2002:B14).

1.4.3 Polysaccharide protein conjugates

The causative agents of many bacterial infections contain surface structures made of polysaccharides, against which protective antibodies are directed. Vaccines based on these polysaccharide antigens alone do not induce a T-cell response and consequently are not capable of eliciting immunological memory. The formation of a polysaccharide-protein conjugate, by linkage of the required antibody-inducing polysaccharides with T-

cell activating carrier proteins, induces the desired immune response and enhances long-term protection against the corresponding pathogen. Aerugen® is a polyvalent conjugate vaccine, combining eight prevalent serotypes from *P. aeruginosa* and exotoxin A. It is the first conjugate vaccine to be based on a lipopolysaccharide (LPS) component from the pathogen. The antigenic O-polysaccharide in the LPS is cleaved off its lipid carrier and transferred to exotoxin A. The resulting vaccine contains the polysaccharide-protein conjugate and elicits an immune response to both the relevant *P. aeruginosa* polysaccharides and the toxin. Aerugen® specifically targets the progressive destruction of lungs caused by *Pseudomonas* infections. Immunisation with this vaccine has been shown to preserve lung function in cystic fibrosis patients by preventing infection and progressive colonisation by *P. aeruginosa* (Gluck *et al.*, 2002:B15).

1.4.4 Reverse genetics

A concept called reverse genetics has recently enabled researchers to construct an experimental vaccine against H5N1, a potential pandemic influenza strain, in less than a month. A similar approach could be used to develop vaccines for severe acute respiratory syndrome (SARS). Vaccines against new strains of well-known viruses of emerging infectious diseases used to take many months to develop. However, the increasing availability of genome sequences for pathogens is now expediting the developing of safer, more effective vaccines (Bradbury, 2003:518).

Reverse genetics is being used to develop vaccines for many other viruses, including respiratory syncytial viruses and parainfluenza viruses. If the definition of the technology is broadened to include expression of proteins from engineered genes, then its future applications are virtually boundless. 10 years ago genetics was used minimally in vaccine development, but now researchers would not even try to develop a vaccine without using all the genetic tools available. Speed is only one aspect of this revolution. Even more important is the flexibility that is now available for vaccine design. Many companies are planning to use reverse genetics to produce attenuated flu strains as live

vaccines that should give more protection than current inactivated vaccines. In other cases, technology has facilitated the development of previously elusive vaccines. For example, in 50 years research has failed to develop an effective vaccine for meningococcus B. In less than four years, with the use of reverse genetics researchers went from getting its genome sequence through identifying new antigens to starting clinical vaccine trials (Bradbury, 2003:518).

1.4.5 DNA vaccine delivery

1.4.5.1 Mechanism of action

DNA vaccines contain the gene or gene coding for an antigenic portion of a virus (the viral core of 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). 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 vaccine antigens, which are taken up into cells via phagocytosis or endocytosis and are processed through the MHC class II system pathway, thereby primarily stimulating antibody response (Mor, 1998:1151).

1.4.5.2 Routes of administration

DNA immunisation can be accomplished via intramuscular saline injection or particle bombardment. Intramuscular injection is the most commonly used form of plasmid DNA

delivery. Using chloramphenicol, acetyl transferase, luciferase or B-galactosidase, Wolff *et al.* demonstrated for the first time that the injection of purified DNA results in the expression of the protein within the muscular cell. Particle bombardment involves coating of the plasmid DNA with gold microparticles. Delivery of the DNA into the epidermis is achieved by using a device called a Gene Gun, which creates a shock wave capable of accelerating the DNA-coated gold particles into the target tissue via a controlled discharge. In this way, both the depth of gold particle penetration and the amount of DNA delivered per cell can be regulated. This method appears to be a highly efficient way to achieve immunisation, since a protective immune response can be elicited with considerably less DNA (as little as 40 ng) than what is typically used for intramuscular injection (10-400 mg). The direct intracellular delivery achieved via particle bombardment is largely responsible for the increased efficiency of this method (Mor, 1998:1152).

1.4.5.3 Safety issues

Potential safety concerns particular to DNA vaccines include integration of the plasmid DNA sequences into the host chromosome, the development of antibodies to DNA and the development of neonatal tolerance. Integration has been perceived as a problem because of the potential activation of oncogenes or disruption of normal gene function and regulation. In several preclinical studies, research has not detected plasmid integration using an assay that can detect one integration event in 1×10^6 cells. In addition, antibodies to DNA or antinuclear antibodies have not been observed in several trails. An early publication suggested that neonatal immunisation of mice with pDNA resulted in immune tolerance. Subsequent studies from several laboratories have not substantiated this finding and indicate that the generation of neonatal tolerance is not an issue for DNA vaccines (Shroff *et al.*, 1999:209).

1.4.5.4 Advantages

The advantages of DNA vaccines originate in the chemistry and molecular biology of DNA. The plasmid encoding the sequence for the antigenic protein also serves as the physical vector for the genes. In this way and mimicking viral infections, DNA-based vaccines are able to stimulate the intracellular synthesis of foreign proteins. Another advantage of DNA vaccination is that it results in the *in vivo* production of the purified and structurally intact antigen. This is important since the interaction between molecules and the cells of the immune system is highly dependent on their three-dimensional shape. For this reason, the paradox, in order to be recognised by the immune system, the components of a vaccine must retain their native conformation, yet purification procedures often result in the denaturation and aggregation of the single antigens or antigen fragments of subunit vaccines. Finally, from a public health point of view, DNA is very stable and resists extreme temperatures, thus facilitating the storage, transport and distribution of vaccines. This is of critical importance for countries lacking the infrastructure to provide and to guarantee the proper storage and efficient distribution of vaccines (Mor, 1998:1152).

1.4.5.5 Dangers

Although the immunogenicity of DNA vaccines is well established, concerns have been raised regarding their safety, more specifically their potential to induce toxic 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, 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. Another concern is the possibility that vaccinated newborns may develop tolerance rather than immunity because of the

immaturity of their immune system. This is especially relevant since most vaccines intended for use are administered to infants and children and because numerous studies have demonstrated that in the early stages of postnatal development the immune system is unresponsive to antigenic challenges. Since the protein encoded by a DNA vaccine is produced endogenously and is expressed in the context of self major histocompatibility complex, it is possible for the neonatal immune system to recognise it as self, leading to the development of tolerance (Mor, 1998:1152-1153).

1.5 Conclusion

It was shown in several studies that orally administered particulates could be absorbed in the GI-tract. However, existing evidence for absorption pathways as well as absorption efficiencies is not entirely consistent due to the different experimental methods adopted by different groups. Direct comparisons among different systems are clearly needed before a more comprehensive understanding of the absorption process can be obtained. This will be critical for the optimisation of particle delivery efficiencies. Studies in different animal models suggest that the efficiency of particle absorption can be improved through modification of particle surfaces with targeting molecules such as antibodies or lectins, or through slowing down the intestinal transit of the particles (Chen *et al.*, 1998:348).

Oral delivery is and will be likely to remain the most convenient way for drug administration. Therefore, the future of oral particulate delivery will highly depend on whether a sufficient level of absorption can be achieved. Since vaccines typically require smaller quantities to be effective, oral particulate vaccines may therefore be more promising. Many of the particulate systems can also provide adjuvanticity to the antigens incorporated, which may compensate for the low amounts of antigens absorbed. Oral vaccination will induce mucosal immunity and improve patient compliance. Reduction of operational costs such as trained medical personnel may also drive down the cost of vaccination. All of these make oral particulate vaccines very attractive. The feasibility

of oral particulate delivery of therapeutic protein and peptide drugs, on the other hand, will largely depend on whether substantially improved particle absorption can be achieved in the future (Chen *et al.*, 1998:349).

CHAPTER 2
Characteristics of chitosan, N-trimethyl chitosan chloride (TMC) and Emzaloids™

2.1 Chitosan

2.1.1 Introduction

The obvious disadvantages of invasive injections compared to non-invasive mucosal vaccination, are the low patient compliance and high costs due to the need of a sterile manufacturing process and qualified personnel to administer the vaccine. However, the best advantage of mucosal vaccine delivery is that it facilitates the neutralisation of pathogens at the moment that they enter the body across the mucosae (Van der Lubben *et al.*, 2001:2).

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 the 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. If microparticulate drug delivery systems are used for intestinal drug delivery, the associated compound should be released upon arrival at the epithelium. Only particles smaller than 100 nm have shown to be taken up by Caco-2 cells. Larger microparticles end up in the Peyer's patches after uptake by the M-cells. For vaccine delivery, the lymphoid tissue should be targeted. Access to mucosal lymphoid tissue is provided by antigen sampling cells. These M-cells are located between the epithelial cells and absorb antigens and microparticles smaller than 10 μm. By incorporating the vaccine into microparticulate drug delivery systems, the vaccine is protected against degradation on its way to the mucosal tissue, efficiently

targeted and taken up by the M-cells. Subsequent release in the Peyer's patches may result in the induction of an immune response. For oral vaccination, antigens need to be protected against degradation by incorporation into microparticulate systems (Van der Lubben *et al.*, 2001:201-202).

In recent years significant progress has been made in identifying substances that increase the absorption of drugs through the paracellular pathway. In this regard, chitosan, a linear polysaccharide derived by N-deacetylation of the natural polymer chitin, is of special interest. Chitosan acts as an absorption enhancer by opening the tight junctions between epithelial cells to allow for the paracellular transport of hydrophilic and macromolecular compounds such as peptide drugs. The absorption enhancing ability of chitosan, a mucoadhesive polymer, is mediated by protonated amino groups on the C-2 position of the molecules that induce interaction with the anionic sites on cell membranes to subsequently alter tight junction integrity (Jonker *et al.*, 2002:206).

Oral drug delivery research has demonstrated that significantly higher amounts of macromolecular drugs can be transported after co-administration with chitosan. Besides its ability to facilitate paracellular transport, chitosan can also be used to prepare microparticles or nanoparticles. In contrast to soluble chitosan formulations, which are able to open the tight junctions, particulate vaccine delivery systems are taken up by the M-cells and subsequently biodegraded. Only then the lymphoid tissue will be targeted efficiently. Particulate systems for macromolecular and hydrophilic drug delivery need to be smaller than 200 nm to be taken up by epithelial cells or for release of the drug upon arrival at the mucosae (Van der Lubben *et al.*, 2001:201-202).

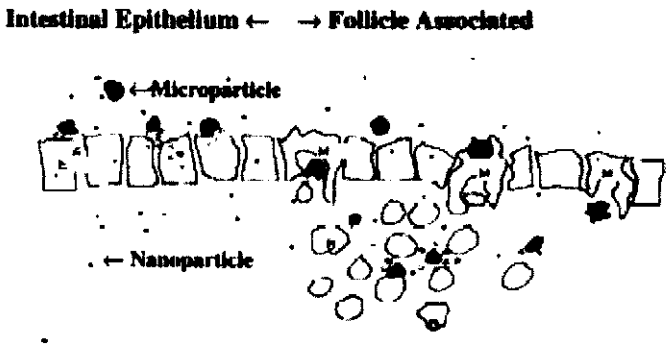


Figure 2.1: A schematic presentation of the intestinal epithelium. M-cells (M) are located between the epithelial cells in the lymphoid associated epithelium. M-cells are the entrance port to the Peyer's patches (PP) and efficiently take up microparticles smaller than 10 μm. Epithelial cells only take up nanoparticles. For oral vaccination, microparticles need to transport the associated vaccine into the Peyer's patches. The vaccine or particle complex is (most often) immediately taken up by phagocytotic cells (Van der Lubben, 2001:202).

2.1.2 Origin of chitosan

Chitin is a major structural polysaccharide found in invertebrate animals and lower plants. It is noticeably present in the outer skeletons of arthropods in particular, for example, in the epidermis of crustaceans such as crabs and prawns (Paul & Sharma, 2000:5).

In the first case, chitin production is associated with the food industries such as shrimp canning. In the second case, the production of chitosan-glucan complexes is associated with fermentation processes, similar to those for the production of citric acid from Aspergillus niger, Mucor rouxii and Streptomyces, which involves alkali treatment yielding chitosan-glucan complexes. Depending on the alkali concentration, some soluble glycans are removed. The processing of crustacean shells mainly involves the removal of proteins and the dissolution of calcium carbonate that is present in crab shells

in high concentrations. The resulting chitin is deacetylated in 40 % sodium hydroxide at 120°C for 1-3 hours. This treatment produces 70 % deacetylated chitosan (Majeti & Kumar, 2000:2-3).

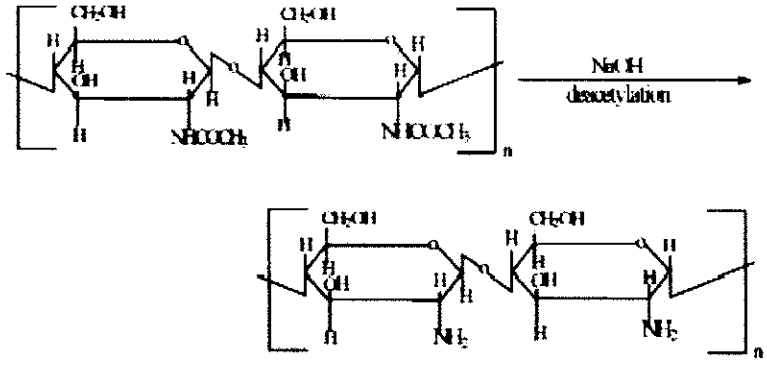


Figure 2.2: The production of deacetylated chitosan (Majeti & Kumar, 2000:3)

When chitin is boiled in a concentrated potassium hydroxide solution, a product was obtained that dissolved in dilute iodine and acids, unlike chitin that only stained brown. Chitosan [α (1-4) 2-amino 2-deoxy B-D glucan], the deacetylated form of chitin, is a mucopolysaccharide having structural characteristics similar to glycosaminoglycans with a chemical formula $(C_6H_{11}O_4N)_n$ (Paul & Sharma, 2000:5).

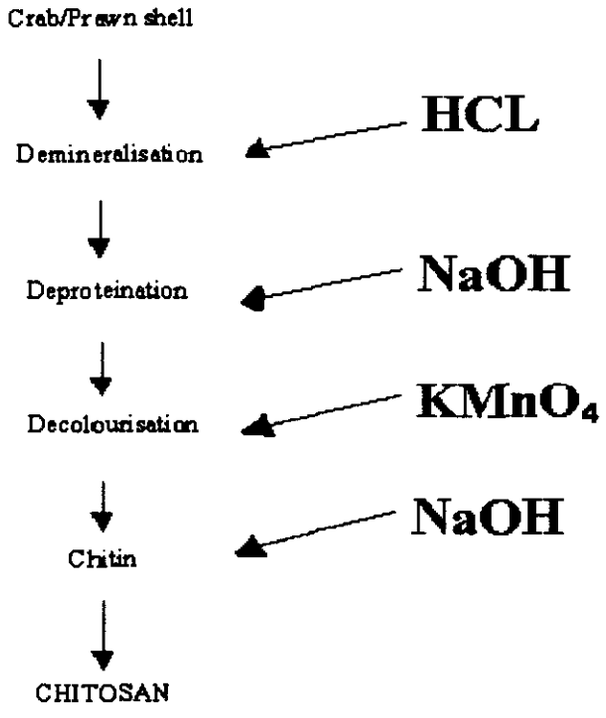


Figure 2.3: Chitosan production flow chart (Paul & Sharma, 2000:5).

2.1.3 Biopharmaceutical orientation

2.1.3.1 Oral route

The bioavailability of drugs has been improved by the use of mucoadhesive dosage forms. By prolonging the residence time of drug carriers at the absorption site, sustained release and improved bioavailability of drugs can be achieved. It is acknowledged that chitosan possesses good mucoadhesive properties and therefore seems suitable for oral administration (Dodane & Vilivalam, 1998:246).

The oral route is the most common, simple, comfortable, convenient and physiological way of administering traditional drugs. However, it is not suitable for the administration

of protein or peptide drugs that degrade in the gastric environment or are poorly absorbed. Chitosan has been thoroughly assessed as a potential oral delivery vehicle. It has been reported that ingestion of chitosan in humans effectively reduces total serum cholesterol, urea and creatinine levels. It also increases serum hemoglobin levels without any clinically relevant symptoms (Paul & Sharma, 2000:7).

2.1.3.2 Parenteral route

Various studies were done using chitosan derivatives in the subcutaneous and intramuscular parenteral routes. These formulations have the ability to prolong the delivery of drugs and hormones such as progesterone for an extended period of time (Paul & Sharma, 2000:7-8).

2.1.3.3 Transdermal route

The advantages of administering drugs through the skin are obvious when considering the first pass metabolism through the liver, enzymatic degradation and the effects on the GI-tract. Chitosan polymers can be incorporated in ointments, creams, lotions and patches. Chitosan and its derivatives are extremely suitable for both membrane and drug reservoirs for transdermal patches (Thacharodi & Roa, 1995:145).

2.1.3.4 Ocular route

The bioadhesiveness of chitosan makes it an excellent candidate for ocular formulations as the application intervals are prolonged significantly. Local activity in the eye is of special interest for preparations, including antiviral and antibacterial agents, where the

use of existing formulations are limited due to lacrimal drainage which prevents delivering drugs for extended periods of time (Genta *et al.*, 1997:737).

2.1.3.5 Nasal route

In contrast to oral administration, nasally administered vaccines have to be transported over a very small distance, remains in the nasal cavity for about 15 min and 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 microparticles, but may also be co-administered as chitosan solution or powder formulations. Systemic and local immune responses were also induced after nasal administration of chitosan and a mutant of diphtheria toxin. No humoral immune responses against chitosan itself were found after nasal or subcutaneous administration (Van der Lubben *et al.*, 2001:142).

2.1.3.6 Chitosan implants

Chitosan is also a good candidate for implants, as it is non-toxic, biodegradable and can be sterilised. It possesses controlled release properties, adequate drug storage capacity and ensures drug stability. It can be cross-linked to present various properties. Research is underway to develop a combined polymer and mineral to produce a material with the toughness and flexibility of a polymer, but with the strength of a mineral deposit (Muzzarelli & Muzzarelli, 2002:233).

2.1.4 Applications of chitosan

Some interesting applications of chitosan is listed below:

- Photography
- Cosmetics
- Chitosan as artificial skin
- Chitin- and chitosan-based dressings
- Food and nutrition
- Water engineering
- Metal capture from wastewater
- Colour removal from textile mill effluents
- Paper finishing
- Solid-state batteries (Majeti & Kumar, 2000:13).

2.1.5 Chitosan as a drug delivery system

Apart from the above mentioned applications, chitosan has also been extensively examined for its potential in the development of controlled release drug delivery systems. These controlled release formulations have been made in the form of gels, tablets, capsules, beads, microspheres and microcapsules. Nanoparticles have a special role in targeted drug delivery, because they have all the advantages of liposomes including a small particle size, but unlike liposomes, nanoparticles have a long shelf life and can usually entrap more drugs than liposomes (Banerjee *et al.*, 2002:94).

Controlled release technology emerged during the 1980's as a commercially sound methodology. The achievement of predictable and reproducible release of an agent into a specific environment, over an extended period of time, has much significant merit. It creates a desired environment with optimal response, minimum side effects and

prolonged efficacy. Controlled release dosage forms enhance the safety, efficacy and reliability of drug therapy. They regulate the drug release rate and reduce the frequency of drug administration to encourage patients to comply with dosing instructions. Conventional dosage forms often lead to wide swings in serum drug concentrations. Most of the drug content is released soon after administration, causing drug levels in the body to increase rapidly, peak and then decline sharply. For drugs whose actions correlate with their serum drug concentration, the sharp fluctuations often cause unacceptable side effects at the peaks, followed by inadequate therapy at the troughs (Figure 2.4) (Majeti & Kumar, 2000:13-14).

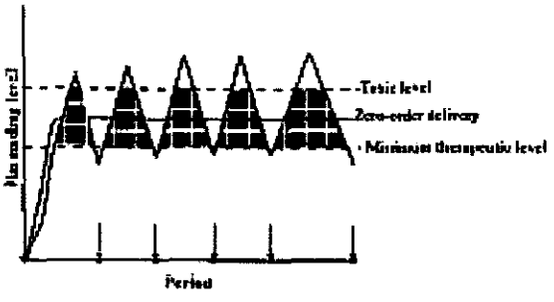


Figure 2.4: Controlled drug delivery versus immediate release (Majeti & Kumar, 2000:14).

Chitosan is non-toxic and easily bioabsorbable, with gel-forming ability at low pH. Moreover, chitosan has anti-acid and anti-ulcer activities that prevent or weaken drug irritation in the stomach. Also, chitosan matrix formulations appear to float and gradually swell in an acid medium. All these interesting properties of chitosan make this natural polymer an ideal candidate for controlled drug release formulations (Majeti & Kumar, 2000:14).

2.1.6 Cross-linking agents

According to several studies, cross-linking the matrix using agents such as glutaraldehyde, NaOH and ethylene glycol diglycidyl ether could control the drug release

from chitosan microparticles. However, these chemical cross-linking agents have the possibility of inducing undesirable effects. Chemically synthesised glutaraldehyde can cause irritation to mucosal membranes due to its toxicity. To overcome this disadvantage of chemical cross-linking, ionic cross-linking interaction has been applied. For example, ionic cross-linking with tripolyphosphate (TPP) produced chitosan beads, micro- or nanoparticles. TPP is non-toxic and a multivalent anion. It can form a gel by ionic interaction between the positively charged amino groups of chitosan and the negatively charged counter-ion of TPP. The charge density of TPP and chitosan could control this interaction, which is dependent on the pH of the solution. The nature of the chitosan matrix could also be depended on the molecular weight (Mw) of chitosan. The higher the molecular weight and degree of deacetylation of chitosan, the lower the release rate from chitosan films (Ko *et al.*, 2002:166).

2.1.7 Chitosan in vaccination

In a previous study, it was observed that large amounts of diphtheria toxoid (the toxoid that will be used in this study), could be incorporated into the chitosan microparticles. The loading efficacy was about 100 % and no diphtheria toxoid (DT) was lost during the loading process. There was no diphtheria toxoid released after storage for 3 months in PBS. The DT was not only associated to the surface, but was also entrapped within the microparticles. This is probable since the microparticles have a very porous structure. Because chitosan microparticles are positively charged and DT is negatively charged, DT can easily associate to the inner surface of the pores. It was found that chitosan microparticles also show excellent loading and release characteristics for the DT, suggesting that these microparticles could be used for multiple vaccines (Van der Lubben *et al.*, 2003:1409).

Fibres did not accompany chitosan microparticles neither forms a gel when resuspended in water, nor were forming big aggregates of microparticles, and all showed reasonable uniformity. Field emission SEM demonstrated that pores are present at the rough surface

of the chitosan microparticles (Figure 2.5a). As evident from Figure 2.5b, the inside of the chitosan microparticles also has a very porous structure (Van der Lubben *et al.*, 2001:693-694).

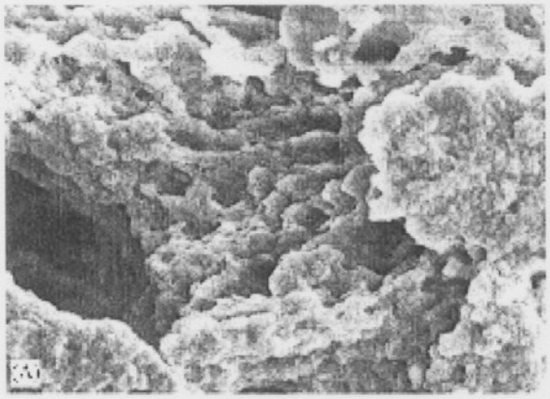


Figure 2.5a: Surface visualisation of chitosan microparticles using field emission SEM (Van der Lubben *et al.*, 2001:674).

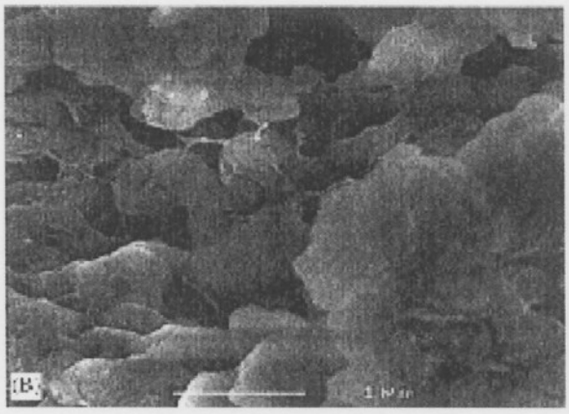


Figure 2.5b: Detail inside a pore of chitosan microparticles. Scale bars are 1µm (Van der Lubben *et al.*, 2001:674).

2.1.8 Encapsulation efficiency of chitosan particles

There are 3 major factors that can have an effect on the encapsulation efficiency of chitosan particles. The first major factor is the chitosan concentration. At high chitosan concentrations encapsulation is extremely difficult and at low concentrations aggregates are formed. Figure 2.6 shows that increased chitosan concentrations decreased the encapsulation efficiency of DT (Xu & Du, 2003:219).

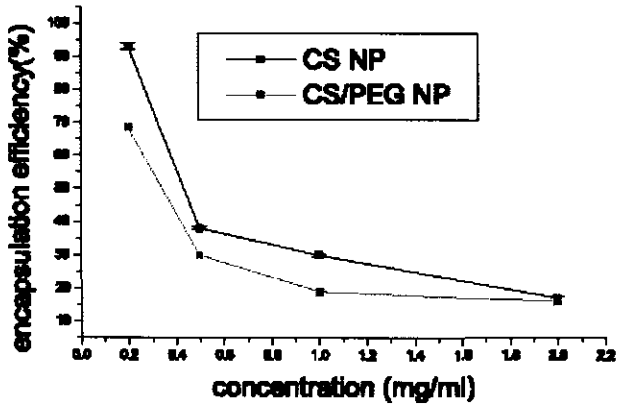


Figure 2.6: The influence of chitosan concentration on the DT's encapsulation efficiency (Xu & Du, 2003:219).

The second major factor is the degree of deacetylation. Figure 2.7 shows that as the degree of deacetylation of chitosan increased, the encapsulation efficiency increased. Chitosan with a higher degree of deacetylation contains more functional groups, which can form complexes with the acid groups of the toxoid and gelate with the tripolyphosphoric groups, resulting that the encapsulation efficiency for the toxoid increases correspondingly (Xu & Du, 2003:219).

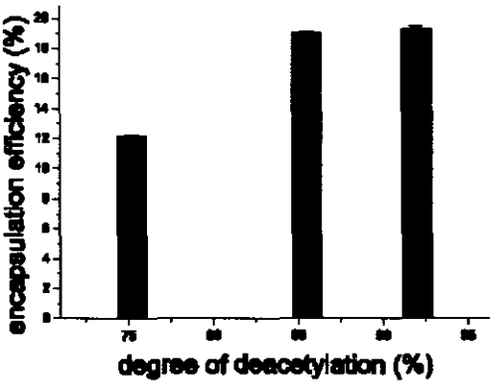


Figure 2.7: The encapsulation efficiency of chitosan nanoparticles with different degrees of deacetylation (Xu & Du, 2003:219).

In another study the temperature dependence of the deacetylation reaction rate yielded interesting results: the deacetylation rate constant was directly proportional to the reaction temperature at 60 and 80 °C; at 100 °C, the rate of reaction was lower than at 80 °C (figure 2.8). This lower reaction rate is most likely the result of the loss of deacetylated polymer due to its increase degradation and fragmentation into water-soluble, non-retainable fractions (Sabnis & Block, 2000:185).

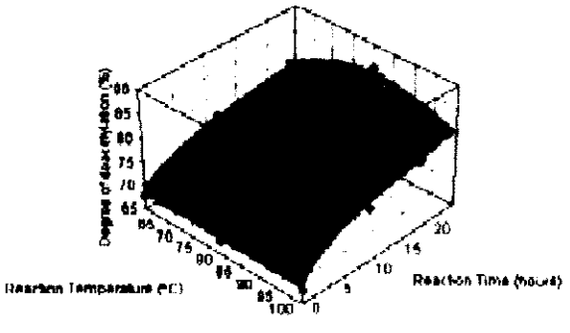


Figure 2.8: Effect of reaction temperature and time on the degree of chitosan deacetylation (Sabnis & Block, 2000:185).

The third major factor is the molecular weight of the chitosan. Figure 2.9, shown that when the molecular weight of chitosan increased, the encapsulation efficiency of the toxoid was increased also. There is sufficient evidence confirming the effect of the molecular weight of chitosan on its complexation behaviour. It was also found that the

effectiveness of chitosan in the coagulation of solids and proteins was inversely proportional to its molecular weight. Depolymerisation provide formation of chitosan having a greater number of amino groups available for interactions with anionic actives (Xu & Du, 2003:220).

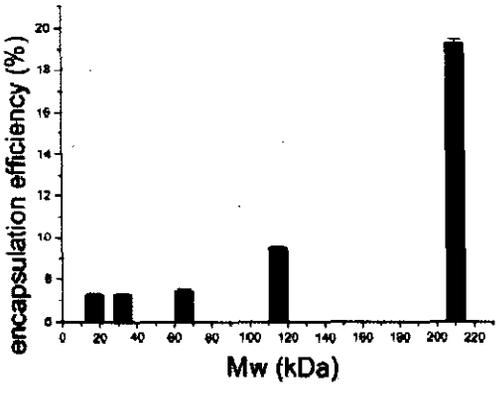


Figure 2.9: The encapsulation efficiency of chitosan nanoparticles prepared from various molecular weights of chitosan (Xu & Du, 2003:220).

2.2 *N*-trimethyl chitosan chloride (TMC)

2.2.1 Introduction

Chitosan and chitosan salts lack the advantage of good solubility at neutral and basic pH values. Chitosan aggregates in solutions at pH values above 6.5 and only protonated chitosan can trigger the opening of the tight junctions, thereby facilitating the paracellular transport of hydrophilic compounds. This property implies that chitosan can be effective as an absorption enhancer, only in a limited area of the intestinal lumen where the pH values are close to or lower than its pK_a. For this reason, chitosan and its salts may not be suitable carriers for targeted peptide drug delivery to specific sites of the intestine, for instance the jejunum or ileum. To overcome this problem, the chitosan derivative *N*-

trimethyl chitosan chloride (TMC) has been previously synthesised and characterised (Figure 2.10). This quaternised chitosan derivative shows much higher aqueous solubility than chitosan in a much broader pH and concentration range. The reason for this improved solubility is the substitution of the primary amine with methyl groups and the prevention of hydrogen bond formation between the amine and the hydroxylic groups of the chitosan backbone (Thanou *et al.*, 2001:121-122).

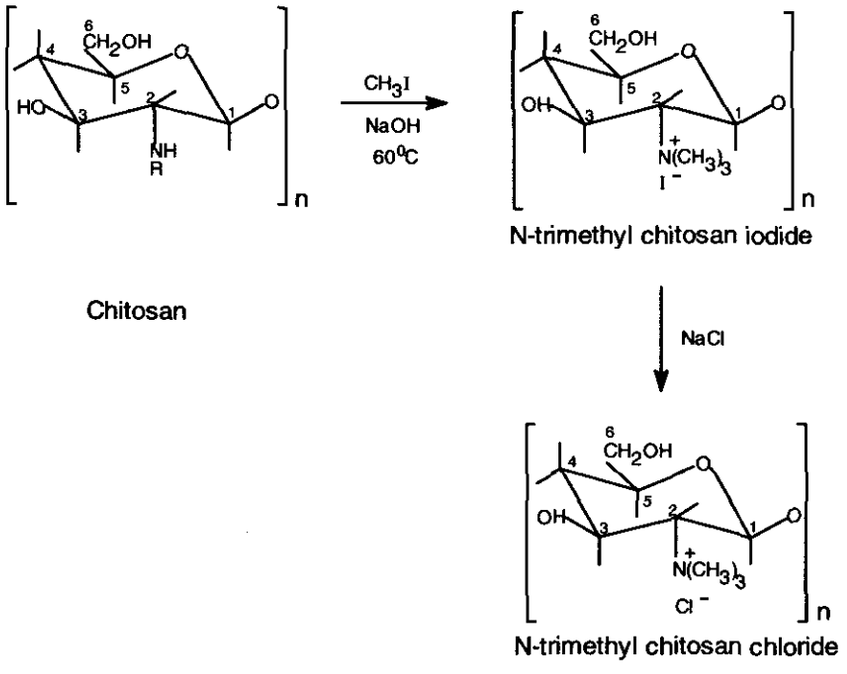


Figure 2.10: Synthesis of *N*-Trimethyl chitosan chloride (TMC) (Thanou *et al.*, 2001:122).

2.2.2 Degree of quaternisation

Previous studies have shown that TMC is a potent absorption enhancer for hydrophilic and macromolecular compounds across mucosal surfaces. TMC proved to be effective in neutral and basic pH environments where the absorption enhancing ability of chitosan is severely hampered by its insolubility in these environments. Different degrees of quaternisation were obtained by varying the number and duration of the reaction steps in

the synthesis process of TMC. The degree of quaternisation of TMC has been shown to play an important role on the absorption enhancing properties of this polymer, especially in neutral and basic pH environments. TMC with a degree of quaternisation of 61.2 % and TMC with a degree of quaternisation of 12.3 % were evaluated in Caco-2 monolayers using the hydrophilic model compound [^{14}C] mannitol. It has been shown that the higher quaternised chitosan is a potent absorption enhancer at a pH of 7.4 where the lower quaternised chitosan was ineffective as an absorption enhancer. The results were explained by the proportion of quaternary amino groups, on the polymer with the higher degree of quaternisation, which seems to be sufficient to interact with the cell membranes or the negative sites within the tight junctions (Jonker *et al.*, 2002:206).

2.2.3 Mucoadhesive properties

It has been shown that the degree of quaternisation has an effect on the absorption enhancing properties of TMC and it is expected that the same could be true for the mucoadhesive properties of this polymer. During the synthesis of TMC, the amount of fixed positive charges on the polymer chain is increased, probably causing expansion of the polymer in solution. As the mucoadhesive properties of a polymer are a function of the chain flexibility it is expected that the degree of quaternisation will have an effect on its mucoadhesive properties as an increase in the degree of quaternisation decreases this chain flexibility. Furthermore, during synthesis, degradation of the polymer chain also occurs, due to factors such as the strong alkaline environment and elevated temperatures. The molecular weight of a polymer has an effect on its mucoadhesive properties up to a value of 100000 g/mole, thereafter any further increases has no noticeable effect. In the development of mucoadhesive drug delivery systems, the contact time at the site of absorption of the intended drug molecule is important for the adhesive effect of an excipient in a controlled release mucoadhesive dosage form. This should decrease the rate of clearance and movement of the drug from the mucosal epithelia, such as in the nasal cavity and gastrointestinal tract, to allow for longer contact time with the absorptive epithelium. This extension of contact with the absorbing epithelia may be beneficial,

especially for hydrophilic drugs or for drugs with a high molecular weight such as peptides and proteins (Snyman *et al.*, 2003:62).

2.2.4 The effect of TMC on Transepithelial electrical resistance (TEER)

Measurement of TEER values is believed to be a good indication of the tightness of the junctions between cells. The results in figure 2.11 clearly demonstrate that TMC-L (12.6 % quaternised) and TMC-H (19.9 % quaternised) are able to decrease the TEER of Caco-2 monolayers (Kotze *et al.*, 1999:272).

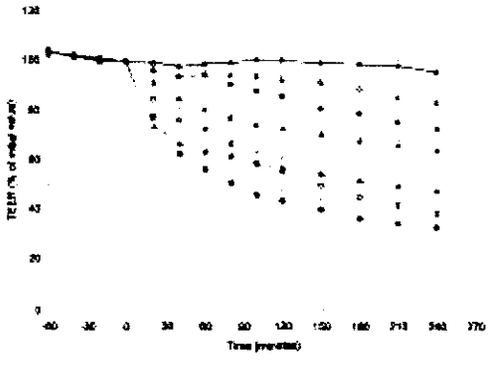


Figure 2.11: The effect of TMC-L and TMC-H on the TEER of Caco-2 monolayers. Each point represents the mean ± S.D. of three experiments. Control (♦), TMC-L 1.5 % (O), TMC-H 1.5 % (●), TMC-L 2.0 % (Δ), TMC-H 2.0 % (▲), TMC-L 2.5 % (□), TMC-H 2.5 % (■) (Kotze *et al.*, 1999:272).

The results presented here show that TMC-H, at similar concentrations, is more effective in reducing the TEER than TMC-L. The difference in effect at similar concentrations of TMC could be explained by the difference in the degree of quaternisation of these polymers. The degree of quaternisation gave an indication of the charge density on the respective polymers. TMC-H, with the highest degree of quaternisation, has more positively charged amino groups for interaction with the negative sites on the cell membranes and is therefore more effective in reducing the TEER of the monolayers at similar concentrations of TMC-L. Several studies have shown that chitosan acts

primarily by an interaction with the positively charged sites on the cell membranes and/or tight junctions. TMC is most likely to act by the same mechanism as chitosan salts. Previous studies have shown that it is unlikely, because of the high viscosity and mucoadhesive character of chitosan and TMC, that all the polymer solution could be removed from the monolayers without damaging the cells. Only a gradual reversibility of the effect of these polymers on the TEER was demonstrated while complete reversibility towards initial TEER values, could not clearly be established.

In conclusion, TMC reduces the TEER of intestinal epithelial cells, thereby opening the tight junctions to allow for the paracellular transport of these hydrophilic molecules. The degree of quaternisation of TMC plays an important role in determining the ability and effectiveness of TMC to open the tight junctions, which regulates permeation through the paracellular transport pathway. The degree of quaternisation determines the amount and density of the positive charges on the C-2 position of this derivative, which could result in different effects on the TEER and permeability of Caco-2 cells (Kotze *et al.*, 1999:272).

2.2.5 Toxicity

Chitosan is considered as a biocompatible, biodegradable and non-toxic polymer. It does not have a damaging effect on nasal membranes in rats and also does not affect the mucociliary clearance rate after daily nasal application in human volunteers. Even when chitosans of different molecular weights were applied chronically on guinea pig nasal epithelia, they had a mild effect on the nasal ciliary beat frequency of the excised tissue. However, in one study using a murine melanoma cell line (B16F10) and rat erythrocytes it has been found that chitosan affected the cell viability and increased the haemoglobin release in a concentration-dependent way (Thanou *et al.*, 1999:74). Because of the high molecular weight of chitosan and TMC these polymers are generally regarded as safe compounds.

2.3 Emzaloid™ (Emzaloid/Emzaloids)

2.3.1 Introduction

Emzaloids are an oil/water emulsion type preparation initially made by MeyerZall Labratories PTY (LTD). An Emzaloid is a stable structure within a system that can be manipulated in terms of morphology, structure, size and function. Emzaloids can entrap, transport and deliver pharmacologically active compounds and other useful molecules. They consist mainly of plant and essential fatty acids (Saunders *et al.*, 1999:99).

Emzaloids have a few advantages that make it a more effective delivery system than other lipid-based delivery systems. These are listed below:

- Increased delivery of active compounds
- Decreased time to onset of action
- Reduction in cytotoxicity
- Penetration of most known barriers in the body and cells
- Ability to target treatment areas
- Lack of immunological response
- Ability to transfer genes to cell nuclei
- Reduction of drug resistance.

Although, all Emzaloid-based products currently on the market are topical products, recent research and development concerned itself with other applications of Emzaloid technology in oral and parenteral administration. In several previous studies, Emzaloid showed better results than currently used medicine. For example, tuberculosis (TB) therapy is a long and intense course of drugs that need to be taken consistently, and the patient compliance is very low. With the use of Emzaloid drug therapy the treatment may possibly be shortened from 6 months to 2-3 months, intervals between dose administrations may be increased, and an increase in compliance and fewer chances of drug resistance may be obtained (Grobler, 2004:3).

2.3.2 Characteristics of Emzaloid

Emzaloids are a unique delivery system and can be manipulated in a very specific manner to ensure its high entrapment capabilities, very fast rate of transport, delivery and stability. With these characteristics, the absorption capabilities and drug release can be controlled (Gorbler, 2004:3).

In the formulation of the Emzaloid, the fatty acids that are incorporated cannot be manufactured by the human cells, but are necessary for various cell functions and have to be ingested. Some of the advantages of these fatty acids are:

- Maintenance of membrane integrity of cells
- Energy homeostasis
- Modulation of the immune system through prostaglandins/leukotriens (Grobler, 2004:4).

2.3.2.1 Decreased time to onset of action

A potentially faster relief from symptoms was shown with the administration of Emzaloids. The Emzaloid delivery system crosses most physiological barriers and delivers the active. An active delivered via the Emzaloid has been shown to act significantly quicker than that same active delivered via a conventional approach (Grobler, 2004:9).

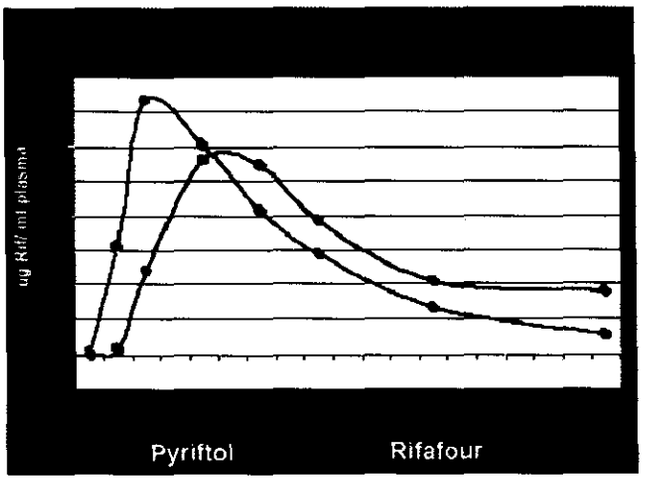


Figure 2.12: The curves illustrate the average plasma levels of rifampicin for 14 healthy volunteers after oral administration of combination anti-tuberculosis DOTS treatment, with and without the Emzaloid (Pyriftol and Rifafour respectively). The time needed to achieve plasma C_{max} is halved by entrapment in Emzaloid when compared to that of one of the preferred comparative products (Grobler, 2004:9).

2.3.2.2 Increased delivery of active compounds

Both *in vitro* and *in vivo* studies showed that the percentage active compound delivered was enhanced by entrapment in Emzaloid (Grobler, 2004:9).

Table 2.1: Release rates and percentage release per label claim for product tested (Grobler, 2004:9).

Active Agent	% Active/product	Release Rate ($\mu g/cm^2/h$)	% Release per label claim
Acyclovir EMZ	0.5	69.1533	0.1214
Acyclovir COM	0.5	54.0942	0.0952
Miconazole Nitrate EMZ	2	389.9238	6.8155
Miconazole COM	2	111.2222	1.9466

2.3.2.3 Reduction of minimum inhibitory concentration

Research has shown that, for certain actives, using as little as 1/40 th of the active entrapped compound may result in an effective Emzaloid formulation. In practice, this characteristic would translate into reduction of patient side effects and cost savings in treatment (Grobler, 2004:10).

2.3.2.4 Increased therapeutic efficacy

In all cases tested, the formulation of an active compound in Emzaloid increased the efficacy. Examples of enhancement of the action of anti-infective agents, as determined by zone inhibition, are given in table 2.2 (Grobler, 2004:10-11).

Table 2.2: Zone of inhibition study: Five commercial anti-infective products against Emzaloid-formulations of the same active compound (Grobler, 2004:11).

Active agent	EMZ/ COM	Dose (mg/5ml)	S. Aureus	P. Aerugin	B. Cereus	E. Coli	A. Niger	C. Albicans
Cloxacillin	EMZ	125	30.74	23.96				
Cloxacillin	COM	125	29.45	19.78				
Erythromycin	EMZ	250	26.7		29.89			
Erythromycin	COM	250	25.84		27.78			
Ciprofloxacin	EMZ	250	33.05			35.78		
Ciprofloxacin	COM	250	30.14			33.4		
Cotrimoxazole	EMZ	240	13.95			24.64		
Cotrimoxazole	COM	240	11			22.83		
Itraconazole	EMZ	50					16.03	14.28
Itraconazole	COM	50					11.47	10.21
Control			9	9	9	9	9	9

2.3.2.5 Reduction in cytotoxicity

The Emzaloid system has the potential to minimise cellular damage that occurs as a result of membrane damage caused by active compounds. Side effects of the drugs are, in most instances, the results of cellular damage (Grobler, 2004:11).

2.3.2.6 Immunological responses

Some drugs, such as proteins or peptides, may induce an immunologic response or adverse intolerance reaction. Masking of the compounds by Emzaloids may reduce recognition by the patient's immune response. Frequency of dosing can be reduced without diminishing potency, or higher doses can be given to enhance therapeutic impact (Grobler, 2004:12).

2.3.2.7 Ability to entrap and transfer genes to cell nuclei and expression of proteins

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

2.3.2.8 Reduction and suggested elimination of drug resistance

The Emzaloid has been shown to reduce or eliminate drug resistance *in vitro*. Analysis of bacterial growth of multidrug resistant TB have shown that formulations containing the standard antimicrobial, rifampicin, entrapped in Emzaloid, obviated pre-existing drug

resistance. The ability to potentially revive the effectiveness of antibiotics such as penicillin has widespread application in the healthcare industry (Grobler, 2004:13).

2.3.3 The Emzaloid versus other lipid based delivery systems

Conventional macromolecular carriers, such as liposomal delivery systems, are substantially different from the Emzaloid delivery system. Table 2.3 provides a comparison of the similarities, differences and key advantages of the Emzaloid and other lipid-based or liposomal drug delivery systems:

Table 2.3: Similarities and differences of Emzaloid and lipid-based delivery systems (Grobler, 2004:6).

Emzaloid	**Other lipid-based delivery systems**
Consists mainly of essential fatty acids, a natural and essential ingredient of the body	Generally contain substances foreign to the body
Cytokine studies demonstrated that the Emzaloid elicits no immune responses in man	Some liposomal formulations have been shown to elicit immune responses
Emzaloid can be manipulated in terms of size, charge, lipid composition and membrane packing	Problems with the degree of repeatability of liposomal systems, liposomal types and sizes have been described
Emzaloid consists of fatty acids and an affinity exists between the Emzaloid and cell membranes	Specific binding and uptake mechanisms have not been described for other delivery systems
The Emzaloid is polyphilic and drugs that have different solubilities as well as insoluble drugs can be entrapped	Most delivery systems are either lipophilic or hydrophilic
Sterically stabilised without the disadvanta-	Delivery systems generally need to be

ges of increased size or decreased elasticity	sterically stabilised
Entrapment in Emzaloid changes the pharmakinetics of active compounds, resulting in a decrease in the time needed to achieve maximum concentration levels	Liposomes have similarly been shown to change the pharmacodynamics of active compounds
The type of Emzaloid formulated for a specific compound determines the loading capacity of that Emzaloid	The loading capacity of most lipid-based delivery systems is dependent on the interior or intra-membrane volume and is therefore limited
The Emzaloid showed *in vivo* stability during vaccine animal studies and in initial phase I volunteer trails	Both product and *in vivo* chemical and physical instability are problematic for some lipid-based delivery systems

2.3.4 The role of Emzaloid in vaccination studies

Previously, smallpox could only be cured by vaccination. The problem with vaccination is that it only gives systemic protection against viral diseases. With the new Emzaloid technology for oral vaccination, it is intended to give systemic as well as humoral protection. A few experimental vaccines have already been developed with the new Emzaloid technology, for example:

- A virus-based vaccine against rabies.

More than 2.5 billion people live in regions where rabies is endemic and more than 10 million receive post-exposure vaccination against this disease. Each year at least 50 000 people die from rabies. The inactivated virus is used in the formulation of rabies vaccines. An Emzaloid-adjuvanted rabies vaccine showed a 9-fold increase in antibody response in comparison to the unadjuvanted sample (Grobler, 2004:18).

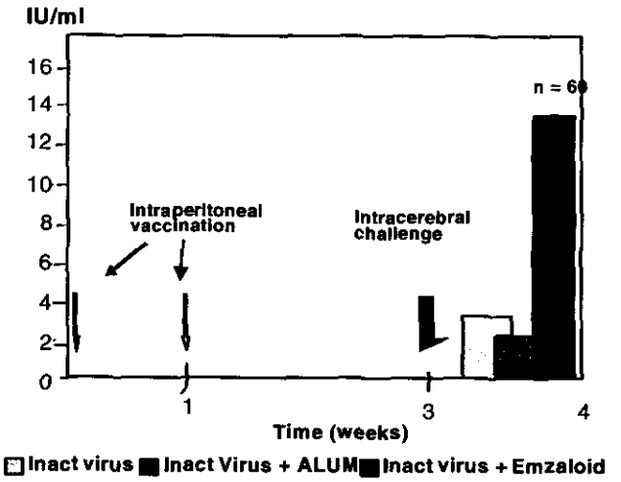


Figure 2.13: The results of an animal trial with the Emzaloid-based proposed rabies vaccine (Grobler, 2004:18).

- A peptide-based vaccine against hepatitis B

  Emzaloid is based on a micro-colloidal carrier system that conferred marked advantage in drug delivery over competitive products. Non-recombinant hepatitis B vaccines are generally based on the use of one of the surface molecules of the virus as antigen. The induction of an antibody response was monitored in a mouse study, following the entrapment of this peptide in Emzaloids. The results are given in figure 2.14 (Grobler, 2004:18-19).

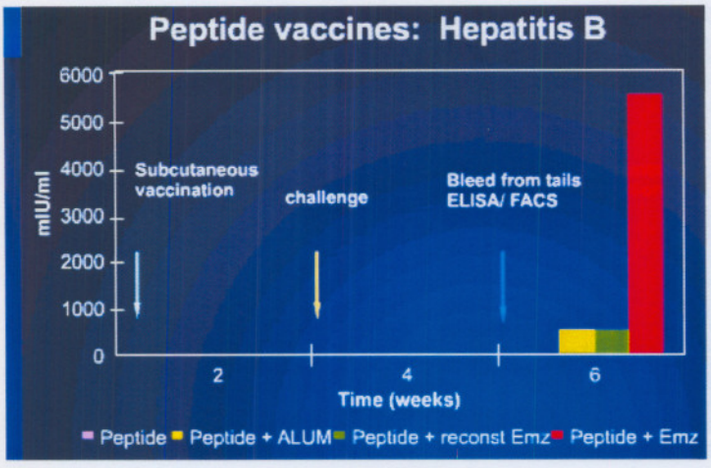


Figure 2.14: The increase in antibody production against hepatitis B after inoculated mice were challenged with the virus. Various vaccine formulations were used to inoculate mice subcutaneously, and the antibodies present in their blood, against hepatitis B, were determined. The enhancement in specific immune response obtained in the Emzaloid-based vaccine was dramatic (Grobler, 2004:19).

2.3.5 The different types of Emzaloids

Figure 2.15 show, confocal laser scanning microscopy (CLSM) micrographs of active compounds entrapped in several Emzaloid types. Each type has a specific composition. The size and shape of the vesicles can be reproducibly controlled.

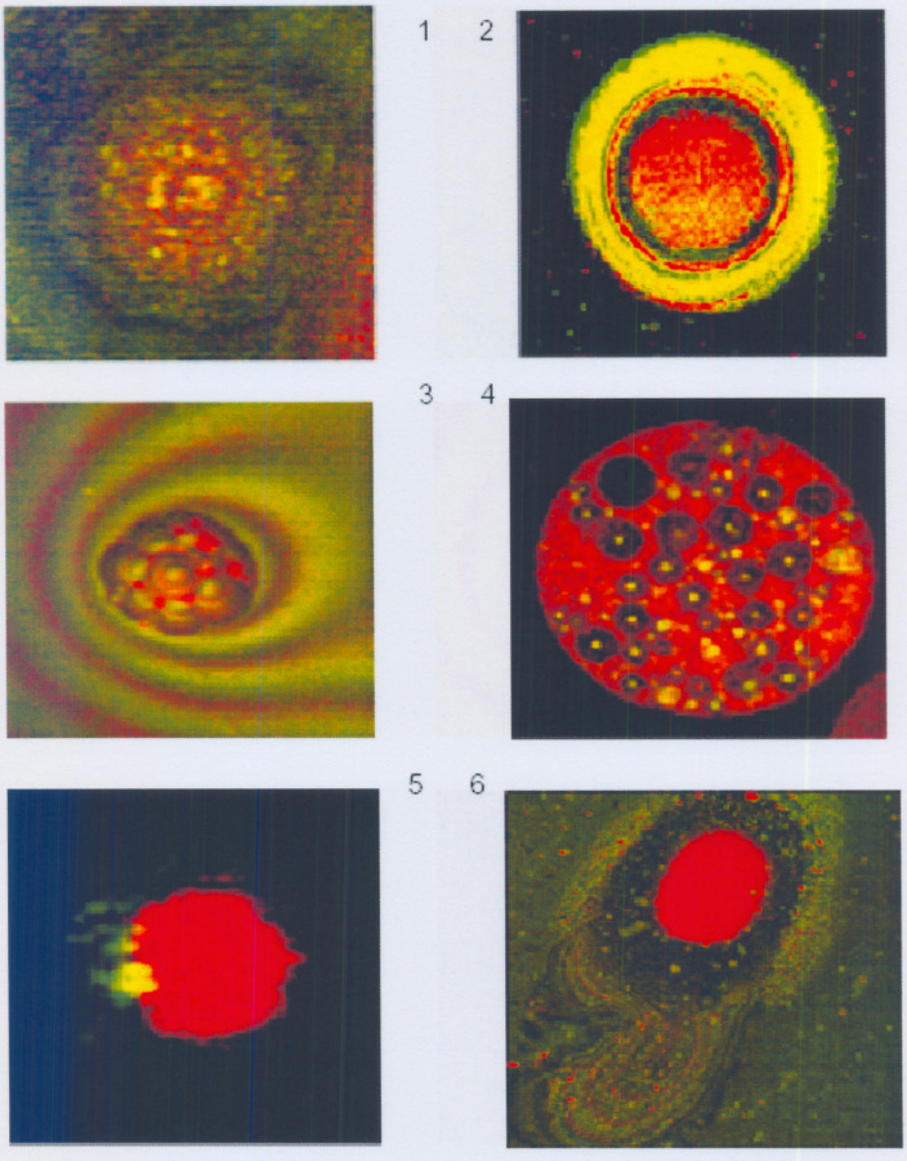


Figure 2.15: Some of the basic Emzaloid types

1. A bilayer membrane vesicle with diameter of 100 nm containing rifampicin.

2. Highly elastic or fluid bilayered vesicles with loose lipid packing containing rifampicin.

3. The formation of small pro-emzaloids. The formulation is used for some oral administrations.

4. The reservoir contains multiple particles of coal tar. Reservoirs have a large loading capacity to surface area ratios and are good entrappers of insoluble compounds. General size is 1-10 μm.

5. This Emzaloid is in the process of entrapping fluorescently labelled water-soluble diclofenac. It is very small and the membrane packing is sponge-like.
6. A depot with a hydrophobic core containing a pro-emzaloid formulation, a surrounding hydrophilic zone and an outer vesicle-containing zone. Selective addition of fluid results in the release of vesicles from a release zone. The depots are used for sustained release according to a concentration gradient and can range in size from 5 to 100 μm. The sizes of Emzaloid reflected above are not all to scale (Grobler, 2004:5-6).

2.3.6 Pro-emzaloid

The pro-emzaloid consists of increased concentrations and larger polymers. All Emzaloid systems contain a small polyethylene glycol (PEG) component. PEG is a non-reactive and non-toxic polymer that is frequently used in food and pharmaceutical products. PEG has been shown to contribute to the following aspects of drug administrations

- Increased bioavailability
- Increased drug stability and extended circulating life
- Lower toxicity
- Enhanced drug solubility

PEG has been shown to render a protein therapeutically effective, where the unmodified form had not been effective (Grobler, 2004:12).

2.4 Conclusion

The oral absorption of biotechnology products remains a major challenge, but considerable progress has been made over the past five years in developing innovative

technologies for promoting absorption across the gastrointestinal tract (Brayden & O'Mahony, 1998:291).

A number of significant advantages involving vaccination in the oral route is most notably, easy administration and improved safety. In addition, unlike systemic immunisation, mucosal delivery results in the induction of the secretory immune response, through secretory IgA. A significant amount of research in recent years has involved the encapsulation of antigens into PLG polymers. More recently, several similar approaches have been applied, involving the micro-encapsulation of antigens into a range of alternative polymers. These alternative approaches are mainly being employed because PLG polymers are deemed unattractive for some antigens, due to the use of organic solvents during the manufacturing of microparticles (O'Hagan, 1998:316).

Previous studies have shown that chitosan microparticles represent a new vaccine delivery system for both oral and nasal immunisation. Systemic (IgG) and local (IgA) immune responses against diphtheria, associated with chitosan microparticles, were strongly enhanced after oral vaccination delivery in mice. Furthermore, dose-dependent systemic immune responses could be elicited and enough antitoxin was produced to provide protection against the harmful effects of the diphtheria toxin. It may be concluded that chitosan microparticles can be suitable for oral vaccine delivery (Van der Lubben *et al.*, 2003:1411-1412).

Some chitosan salts and a derivative of chitosan, TMC, are able to increase the transport of large hydrophilic compounds. These polymers reduce the TEER of intestinal cell monolayers in a reversible way to allow for paracellular transport. Confocal laser scanning microscopy confirms that chitosan salts and TMC open the tight junction that regulates permeation through this alternative transport pathway. The charge, charge density and structural features of the chitosan salts and TMC play an important role in their ability to act as absorption enhancers (Kotze *et al.*, 1998:44-45). The degree of quaternisation of these polymers also plays an important role in the enhancement of the paracellular transport (Thanou *et al.*, 2000:24).

In previous studies, the Emzaloid delivery system was investigated as a vehicle for the delivery of antigens in an effort to enhance the efficacy of existing vaccines. According to these studies, the Emzaloid delivery system proved to have a dual role in vaccines. Firstly, as a delivery system for disease specific antigens, and secondly, as an immuno-stimulatory adjuvant. Results showed that experimental Emzaloid-based vaccines were 7 to 9 times more effective than the tested commercial vaccines. The use of Emzaloid technology showed a more than ten fold increase in the efficacy of the peptide-based hepatitis B vaccine. Thus, by changing existing vaccines to Emzaloid-based vaccines, a more effective vaccine could be produced and considerable development time and cost could be saved (Grobler, 2004:19).

Chapter 3
Preparation and characterization of chitosan, TMC and Emzaloid™ microparticles and nanoparticles

3.1 Introduction

Adjuvants are substances that enhance the ability of antigens to elicit an immune response. Recombinant proteins are potentially better defined and safer components for both veterinary and human vaccines. However, recombinant proteins are often weakly immunogenic and require adjuvants that are superior to aluminium to make them effective vaccines (Seferian & Martinez, 2001:661).

Chitosan, the deacetylated form of chitin, open tight junctions and allow paracellular transport across the epithelium. Oral drug delivery research has demonstrated that significantly higher amounts of macromolecular drugs can be transported after co-administration with chitosan. Besides its ability to facilitate paracellular transport, chitosan can also be used to prepare microparticles and nanoparticles (Van der Lubben *et al.*, 2001:201).

It has also been demonstrated that chitosan is a very good delivery system for oral vaccines. Previous studies have shown that chitosan can entrap vaccines, and that the loading and release properties of chitosan particles is very good. The only requirement is that the particles must be smaller than 10 μm for absorption by the M-cells of the Peyer's patches. These microparticles are then suitable to serve as vaccination systems. Chitosan microparticles have a very porous structure that facilitates entrapment of the vaccines. Previous studies have shown that the release of the vaccine is very low within 4 hours and most of the vaccine remained entrapped in the particles (Van der Lubben *et al.*, 2001:698).

Despite the fact that chitosan microparticles are a good delivery system, chitosan nanoparticles are more effective because of their small size. Chitosan nanoparticle sizes range from 50-500 nm, and particles smaller than 100 nm have shown to be taken up by Caco-2 cells, whereas the microparticles end up in the Peyer's patches after uptake by the M-cells. For effective vaccine delivery, the lymphoid tissue must be targeted (Van der Lubben *et al.*, 2001:201).

Despite all these properties, chitosan is still a polymer that lacks the advantage of good solubility at physiological pH values. It aggregates in solutions with pH values above 6. The polymer is therefore only soluble in acidic solutions (pH 1-6), where most of the amino groups are protonated. To overcome this problem a chitosan derivative, *N*-trimethyl chitosan chloride (TMC), has been synthesised previously. In this study TMC was also investigated as an oral vaccine delivery system (Thanou *et al.*, 2000:15).

The Emzaloid system, based on Emzaloid technology, is able to enhance the absorption of various categories of drugs. The greatest advantage of Emzaloid in contrast to chitosan and TMC is the method of preparation. Larger quantities can be produced in a much shorter time. The loading of particles are much more effective and quicker than that of other formulations. Experimental Emzaloid-based vaccines were found to be between 7 and 9 times more effective than the tested commercial vaccines. Thus, reformulation of existing vaccines in Emzaloid should save considerable development time and cost, whilst at the same time producing a more effective vaccine (Grobler, 2004:6).

This chapter describes the preparation and characterisation of chitosan, TMC and Emzaloid micro- and nanoparticles. The loading and release properties of these particles for diphtheria toxoid were also investigated. The stability of chitosan and TMC particles are also described. These particles were used for an *in vivo* vaccination study in mice with diphtheria toxoid.

3.2 Preparation and characterisation of chitosan micro-particles

3.2.1 Materials

Chitosan was a generous gift from Leiden University, The Nederlands and was obtained from Primex Ingredients (Germany). The viscosity of the chitosan used was measured as a 1 % (w/v) chitosan in a 1 % (v/v) acetic acid solution in deionised water on a rotation viscosimeter (Haake, Germany) and was 20 mPa.S. The degree of deacetylation was given as 93 % and the solubility was ± 95 %. Soduim sulphate and Tween 80® were obtained from Sigma (Germany). Acetic acid was obtained from Saarchem (South Africa). All other reagents were of analytical grade.

3.2.2 Method

A 0.25 % (w/v) chitosan solution was prepared in a mixture of 2 % (v/v) acetic acid and 1 % (w/v) Tween 80® in deionised water. To this solution 2 ml of a 10 % (w/v) sodium sulphate solution was added drop-wise (about 1 ml/min) to 200 ml chitosan solution under magnetic stirring and continuous sonication (Branson sonifier 250 at 55 Watt and a Horn frequency of 19.85-20.05 kHz. The tip used in the particle preparation was a Branson flattip ½). After adding the sodium sulphate solution, stirring and sonication was continued for 20 min. The microparticle suspension was subsequently centrifuged for 25 min at 2750 rpm. The pellet was resuspended in deionised water to wash the microparticles and centrifuged again. This washing procedure was repeated twice before freeze-drying of the pellet overnight. Freeze-drying was performed with a Freezemobile 6 (Virtis Gardiner, USA) freeze-dryer.

3.2.3 Characterisation

The size of the chitosan microparticles was determined with an Accusizer 770 (PSS, USA). Surface visualisation of the microparticles was done with field emission scanning electron microscopy (SEM) with a Quanta 200 SEM (Carl Zeiss, Germany). The zeta potential of the chitosan microparticles was measured in demineralised water at neutral pH with a Malvern 2000 zetasizer (Malvern, UK).

3.2.4 Results

The results of the size analysis of different batches of the prepared chitosan microparticle formulations are given in table 3.1. From table 3.1 it is clear that there are dramatic differences in size when the particle size was measured directly after suspension in the sample bath of the apparatus and after another analysis, on the same sample, 5 min after initial suspension. The size decreases dramatically after stirring and the standard deviation is considerably smaller for the particles that were stirred for 5 minutes.

This result is a clear indication that the particles tend to aggregate in the dry state and that stirring in solution result in deaggregation of the particles. Eventually the four different batches prepared were mixed and used for the vaccination study in the mice. From table 3.1 it is clear that the microparticles are smaller than 10 μm and this is considered suitable for oral vaccination.

Table 3.1: Size analysis of the chitosan microparticles.

Batch	Time (min)	d(0.1) (μm)	d(0.5) (μm)	d(0.9) (μm)	Annexure
C1	0	17.38	63.59	203.96	Annexure 1

C1	5	1.55	2.12	3.88	Annexure 1
C2	0	11.85	55.51	199.35	Annexure 2
C2	5	1.83	2.75	6.39	Annexure 2
C3	0	6.85	29.88	127.02	Annexure 3
C3	5	1.92	3.04	6.74	Annexure 3
C4	0	4.36	14.32	40.46	Annexure 4
C4	5	1.43	2.38	5.33	Annexure 4
Mean	0	10.11 ± 5.76	40.83 ± 22.70	142.69 ± 76.73	_
Mean	5	1.68 ± 0.23	2.57 ± 0.40	5.59 ± 1.29	_

It proved difficult to measure the zeta potential of the particles and consistent values were not obtained. However, zeta potential values in the positive mV range were obtained for the microparticles. In figure 3.1 and 3.2 micrographs of the microparticles, obtained with a SEM, are depicted.

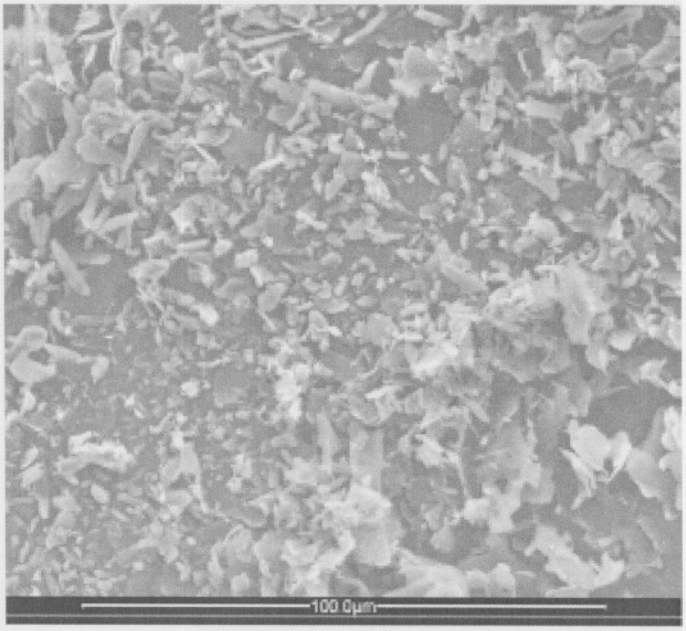


Figure 3.1: Surface visualisation of the chitosan microparticles.

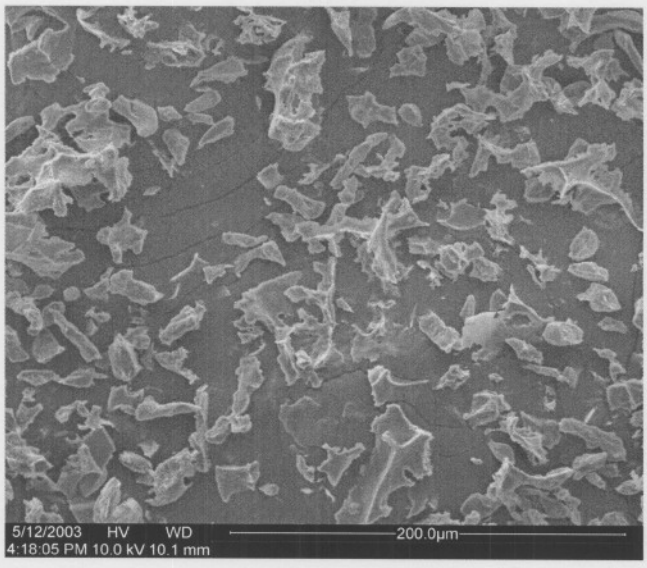


Figure 3.2: Detail of the surface of the chitosan microparticles

3.3 Preparation and characterisation of chitosan nanoparticles

3.3.1 Materials

The viscosity of the chitosan (Primex, Germany) used, was measured as a 1 % (w/v) chitosan in 1 % (v/v) acetic acid solution in deionised water on a rotation viscosimeter (Haake, Germany) and was 15 mPa.S. The degree of deacetylation, as determined by the supplier was 97 % and the solubility was 99.2 %. Tripolyphosphate (TPP) (Sigma, Germany) and acetic acid (Saarchem, SA) was also used in the preparation of the nanoparticles.

3.3.2 Method

A 0.25 % (w/v) chitosan solution was prepared in 100 ml deionised water containing 500 µl acetic acid (solution A) and a 0.10 % (w/v) TPP solution was also prepared in deionised water (solution B). Of solution B 8 ml were added drop-wise to solution A

while stirring at a 1000 rpm on a magnetic stirrer (Heidolph, Germany). The resulting suspension was then centrifuged at 6500 rpm in an Eppendorf 5415C Centrifuge (Eppendorf, Germany) for 10 min. The pellet was washed 4 times with deionised water and then put in a sonication bath for an hour. This suspension was freeze-dried overnight.

3.3.3 Characterisation

The size distribution of the prepared chitosan nanoparticles could not be determined with an Accusizer 770 (PSS, USA) because the optical lense and laser capacity of this particular apparatus only allow detection of particles larger than 500 nm. Visualisation of the prepared particles was done with a transmission electron microscope (TEM) (Phillips, The Netherlands).

3.3.4 Results

Figure 3.3 to 3.5 depicts typical TEM micrographs of the prepared chitosan nanoparticles. An accurate quantitive analysis of the mean particle size, with the TEM was not possible due to statistical reasons. However, it was clear that the size of the nanoparticles, as measured with the scale bar of the TEM, was between 50 – 450 nm. This was a clear indication that the method used to prepare the nanoparticles was suitable for preparation of chitosan nanoparticles smaller than 500 nm. As was observed with the chitosan microparticles, aggregation also occurred between the dry nanoparticles but it could also be assumed that deaggregation will occur in solution with stirring. Since TEM is done in static samples this could not be verified.

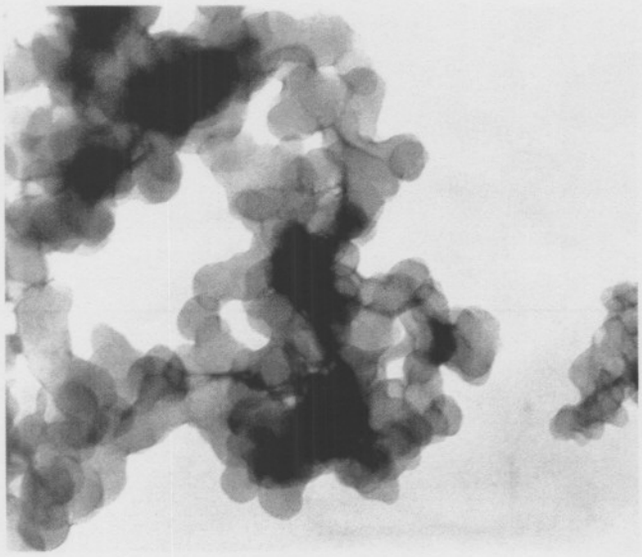


Figure 3.3: TEM micrograph of freshly prepared (wet) chitosan nanoparticles.

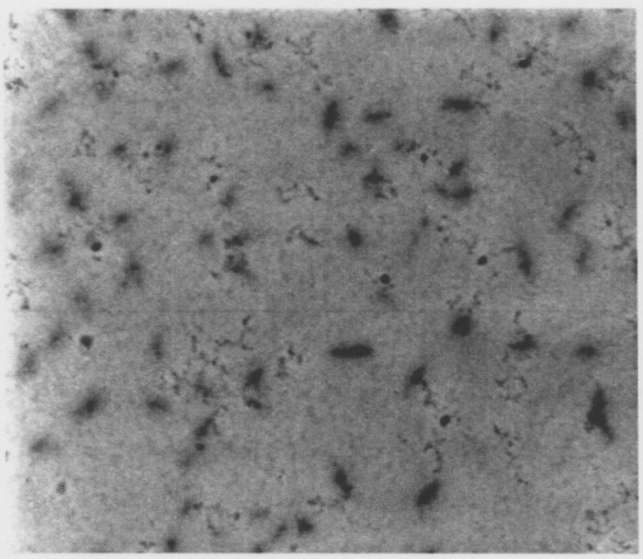


Figure 3.4: A low magnification (×73000) micrograph of the prepared chitosan nanoparticles.

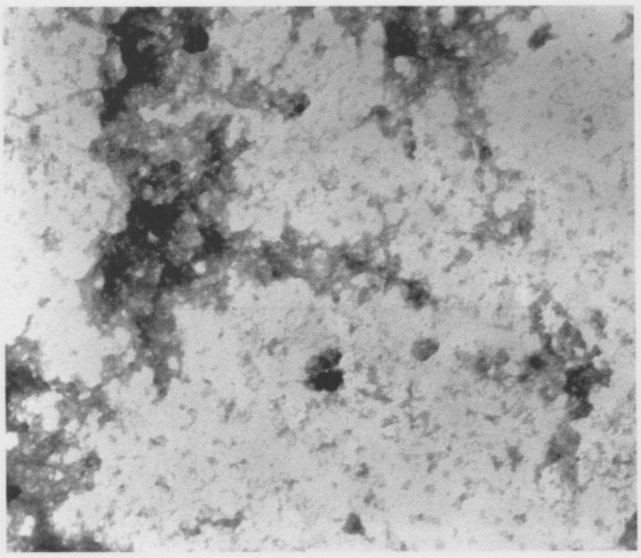


Figure 3.5: Evidence of aggregation between chitosan nanoparticles.

3.4 Preparation and characterisation of TMC microparticles

3.4.1 Materials

TMC was synthesised with the same batch of chitosan that was used for the preparation of chitosan nanoparticles (3.3.1). Sodium iodide, sodium hydroxide, methyl iodide, N-methyl-2-pyrrolidinone, NaOH, NaCl (Sigma, Germany), ethanol and diethylether (Saarchem, SA) was also used to synthesise TMC.

3.4.2 Method

TMC was synthesised in a one-step reaction according to the method of Sieval *et al.* (1998:157). In the chemical reaction used the primary amino group on the C-2 position of chitosan is changed to a quaternary amino group by substitution of the hydrogen atoms with methyl groups. The number and duration of the reaction steps involved in the

synthesis process could control the degree of quaternisation of TMC. By controlling these factors during the synthesis procedure, TMC polymers with different degrees of quaternisation can be synthesised. For the purpose of this study, only a one-step reaction was used to synthesise TMC with a low degree of quaternisation.

Chemical reaction: A mixture of 2 g chitosan, 4.8 g of sodium iodide, 11 ml of a 15 % aqueous sodium hydroxide solution and 11.5 ml of methyl iodide in 80 ml of N-methyl-2-pyrrolidinone was magnetically stirred for 1 hour on a water bath at 60 °C. Special care was taken to keep the methyl iodide in the reaction mixture by using a Liebig condenser. The product was precipitated with ethanol and isolated by centrifugation.

Ion exchange step: After washing the final product with ethanol and diethyleter, the product was dissolved in 40 ml of a 10 % NaCl solution to exchange the iodide-ion with a chloride-ion and once again precipitated with ethanol.

Purifying step: To remove any residual NaCl, the polymer was dissolved in 40 ml of water and precipitated from the solution with ethanol and diethyleter. The final product was dried in a vacuum oven at 40 °C.

Preparation of microparticles: A 0.25 % (w/v) TMC solution was prepared in deionised water containing 1 % Tween 80®. Then 5.5 ml of a 5 % (w/v) tripoly 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 solution, stirring and sonication was continued for 25 minutes at 2750 rpm. The pellet was resuspended in deionised 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 using a Christ freeze-dryer (Osterode am Harz, Germany).

3.4.3 Characterisation

The degree of quaternisation of the prepared TMC was determined from a ^{1}H-NMR spectrum obtained with a 600 MHz Bruker NMR apparatus (Bruker, Switzerland) in D_2O at 80 °C with suppression of the water peak. The degree of quaternisation (DQ) was calculated with the following equation:

$$DQ\ (\%)=[(\frac{\int TM}{\int H}) \times \frac{1}{9}] \times 100$$

Where $\int^{TM}$ is the integral of the trimethylated peak and $\int^{H}$ is the integrals of ^{1}H.

The size of the TMC microparticles was determined with an Accusizer 770 and surface visualisation was done with a SEM as described in section 3.2.3 for the chitosan microparticles. No zeta potential measurements were performed.

3.4.4 Results

Figure 3.6 depicts the ^{1}H-NMR spectra of the synthesised TMC. Sieval *et al.*, (1998:158) noted that the peak at 3.3 ppm was assigned to the trimethyl amino group [$N(CH_3)_3^+$] and the peaks between 4.7 and 5.7 ppm were assigned to ^{1}H. The integrals of those peaks form the ^{1}H-NMR spectra of each product were substituted into the equation given in 3.4.3 and the degree of quaternisation of the synthesised polymer were calculated. The synthesised TMC has a degree of quaternisation of 22.53 %. This low degree of quaternisation is due to the fact that it was only a 1-step synthesis procedure.

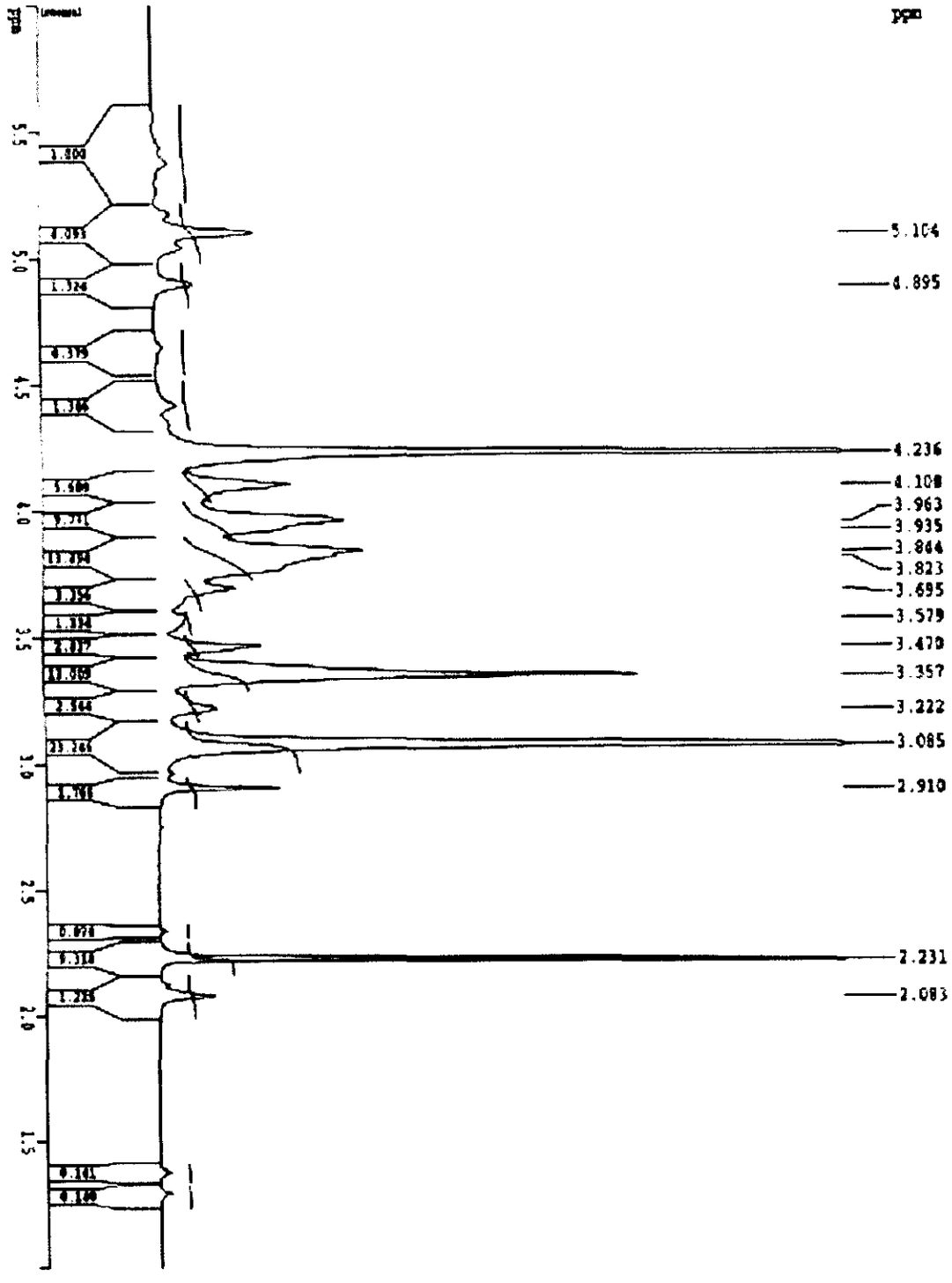


Figure 3.6: ^{1}H-NMR spectrum of TMC with a degree of quaternisation of 22.53 %

Table 3.2 shows the results of the size analysis of the two prepared batches of TMC microparticles. From table 3.2 it is clear that there are dramatic differences in size when the particle size was measured directly after suspension in the sample bath of the apparatus and after another analysis, on the same sample, 5 min after initial suspension. The size decrease dramatically after stirring and the standard deviation is considerably smaller for the particles that were stirred for 5 minutes.

This result is a clear indication that the particles tend to aggregate in the dry state and that stirring in solution result in deaggregation of the particles. Eventually the two different batches prepared was mixed and used for the vaccination study in the mice. From table 3.2 it is clear that the microparticles are smaller than 5 μm and this is considered suitable for oral vaccination.

Table 3.2: Size analysis of the TMC microparticles.

Batch	**Time (min)**	**d(0.1) (μm)**	**d(0.5) (μm)**	**d(0.9) (μm)**	**Annexure**
T1	0	5.47	19.05	988.42	Annexure 5
T1	5	1.06	1.85	4.77	Annexure 5
T2	0	5.64	36.57	270.90	Annexure 6
T2	5	1.43	2.27	4.83	Annexure 6
Mean	0	5.56 ± 0.12	27.81 ± 12.39	629.66 ± 507.30	_
Mean	5	1.25 ± 0.26	2.06 ± 0.29	4.80 ± 0.04	_

In figure 3.7 and 3.8 micrographs of the prepared microparticles are depicted. The SEM confirmed that the particle size ranges between 1 – 5 μm. TMC nanoparticles were prepared but were not suitable for an oral vaccination study, because the TMC nanoparticles agglomerated and formed extremely hard sediments that could not be vortexed to form a suspension again. For this reason TMC nanoparticles were excluded from the *in vivo* vaccination study.

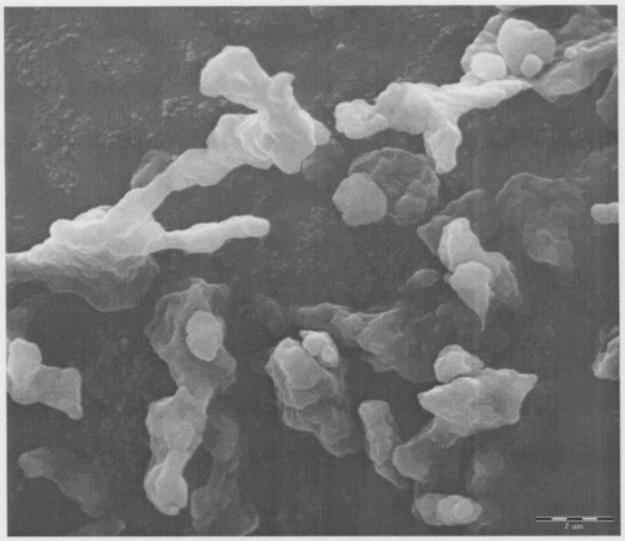


Figure 3.7: Detail of the surface of the TMC microparticles

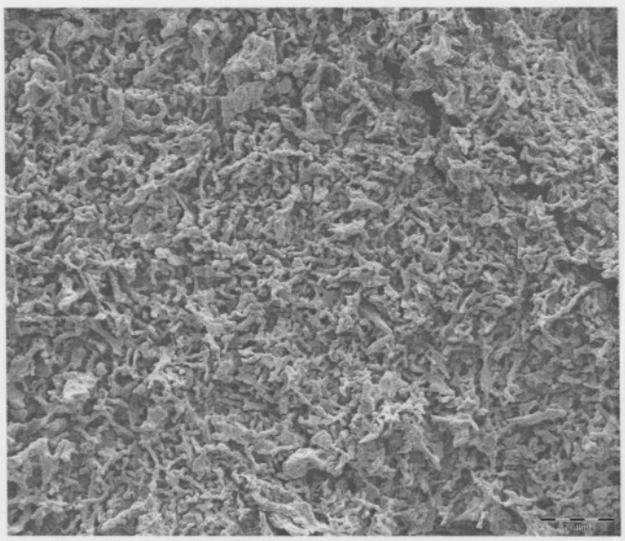


Figure 3.8: Surface visualisation of the TMC microparticles.

3.5. Preparation and characterisation of Emzaloid micro-particles

3.5.1 Materials

In the preparation of the Emzaloid microparticles, the following materials were used: Vitamin F ethyl ester (Kurt Richter Pharma, Germany) 1.5 g, Cremophor RH-40 (BASF, Germany) 0.45 g, dL-α tocopherol (Sigma, Germany) 0.2 g and N_2O saturated water 97.85 g.

3.5.2 Method

Step 1: Weigh off the Vitamin F ethyl ester, dL-α tocopherol and cremophor RH-40 in a closable container. This is the oil phase.

Step 2: Mix and heat the oil phase to 70 °C in a waterbath.

Step 3: Add 300 ml water in a glass beaker and weigh off the nitrous oxide water and close the top of the beaker and heat on a warm plate to 70 °C.

Step 4: While maintaining the N_2O saturated water at 70 °C, add the heated oil phase from Step 2 to the water. This must be done quickly.

Step 5: Mix with a Braun mixer, on speed 2 until the emulsion is homogenous. This must also be done quickly. Transfer the emulsion to a glass schott bottle and shake until room temperature is reached.

Step 6: Filter the Emzaloids with a 0.22 μm GD/X filter and seal in a sterile airtight container in a laminar flow hood.

3.5.3 Characterisation

The size distribution of the Emzaloid microparticles was determined by a confocal laser scanning microscope (CLSM) (Nikon PCM 2000 with digital camera DMX 1200, The Netherlands). The CLSM was used to determine the sizes of the particles. A He/Ne laser was used with an objective of 60 ×, with an emmision of 568 nm. The pinhole size of the CLSM was smaller than 5 μm, and an oil emulsion apoPlan 1.4 neumerical appitune was used. No neutral density filters was used. Surface visualisation of the microparticles was also done with the confocal laser scanning microscope (CLSM). The Emzaloid microparticles were stained with Nile Red at a concentration of 50 nM. The stained Emzaloids were placed on a glass slide and covered with a glass cover slip. The glass cover slip was sealed together using adhesive to prevent the Emzaloids form drying out. No zeta potential measurements were performed.

3.5.4 Results

Figure 3.9 depicts a typical confocal laser scanning microscope (CLSM) micrograph of the prepared Emzaloid microparticles. It was clear that the size of the microparticles, as measured with the scale bar of the CLSM, was between 2 – 4 μm. This was a clear indication that the method used to prepare the microparticles was suitable for preparation of Emzaloid microparticles smaller than 5 μm and this was considered suitable for oral vaccination.

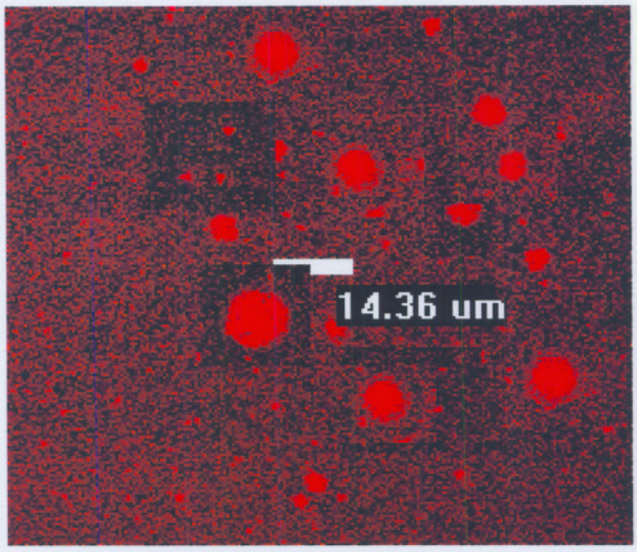


Figure 3.9: Surface visualisation of the Emzaloid microparticles as seen using the confocal laser scanning micrograph.

3.6 Preparation and characterisation of Emzaloid nano-particles

3.6.1 Materials

In the preparation of the Emzaloid nanoparticles, the following materials were used: Vitamin F ethyl ester (Kurt Richter Pharma, Germany) 1.5 g, Cremophor RH-40 (BASF, Germany) 0.45 g, dL-α tocopherol (Sigma, Germany) 0.2 g and N_2O saturated water 97.85 g.

3.6.2 Methods

Step 1: Weigh off the Vitamin F ethyl ester, dL-α tocopherol and cremophor RH-40 in a closable container. This is the oil phase.

Step 2: Mix and heat the oil phase to 70 °C in a waterbath.

Step 3: Add 300 ml water in a glass beaker and weigh off the nitrous oxide water and close the top of the beaker and heat on a warm plate to 70 °C.

Step 4: While maintaining the N_2O saturated water at 70 °C, add the heated oil phase from Step 2 to the water. This must be done quickly.

Step 5: Mix with a Braun Mixer on speed 2 until the emulsion is homogenous. This must also be done quickly. Transfer the emulsion to a glass schott bottle and shake until room temperature is reached.

Step 6: Filter the Emzaloids with a 0.22 µm GD/X filter and seal in a sterile airtight container in a laminar flow hood.

Step 7: Sonicate solution in a waterbath for 30 minutes at 37 °C.

3.6.3 Characterisation

The size distribution of the Emzaloid nanoparticles was determined by the CLSM as described in 3.5.3. No zeta potential measurements were performed.

3.6.4 Results

Figure 3.10 depicts a typical CLSM micrograph of the prepared Emzaloid nanoparticles. It was clear that the size of the nanoparticles, as measured with the scale bar of the CLSM, was between 400 – 500 nm. This was a clear indication that the method used to prepare the nanoparticles was suitable for preparation of Emzaloid nanoparticles smaller than 500 nm. As was observed with the chitosan micro- and nanoparticles, aggregation also occurred between the Emzaloid nanoparticles but it could also be assumed that deaggregation will occur with intake of the particles.

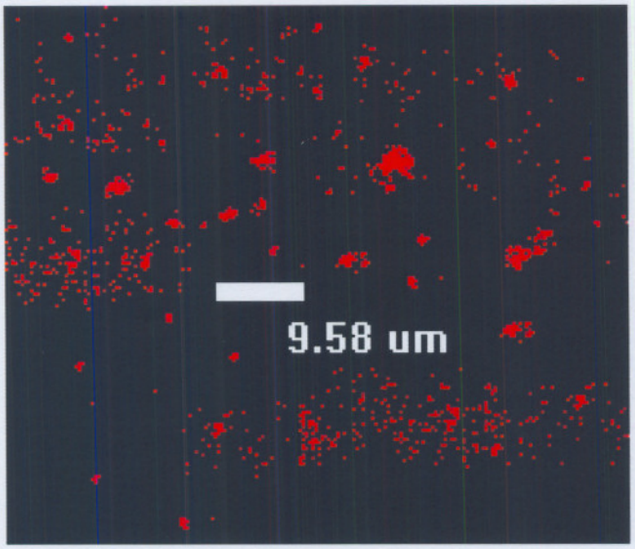


Figure 3.10: Surface visualisation of the Emzaloid nanoparticles.

3.7 Loading and release of Diphtheria toxoid (DT)

3.7.1 Materials

Diphtheria toxoid (DT) was a generous gift from Leiden University, The Netherlands. Chitosan microparticles, chitosan nanoparticles and TMC microparticles were prepared as described in section 3.2, 3.3 and 3.4. Phosphate buffered solution (PBS) (pH 7.30) was freshly prepared for the experiment. A BioRad protein assay (BioRad Labratories, Germany) was used to determine the loading and release of DT into the particles. It was not possible to determine the loading and release of DT into the Emzaloid micro –and nanoparticles with the same protein assay.

3.7.2 Method

The diphtheria toxiod (DT) loaded and released from the chitosan micro- and nanoparticles and the TMC microparticles were determined in PBS (pH 7.30). Loading

of the particles was done by incubating a 1 % (w/v) dispersion of the particles and a 0.25 % (w/v) solution of the toxoid. After incubation for 180 min, the suspension was centrifuged at 3000 rpm for 2 min to remove the free, unloaded DT. The loading degree was determined by quantifying the non-bound toxoid in the supernatant with the BioRad protein assay method. Both loading capacity (LC) and loading efficacy (LE) were determined as follows:

LC = [(total amount Diphtheria toxoid)-(free Diphtheria toxoid)]/Weight of particles
LE = [(total amount Diphtheria toxoid)-(free Diphtheria toxoid)]/Total amount of Diphtheria toxoid (Van der Lubben *et al.*, 2001:689)

Diphtheria toxoid released from the particles was also determined in PBS (pH 7.30). After loading of the particles they were resuspended in PBS to make a 1 % (w/v) particle suspension. Samples of 1 ml were incubated at 37 °C under mild shaking. After 15, 30, 45, 60, 90, 120, 180 and 240 min, the tubes were centrifuge for 1 min at 3000 rpm and samples of 250 µl of the supernatant were taken. These samples were replaced by 250 µl PBS (pH 7.30) and 100 µl was used for analysis. The non-bound toxoid was determined with the BioRad protein assay.

Loading of the Emzaloid micro- and nanopartilces was done by incubating a 1 % (w/v) solution of the particles and a 0.25 % (w/v) solution of the toxoid. This solution was shaken for 180 min to ensure that the particles were loaded. This solution was not centrifuged after loading and the release of the particles could not be measured. This solution was directly used for the oral vaccination study. Figure 3.11 shows that the Emzaloid particles are loaded with the DT as viewed with CLSM.

3.7.3 Results

Table 3.3 gives the results of the loading capacity (LC), the loading efficacy (LE) and the DT release of the particles. This results show that the chitosan microparticles had the best loading capacity and the highest loading efficacy for DT.

Table 3.3: The loading and release results of the different particle formulations

Formulation	Loading capacity (LC) (%)	Loading efficacy (LE) (%)	Release (%)
Chitosan microparticles	25.67 ± 0.60	88.86 ± 1.50	0.00 ± 0.00
TMC microparticles	18.28 ± 0.33	63.11 ± 1.16	0.00 ± 0.00
Chitosan nanoparticles	8.00 ± 0.11	27.74 ± 0.39	0.00 ± 0.00

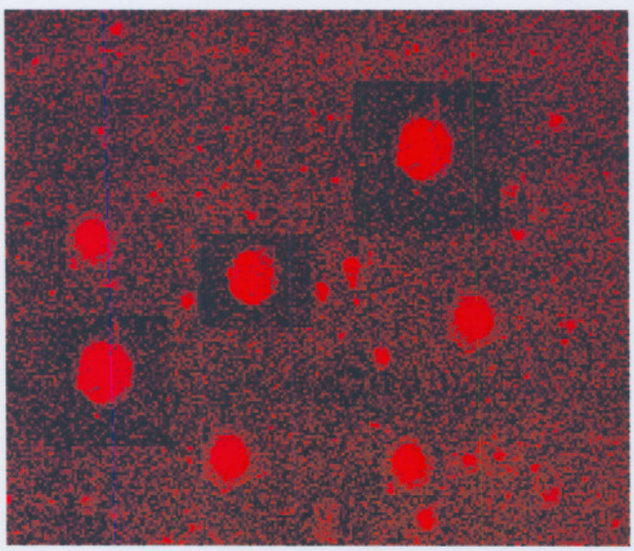


Figure 3.11: Emzaloid microparticles with the DT entrapped in the particles.

3.8 Stability studies on chitosan and TMC particles

3.8.1 Materials

Stability studies were conducted on chitosan and TMC particles loaded with DT and non-loaded particles. It was not possible to determine release of DT in the Emzaloid formulations.

3.8.2 Method

To determine the stability of the prepared chitosan microparticles, chitosan nanoparticles and TMC microparticles over a period of 3 months, DT loaded and non-loaded particles were stored at 4 °C and ambient temperature as 1 % (w/v) suspension in PBS (pH 7.30). Samples were taken from week 0 to week 13 after preparation for analysis of free DT.

3.8.3 Results

Table 3.4 gives a summary of DT release in weeks 0 and 13. Neither at 4 °C nor at ambient temperature were changes in the size of loaded particles observed. The results show that the DT entrapped in the particles are not released, even over a period of 13 weeks. It can be concluded that the particles loaded with DT are very stable and are suitable for vaccination studies.

Table 3.4: The stability of DT loaded particles at 4 °C and ambient temperature over a period of 13 weeks. The particles were loaded in week 0 and the toxoid released was measured every week for 13 weeks.

Formulation	Release: Week 1 (%)	Release: Week 13 (%)
Chitosan microparticles	0.00 ± 0.00	0.00 ± 0.00
TMC microparticles	0.00 ± 0.00	0.00 ± 0.00
Chitosan nanoparticles	0.00 ± 0.00	0.00 ± 0.00

3.9 Conclusion

This chapter describes the preparation and characterisation of chitosan, TMC and Emzaloid micro- and nanoparticles. Chitosan microparticles were prepared by crosslinking with sodium sulphate. The mean particle size was 10 μm. The zeta potential of the particles was difficult to measure but was in the positive mV range. Chitosan nanoparticles were prepared by crosslinking with tripolyphosphate (TPP). The particle size was measured by TEM and the average particle size was 50 - 450 nm. The zeta potential of the particles could not be measured. TMC microparticles were prepared by crosslinking with TPP. The particle size was 5 μm. No zeta potential measurements were performed.

Emzaloid microparticles were also prepared and the particle size was measured with confocal laser scanning microscope (CLSM). The average particle size was smaller than 5 μm. No zeta potential measurements were performed. Emzaloid nanoparticles were prepared and the particle sizes were also measured with CLSM. The average particle size was 400 – 500 nm. No zeta potential measurements were performed.

The above-mentioned chitosan, TMC and Emzaloid particles were also loaded with diphtheria toxoid (DT) and their loading capacity and loading efficacy were determined. Sufficient amounts of DT were loaded into the particles to make them suitable for use in an oral vaccination study. Both the chitosan and TMC formulations show excellent stability over a period of 13 weeks, which further strengthens its use in an experimental vaccination study.

Chapter 4
Oral vaccination in mice with chitosan, TMC and Emzaloid particles loaded with Diphitheria toxoid

4.1 Introduction

As an alternative to traditional parenteral vaccination, immunisation via the mucosal route has gained increasing interest, and the oral route is currently considered as the most desirable and the most challenging mode of vaccination against infectious diseases. Oral vaccines can induce both mucosal and systemic immune responses in contrast to parenteral vaccines that are poor inducers of mucosal immunity. Thus, peroral immunisation offers greater efficacy against strict mucosal pathogens as well as against micro-organisms initiating the infection process at mucosal surfaces. Moreover, oral vaccines would be less expensive to produce and to use, compared to the ones administered by injection, and their application would eliminate the risk of infection accompanying vaccination via a needle (Kesik *et al.*, 2004:197).

Many questions regarding the induction of mucosal and humoral immunity through oral vaccination exist. Efficacy is dependent on the physicochemical properties of the antigen, the gastrointestinal environment, the presence of adjuvants and the mode of delivery. Understanding how these factors interrelate will be critical to the development of new oral vaccines (Shalaby, 1995:127).

The intestine comprises 70 to 80 % of all immunoglobulin-producing cells in the body and produces more s-IgA per day than the total production of IgG per day. The inductive site for s-IgA production occurs in the Peyer's patches. The Peyer's patches contain a dome region at its luminal surface that is enriched with lymphocytes, macrophages and plasma cells. Phagocytic cells called microfold or M-cells cover the dome region, which transport luminal antigens to underlying follicles via uncoated and coated pits. Morphologically, the absences of a brush border, a thin glycocalyx on the apical surface

and the presence of an invaginated basolateral membrane characterise the M-cells. The small distance between the apical and basolateral membranes facilitates rapid transcytotic movement of endocytic vesicles. Endocytosis does not result in significant antigen digestion presumably due to the lack of fusion with lysosomes (Shalaby, 1995:127).

Recent use of lower particle numbers in intestinal loops and improved confocal assessment at the single cell level, suggest that less than 0.01 % of biodegradable PLG microparticles administered to intestinal loops actually bind to the gastrointestinal tract wall. Handfuls of these few micron-sized particles were seen in M-cells of the follicle-associated epithelium (FAE) rather than in villous or FAE-associated enterocytes (Brayden, 2001:183).

In chapter 3 the preparation and characterisation of chitosan, TMC and Emzaloid particles were described. It was also show that it is possible to load sufficient amounts of Diphtheria toxoid into these particles and that these loaded particles show excellent stability properties over a period of 13 weeks. This chapter describe the oral administration of the particles in mice to determine their local and humoral vaccination response.

4.2 Preparation of particles

The loading of the particles with the Diphtheria toxoid (DT) was done as described in chapter 3 (section 3.7). The particles were loaded for 3 hours and the loaded particles were then given orally for Balb/c mice.

4.3 Animals

All procedures, using animals, were approved by the university's ethics committee and were strictly adhered to during this study. Female Balb/c mice (9-12 weeks) were bred

and kept at the Animal Research Centre of the North-West University. The mice were bred and kept under artificial conditions to ensure an ideal environment for optimal growth, as well as good health of the animals. Infection with pathogenic organisms was also minimised through breeding the mice in a germ free room with constant air movement. All the variables in the Animal Research Centre were kept constant and all the conditions, to which the mice used in this study were exposed to, are shown in table 4.1.

Table 4.1: Conditions at the Animal Research Centre of the North-West University

Condition	Recommended value*	Real value
Temperature	19 ± 2 °C	21 ± 2 °C
Relative humidity	55 ± 15 °C	55 ± 10 °C
Rate of ventilation/air movement	15 – 20 changes per minute	18 changes per minute
Light intensity	350 - 400 lux one meter above floor level	350 - 400 lux one meter above floor level
Light period	12 hours light and 12 hours dark	12 hours light and 12 hours dark

* Values recommended by the Animal Research Centre of the North-West University according to international standards.

4.4 Systemic immune response (IgG)

4.4.1 Materials

ELISA plates (Microlon 96W high binding microtiter plates, polystyrene 96 Well*F) were obtained from Sigma (Germany). The Carbonate buffer consists of sodium hydrogen carbonate and sodium carbonate (Merck, SA). Tween 20®, BSA and anti-

mouse IgG peroxidase conjugate were obtained from Sigma (Germany). TMB solution consists of acetate buffer (pH 4.60). Hydrogen peroxide (H_2O_2), ethanol, H_2SO_4 and dimethyl sulfoxide (DMSO) were obtained from Sigma (Germany).

4.4.2 Method

In this oral vaccination study, 12 groups of 6 mice were vaccinated orally by intragastric feeding, using the following formulations:

Group 1: Chitosan microparticles associated with DT (CMP)
Group 2: Chitosan microparticles without DT
Group 3: TMC microparticles associated with DT (TMC)
Group 4: TMC microparticles without DT
Group 5: Chitosan nanoparticles associated with DT (CNP)
Group 6: Chitosan nanoparticles without DT
Group 7: Emzaloid microparticles associated with DT (EMP)
Group 8: Emzaloid microparticles without DT
Group 9: Emzaloid nanoparticles associated with DT (ENP)
Group 10: Emzaloid nanoparticles without DT
Group 11: PBS associated with DT (PBS)
Group 12: Alum associated with DT (vaccinated subcutaneously) (ALUM)

In all vaccination studies, mice were vaccinated on day 1, 2 and 3, and again on day 15, 16 and 17, i.e. on three consecutive days in week 1 and 3. They received 40 Lf DT per week in a total volume of 300 µl. Blood samples of about 100 µl from the tail vein were taken at week 1 (prior to the priming), at week 3 (prior to boosting) and subsequently at weeks 4, 5 and 6. Blood samples were centrifuged for 10 min at 12 000 rpm in an Eppendorf centrifuge at 4 °C. The obtained sera of each group of the first five weeks were pooled and stored at –20 °C until analysis for IgG titers. The sera obtained from the last week were kept separate and also centrifuged and stored until analysis.

DT-specific IgG antibodies were measured by an adapted method enzyme-linked immunosorbent assay (ELISA). DT (1 Lf/ml in 0.04 M carbonate buffer (pH 9.60) was adsorbed to 96-wells plates by overnight incubation at 20 °C. Plates were then washed four times with a PBS/Tween 20® (0.1 %) solution (200 µl/well). Plates were then blocked with 200 µl of a BSA 1 %/PBS solution for 45 min. The plates were then again washed four times with the PBS/Tween 20® (0.1 %) solution (200 µl/well). Two-fold serial dilutions of the samples were done in diluent (PBS) and incubated for 2 hours at ambient temperature. The plates were washed four times with the PBS/Tween® (0.1 %) solution and the peroxidase-conjugated anti-IgG was added. The plates were incubated for 2 hours at ambient temperature and then washed four times with the PBS/Tween® (0.1 %) solution. Then 100 µl of a TMB solution (30 mg/5 ml DMSO) was added to each well. After 15 min, by adding 50 µl 2M H_2SO_4 to each well, the reaction was stopped. The optical density (OD) at 450 nm was measured and IgG titres of samples were calculated from dilutions with an OD of 0.3.

4.4.3 Results

Figures 4.1 to 4.4 give the IgG titers after weeks 4, 5 and 6 respectively. From figure 4.1 it is clear that the IgG titer for the chitosan nanoparticles is the highest and can compare to the immune response of the Alum results. From figure 4.2 it is also clear that the immune response of the chitosan nanoparticles is still similar to the Alum immune response. The immune response of the Emzaloid nanoparticles was lower and the immune response of the Emzaloid microparticles rose from week 4 to week 5.

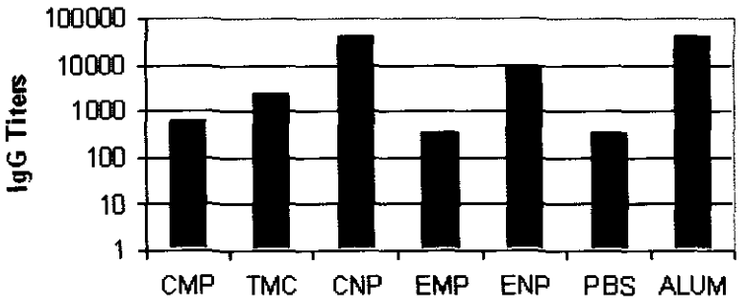


Figure 4.1: *The comparative results of the IgG titers in week 4.*

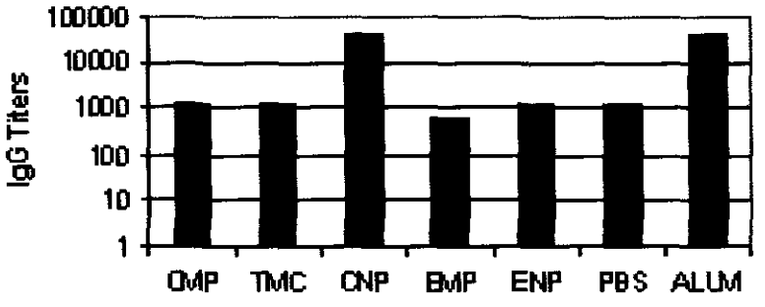


Figure 4.2: The comparative results of the IgG titers in week 5.

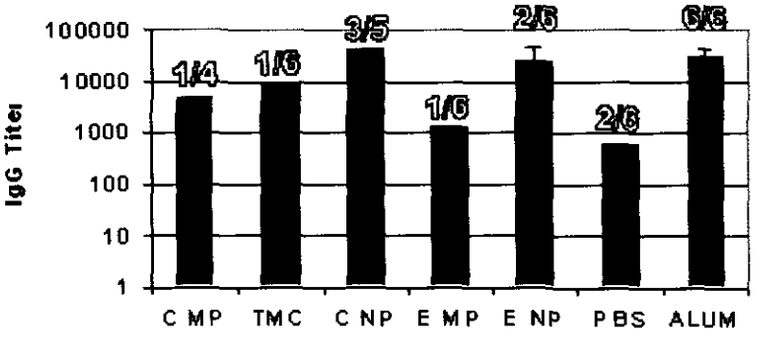


Figure 4.3: The comparative results of the IgG titers in week 6. Mice were vaccinated in both week 1 and week 3 on three consecutive days. IgG titers are expressed as mean ± SEM calculated at an OD of 0.3. The numbers above the column present the number of sera, giving a positive response per number of animals tested.

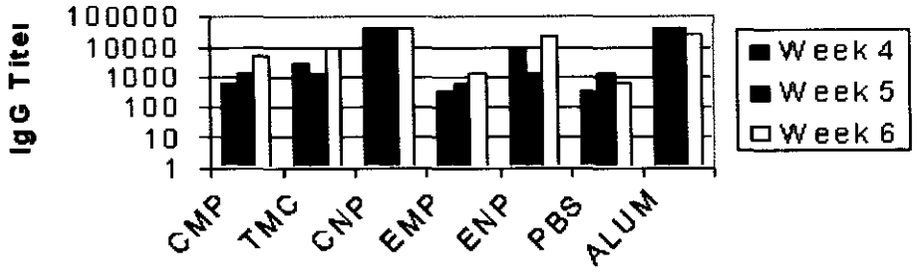


Figure 4.4: The IgG titers of all the formulations at week 4, week 5 and week 6, after vaccination in week 1 and week 3 with 40 Lf DT. The Alum is associated with DT and was given subcutaneously (positive control).

From figure 4.3 it can be seen that the chitosan nanoparticle titers and the Alum titers were comparable, but the Alum titers showed a better end result. In the case of the chitosan nanoparticle titers, only 3 out of the 5 mice still had an immune response, where as in the case of Alum, all 6 mice still had an immune response.

Two of the mice with the PBS formulation had a small immune response. Injury in the gastrointestinal tract could have caused the PBS formulation to enter the bloodstream, which could cause a systemic immune response. The TMC microparticle formulation had a slightly higher immune response than that of the chitosan microparticle formulation. This wasn't expected, because in a previous study the chitosan microparticles had a much higher immune response than that of the TMC microparticles (Van der Lubben *et al.*, 2001:202).

The Emzaloid immune response was also measured in week 4, 5 and 6. In these weeks the immune response was much lower than most of the other formulations. It is suggested that the peak immune response occurred before week 3, and should rather be measured prior to week 4, 5 and 6. Another reason for the low Emzaloid immune response is that a pro-emzaloid formulation should have been used. The difference between the Emzaloid used and the pro-emzaloid is that the pro-emzaloid has no water phase. The water needed for the pro-emzaloid to be active is obtained from the stomach fluid and this could stabilise the formulation. With the Emzaloid formulations the pH of

the formulations was 6.20 and it should have been adjusted to the pH value of the PBS, namely 7.30. This caused the Emzaloid formulation to be unstable and it could have been toxic. The pH value lowered even more in the stomach acid and this could be another reason why the immune responses were low.

Due to the fact that the results from week 4 and week 5 could not be compared because the blood was pooled in these 2 weeks, the variation between the different formulations in week 6 has been determined. With this calculation it was possible to determine if the different formulations are comparable and if the formulations have a medium or high effect. The formula used to determine this is as follows:

$$D = \frac{\text{The average titre of a formulation} - \text{The average titre of the next formulation}}{\text{Maximum standard deviation}}$$

where D = variation.

Table 4.2: The average and standard deviation of all the formulations in week 6.

Formulation	Average
Chitosan microparticles	5120.00 ± 0.00
TMC microparticles	10240.00 ± 0.00
Chitosan nanoparticles	40960.00 ± 22434.00
Emzaloid microparticles	1280.00 ± 0.00
Emzaloid nanoparticles	25600.00 ± 16405.00
PBS	640.00 ± 0.00
Alum	29013.00 ± 13610.00

Table 4.3: The variation (D) of all the formulations tested.

Formulation vs Formulation	Variation (D)
Chitosan microparticles vs Alum	-1.7
TMC microparticles vs Alum	-1.3
Chitosan nanoparticles vs Alum	0.5
Emzaloid microparticles vs Alum	-2.0
Emzaloid nanoparticles vs Alum	-0.2
PBS vs Alum	-2.1

According to this statistical analysis, a variation of ≈ 0.5 has a medium effect and could show a statistical significant difference. If the variation is ≈ 0.8 it has a big effect and shows a definite statistical significant difference. Table 4.3 shows that the chitosan nanoparticles have a medium effect and are better than the Alum formulation that is currently used. The Emzaloid nanoparticles have a negative effect and give a smaller response than the conventional method used but the result is only –0.2 and thus very comparable with the Alum formulation. All the other formulations are not comparable and give a much smaller response than the Alum.

4.5 Local immune response (IgA)

4.5.1 Materials

The homogenisation buffer consists of ethlene diamine tetraacedic acid (EDTA), bovine serum albumin (BSA), trypsin inhibitor and phenyl methane sulfonyl fluoride (PMSF) (Sigma, Germany). Anti-mouse IgA peroxidase conjugate was obtained from Sigma (Germany).

4.5.2 Method

The same mice were used for the systemic immune response and the local immune response. The faeces were collected from each mouse at 1, 3, 4, 6, 14 and 20 days after the second immunisation in week 3. After freeze-drying the faeces overnight, the IgA was isolated by incubating the pellets for 15 min in a homogenisation buffer (50 mM EDTA, 1% BSA, 0.1 mg/ml trypsin inhibitor and 2 mM PMSF in PBS (pH 7.30); 20 µl buffer per/mg faeces) on ice, followed by mashing it with a blunt needle. The IgA containing supernatants, obtained by centrifuging the suspension for 20 min at 14 000 rpm at 4 °C, were stored at –20 °C until analysis for IgA titers. DT-specific IgA antibodies were also measured by ELISA with the same procedure as described in section 4.4.2 for IgG, with exception that peroxidase-conjugated anti-IgA was used.

4.5.3 Results

In figure 4.5 it is shown that the chitosan nanoparticles are the only formulation that showed a local immune response. In all the other formulations no local immune response were detected.

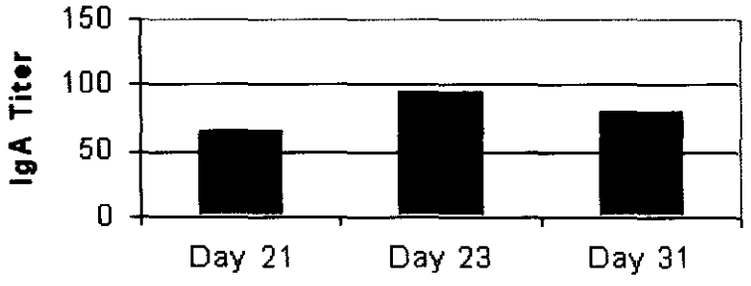


Figure 4.5: The IgA-titers for the chitosan nanoparticles on day 21, 23 and 31.

4.6 Conclusion

The results show that the chitosan nanoparticles had the best results of all the formulations. The production of the chitosan nanoparticles is not very cost- and time efficient. For further development of this product more research need to be done.

The Emzaloid nanoparticles also showed relatively good results and the production of this formulation is much more cost– and time efficient. In this study, a normal Emzaloid formulation was used, but in future studies it is suggested that a pro-emzaloid formulation should be used for oral vaccination studies.

Although the study was conducted successfully, a lot of changes need to be made to make it a more accurate analysis. Firstly, the blood samples from the first 5 weeks must not be pooled. If this is done an inaccurate conclusion can be drawn. The study also needs to be adapted to include larger groups of mice so that mice can be sacrificed in week 4, 5 and 6 to get accurate results.

Summary and future prospects

IgG Titers

Although chitosan nanoparticles provide a good immune response and show the same results as existing parenteral vaccines, the Alum formulation is still the most desirable formulation for vaccination. The manufacturing of chitosan nanoparticles is very time consuming and only small volumes can be prepared at a time, and is therefore not yet practical for industrial manufacturing. The formulations used in this study show promising results for the future, but further studies are required to obtain oral vaccination delivery systems.

The method used by Van der Lubben *et al.*, (2001:202) was applied to this study, where blood samples are collected in week 4, 5 and 6. The mice were only sacrificed in week 6, and blood samples were kept separately, whereas the blood taken from week 4 and 5 was pooled. Due to the fact that blood from different mice was pooled, no individual immune responses could be identified, but rather an average response per group. It is suggested that blood from individual mice should not be pooled, as blood samples could react with one another. The blood samples collected in week 6 showed far more accurate results. It is also suggested that mice should be sacrificed evenly over the 3-week period (week 4, 5 and 6) so that a more accurate analysis can be done. However, this study proved that there are many possibilities in oral vaccination for the future.

IgA titers

A few problems occurred while analysing the IgA titers. During the study problems were experienced with solubility of the EDTA and technical problems with the freeze dryer also occurred. This led to inadequate results and no local immune response could be

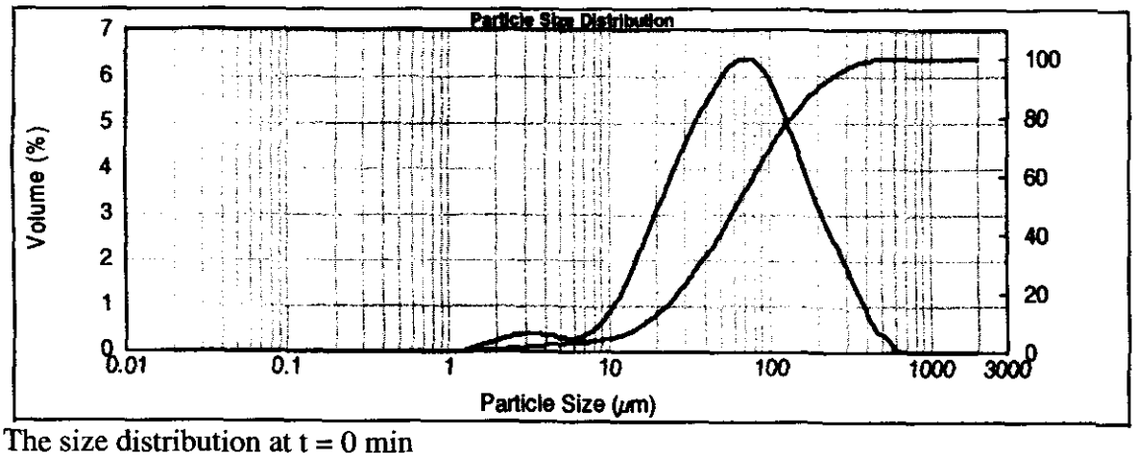
detected in most of the formulations. Although chitosan nanoparticles showed a local immune response, it is suggested that a higher response could have been detected, if the above-mentioned problems did not occur.

General

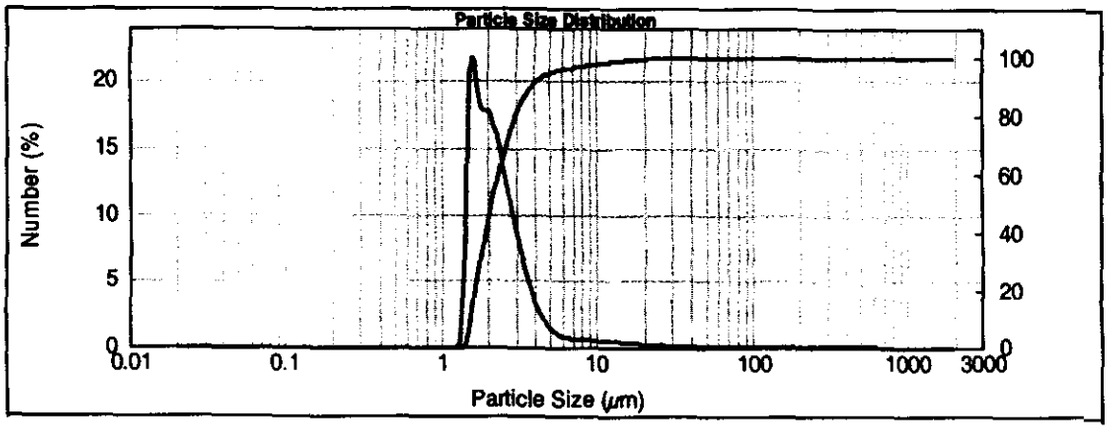
It is suggested that the study of the systemic and local immune response should be repeated in the future, and that the following recommendations should be considered:

- Larger groups of mice should be included in a future study so that mice can be sacrificed in all three weeks to determine more accurate systemic immune responses. It is recomended that individual blood samples must be taken and analysed.
- Pro-emzaloid formulations need to be used, instead of Emzaloid formulations: in previous studies it was shown that pro-emzaloids are a better delivery system for oral vaccination because this formulation has no water phase and is more stable in the stomach acid.
- The pH of the Emzaloid formulation should be adjusted to the pH value of the PBS used in the study for a more stable formulation.

Annexure 1
The particle size distribution of chitosan microparticles: Batch 1

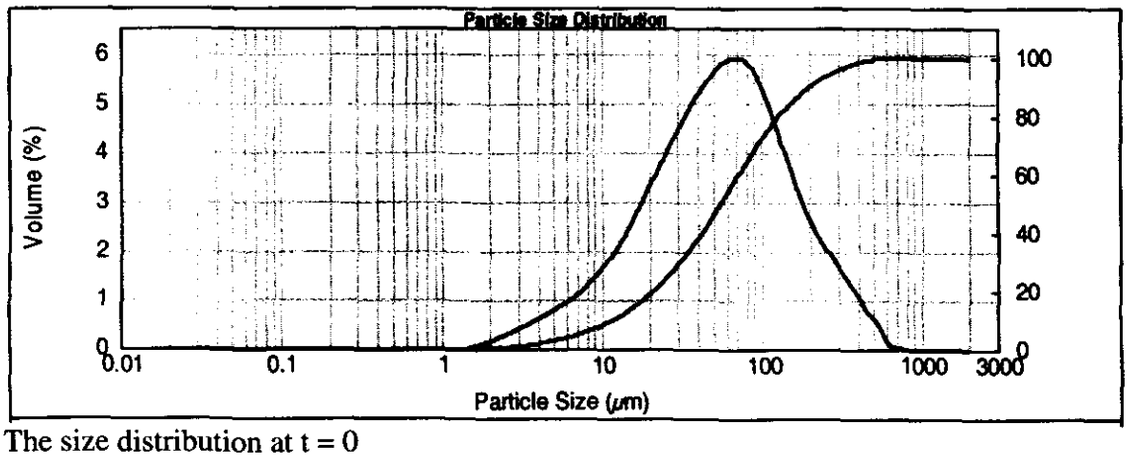


The size distribution at t = 0 min



The size distribution at t = 5 min

Annexure 2
The particle size distribution of chitosan microparticles: Batch 2



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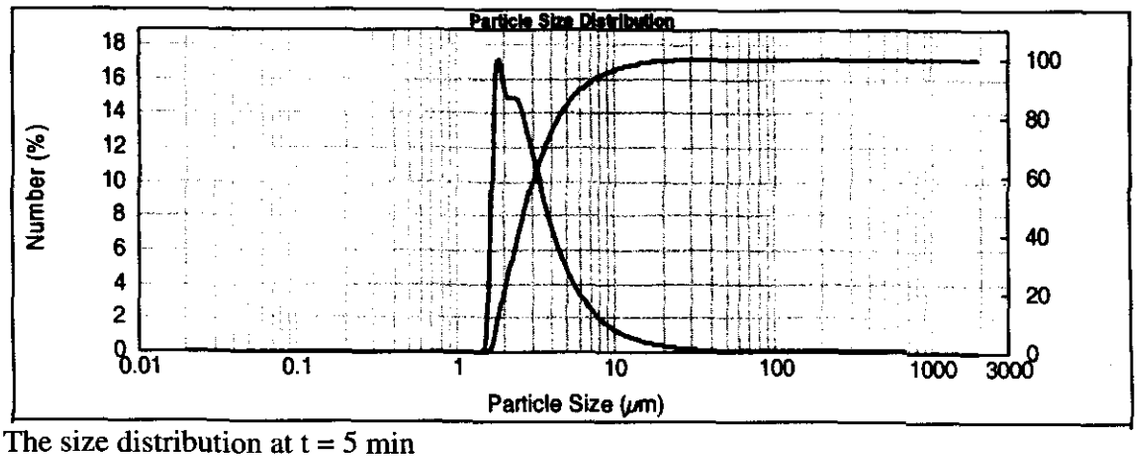
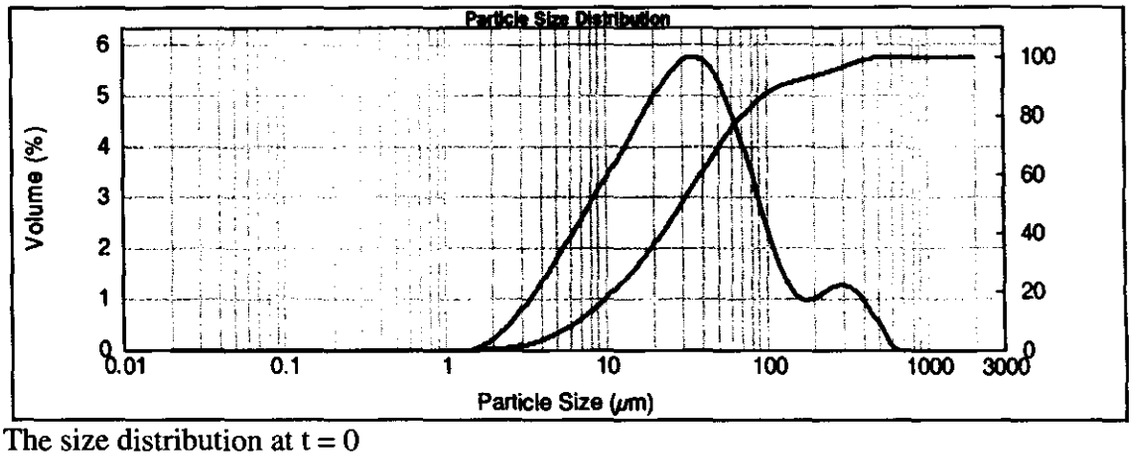
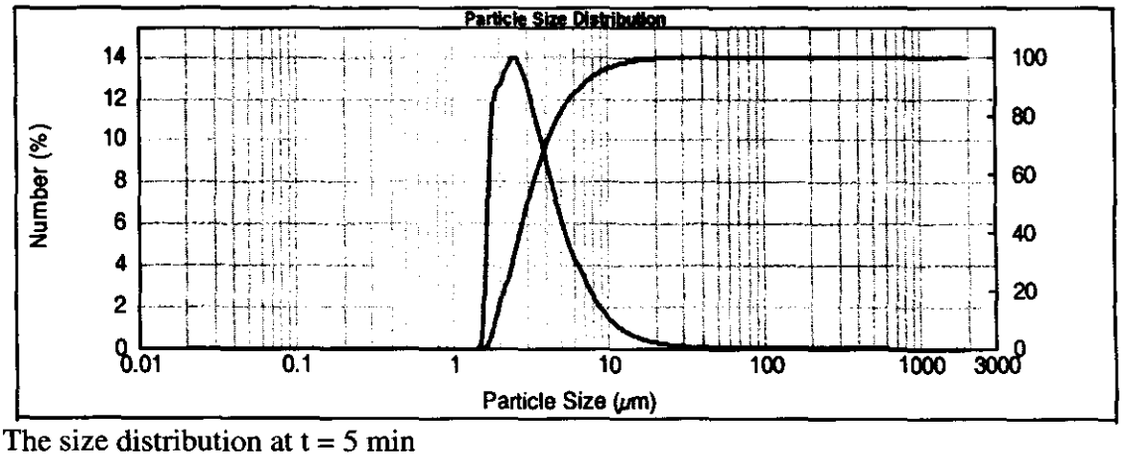


The size distribution at t = 5 min

Annexure 3
The particle size distribution of chitosan microparticles: Batch 3

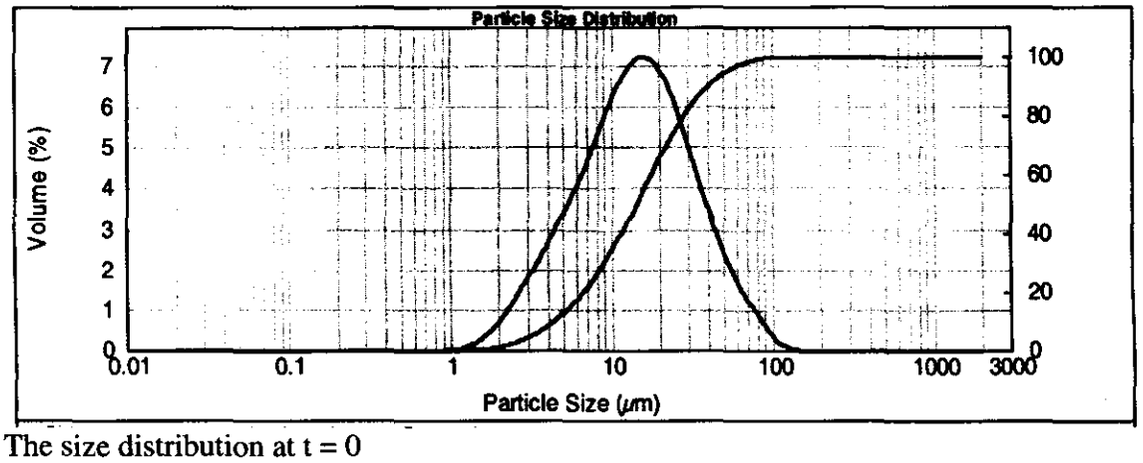


The size distribution at t = 0



The size distribution at t = 5 min

Annexure 4
The particle size distribution of chitosan microparticles: Batch 4



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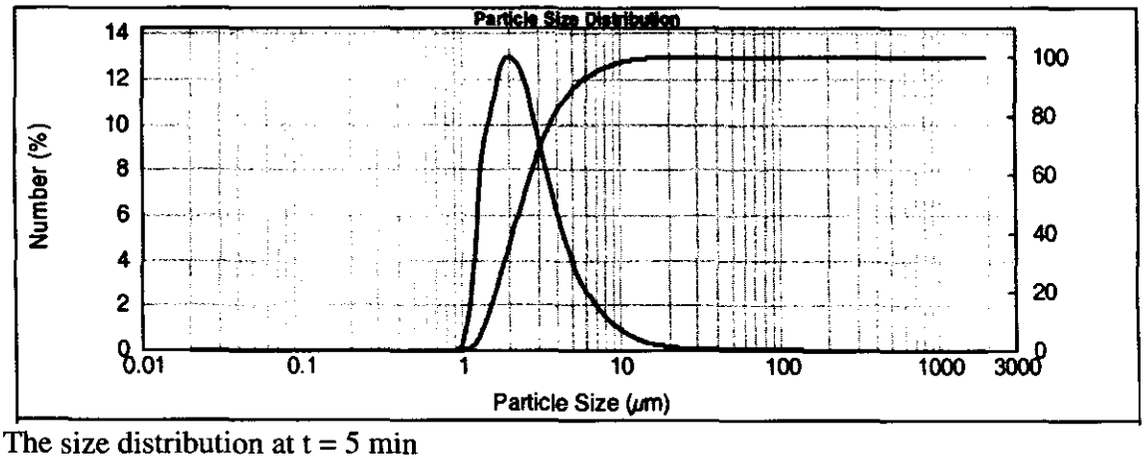
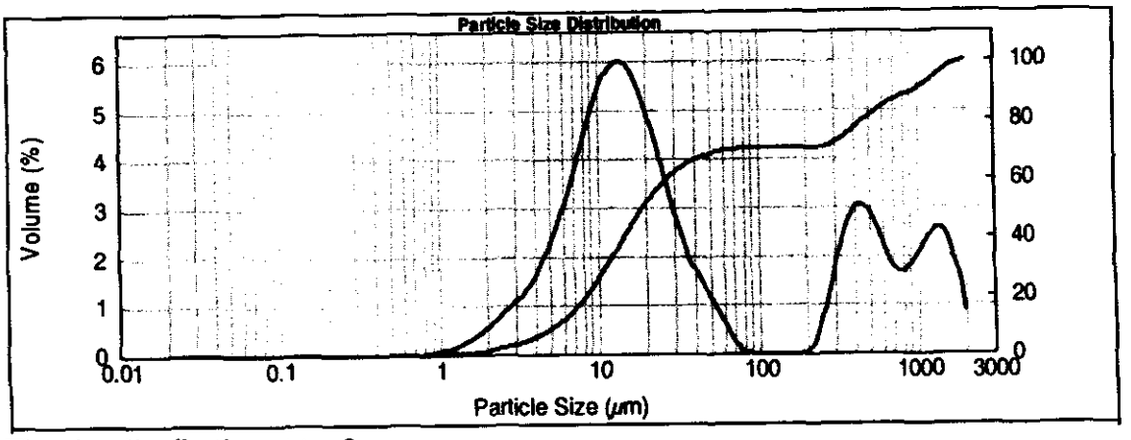


The size distribution at t = 5 min

Annexure 5
The particle size distribution of TMC microparticles: Batch 1



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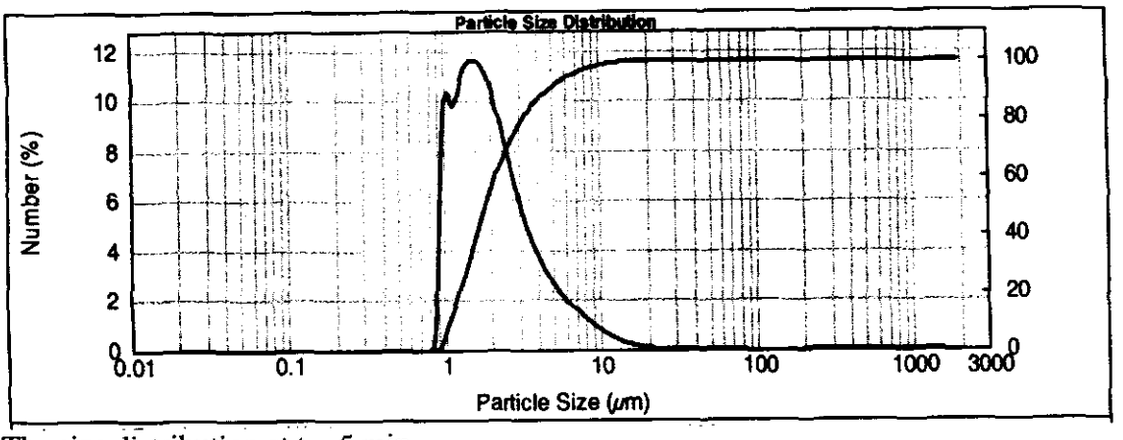
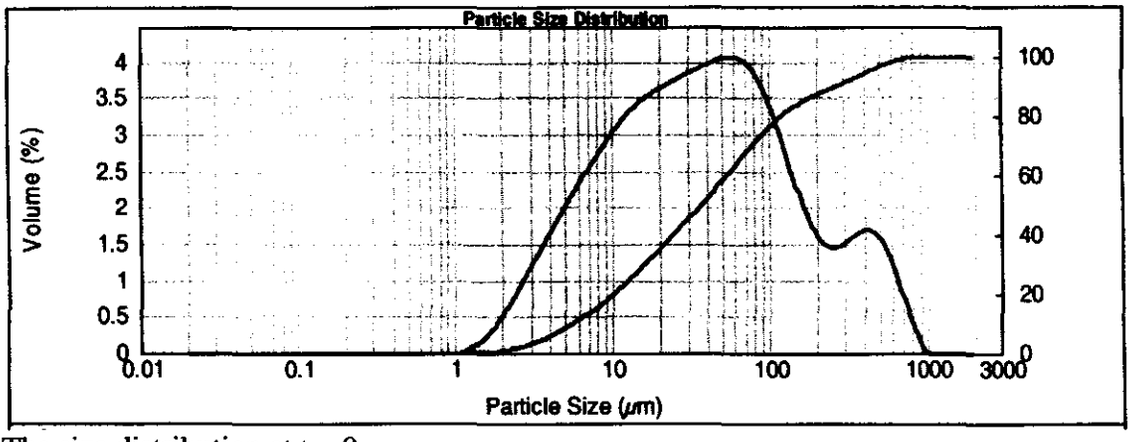


The size distribution at t = 5 min

Annexure 6
The particle size distribution of TMC microparticles: Batch 2



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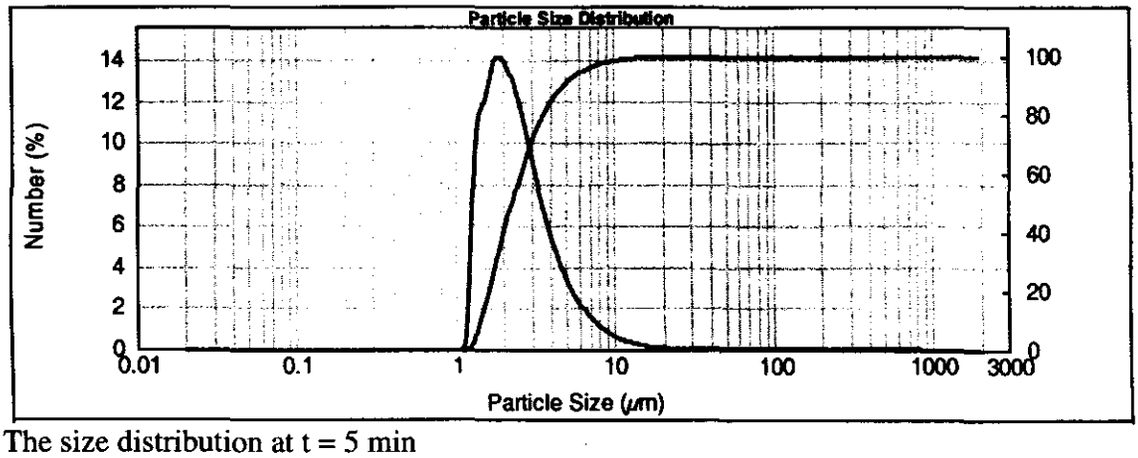

The size distribution at t = 5 min

References

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

ANON. 2003. Nurse triggers aids scare in botswana. ***Cape times,*** 7 march 2003:3. [Web:] http://www.journ-aids.org/reports/08032003b.htm [Date of access: 6 Oct. 2004].

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

BANERJEE, T., MITRA, S., SINGH, A.K., SHARMA, R.K. & MAITRA, A. 2002. Preparation, characterisation and biodistribution of ultrafine chitosan nanoparticles. ***International journal of pharmaceutics,*** 243:93-105.

BRADBURY, J. 2003. Custom-made vaccines at speed. ***Drug discovery today,*** 8:518-519.

BRAYDEN, D.J. 2001. Oral vaccination in man using antigens in particles: current status. ***European journal of pharmaceutical science,*** 14:183-189.

BRAYDEN, D.J. & O'MAHONY, D.J. 1998. Novel oral drug delivery gateways for biotechnology products: polypeptides and vaccines. ***PSTT,*** 1:291-299.

CHEN, H. & LANGER, R. 1998. Oral particulate delivery: status and future trends. ***Advanced drug delivery reviews,*** 34:339-350.

CLARK, M.A., JEFSON, M.A. & HIRST, B.H. 2001. Exploiting M-cells for drug and vaccine delivery. ***Advanced drug delivery reviews,*** 50:81-106.

DODANE, V. & VILIVALAM, V.D. 1998. Pharmaceutical applications of chitosan. ***PSTT,*** 1:246-253.

EL-SHABOURI, M.H. 2002. Positively charged nanoparticles for improving the oral bioavailability of cyclosporin-A. ***International journal of pharmaceutics,*** 249:101-108.

GENTA, I., CONTI, B., PERUGINI, P., PAVANETTO, F., SPADARO, A. & PUGLISI, G. 1997. Bioadhesive microsheres for ophthalmic administration of acyclovir. ***Journal of pharmacy and pharmacology,*** 49:737-742.

GLUCK, 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 (UCT)).

GUILLERMO, A., HERRERA, M.D., ZHEN ZHAO, R. & KLEVENS, M. 2001. Variation in vaccination coverage among children of Hispanic ancestry. ***Am journal preventative medicine,*** 20:69-74.

HEYDENREICH, A.V., WESTMEIER, R., PEDERSEN, N., POULSEN, H.S. & KRISTENSEN, H.G. 2003. Preparation and purification of cationic solid lipid nanospheres – effects on particle size, physical stability and cell toxicity. ***International journal of pharmaceutics,*** 254:83-87.

HILLEMAN, M.R. 2000. Vaccines in historic evolution and prospective: A narrative of vaccine discoveries. ***Vaccine,*** 18:1436-1447.

JOHNSON, A.G. 1994. Molecular adjuvants and immunomodulators. ***New approaches to immunisation,*** 7:277-289.

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

KESIK, M., SACZYNSKA, V., SZEWCZYK, B. & PLUCIENNICZAK, A. 2004. Inclusion bodies from recombinant bacteria as a novel system for delivery of vaccine antigen by the oral route. ***Immunology letters,*** 91:197-204.

KO, J.A., PARK, H.J., HWANG, S.J., PARK, J.B. & LEE, J.S. 2002. Preparation and characterisation of chitosan microparticles intended for controlled drug delivery. ***International journal of pharmaceutics,*** 249:165-174.

KOTZÈ, A.F., LUEBEN, H.L., DE LEEUW, B.J., DE BOER, A.G., VERHOEF, J.C. & JUNGINGER, H.E. 1998. Comparison of the effect of different chitosan salts and *N*-trimethyl chitosan chloride on the permeability of intestinal epithelial cells (Caco-2). ***Journal of controlled release,*** 51:35-46.

KOTZÈ, A.F., THANOU, M.M., LUEβEN, B., DE BOER, G., VERHOEF, J.C. & JUNGINGER, H.E. 1999. Effect of the degree of quaternisation of N-trimethyl chitosan chloride on the permeability of intestinal epithelial cells (Caco-2). ***European journal of pharmaceutics and biopharmaceutics,*** 47:269-274.

KURSTAK, E. 2002. Towards the new global vaccinology era in prevention and control of diseases. ***Vaccine,*** 21:580-581.

LAVELLE, E.C., SHARIF, S., THOMAS, N.W., HOLLAND, J. & DAVIS, S.S. 1995. The importance for gastrointestinal uptake of particles in design of oral delivery systems. ***Advanced drug delivery review,*** 18:5-22.

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

MAJETI, N.V. & KUMAR, R. 2000. A review of chitin and chitosan applications. ***Reactive and functional polymers,*** 46:1-27.

MESTECKY, J., MOLDOVEANU, Z., MICHALEK, S.M., MORROW, C.D., COMPANS, R.W., SCHAFER, D.P. & RUSSELL, M.W. 1997. Current options for vaccine delivery systems by mucosal routes. ***Journal of controlled release,*** 48:243-257.

MIDDLEMAN, A.B. 2004. Race/ethnicity and gender disparities in the utilisation of a school-based hepatitis b immunisation initiative. ***Journal of adolescent health,*** 34:414-419.

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

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

MURDAN, S. 2003. Sorbitan monostearate organogels and amphiphilogels for drug and vaccine delivery. ***Scientifically speaking,*** 10:16.

MUZZARELLI, R.A.A. & MUZZARELLI, C., 2002. Natural and artificial chitosan-inorganic composites, a mini-review. (*In* Muzzarelli *et al.* Eds. Chitosan in pharmacy and chemistry. Italy: Atec. P233)

O'HAGAN, D.T. 1998. Microparticles and polymers for the mucosal delivery of vaccines. ***Advanced drug delivery reviews,*** 34:305-320

PASTORET, P.P. & VAN DER PLASSCHEN, A. 2003. Poxviruses as vaccine vectors. ***Comparative immunology, microbiology and infectious diseases,*** 26:343-355.

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

PO, A., ROGERS, E., SHEPPHARD, M. & SCOTT, E.M. 1995. Delivery systems for non-parenteral vaccines. ***Advanced drug delivery reviews,*** 18:101-109.

POLO, S., VEGLIA, F., MALNATI, M.S., GOBBI, C., FARCI, P., RAITERI, R., SINICCO, A. & LUSSO, P. 1999. Longitudinal analysis of serum chemokine levels in the course of HIV-1 infection. ***AIDS,*** 13:447-454.

SABNIS, S. & BLOCK, L.H. 2000. Chitosan as an inabling exipient for drug delivery systems. ***International journal of biological macromolecules,*** 27:181-186.

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

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

SHALABY, S.W. 1995. Development of oral vaccines to stimulate mucosal and systemic immunity: barriers and novel strategies. ***Clinical immunology and immunopathology,*** 74:127-134.

SHROFF, K.E., SMITH, L.R., BAINE, Y. & HIGGINS, T.J. 1999. Potential for plasmid DNAs as vaccines for the new millennium. ***PSTT,*** 2:205-211.

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

THACHARODI, D. & ROA, K.P. 1995. Development and in vitro evaluation of chitosan-based transdermal drug delivery systems for the controlled delivery of propanolol hydrochloride. ***Biomaterials,*** 16:145-148.

THANOU, M.M., KOTZE, A.F., SCHARRINGHAUSEN, T., LUEBEN, H.L., DE BOER, A.G., VERHOEF, J.C. & JUNGINGER, H.E. 2000. Effect of degree of quaternisation of N-trimethyl chitosan chloride for enhanced transport of hydrophilic compounds across intestinal caco-2 cell monolayers. ***Journal of controlled release,*** 64:15-25.

THANOU, M., VERHOEF, J.C. & JUNGINGER, H.E. 2001. Oral drug absorpsion enhancement by chitosan and its derivatives. ***Advanced drug delivery reviews,*** 52:117-126.

THANOU, M.M., VERHOEF, J.C., ROMEIJN, S.G., NAGELKERKE, J.F., MERKUS, W.H.M. & JUNGINGER, H.E. 1999. Effects of n-trimethyl chitosan chloride, a novel absorption enhancer, on Caco-2 intestinal epithelia and the ciliary beat frequency of chicken embryo trachea. ***International journal of pharmaceutics,*** 185:73-82.

VAN DER LUBBEN, I.M., VERHOEF, J.C., PORCHARD, G. & JUNGINGER, H.E., 2001. Chitosan and its derivatives in mucosal drug and vaccine delivery. ***Pharmaceutical science,*** 14:201-207.

VAN DER LUBBEN, I.M., VERHOEF, J.C., VAN AELST, A.C., BORCHARD, G. & JUNGINGER, H.E. 2001. Chitosan microparticles for oral vaccination: preparation,

characterisation and preliminary in vivo uptake studies in murine peyer's patches. ***Biomaterials,*** 22:687-694.

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 LUBBEN, I.M., KERSTEN, G., FRETZ, M.M., BEUFERY, C., VERHOEF, J.C. & JUNGINGER, H.E. 2003. Chitosan microparticles for mucosal vaccination against diphtheria: oral and nasal efficacy studies in mice. ***Vaccine,*** 21:1400-1415.

VON HERTZEN, L.C. & HAAHTELA, T. 2003. Immunisation and atopy: Possible implications of ethnicity. ***American academy of allergy, asthma and immunology,*** 113:401-406.

WIKINGSSON, L.D. & SJOHOLM, I. 2002. Polyacryl starch microparticles as adjuvant in oral immunisation, inducing mucosal and systemic immune responses in mice. ***Vaccine,*** 20:3355-3363.

XU, Y. & DU, Y. 2003. Effect of molecular structure of chitosan on protein delivery properties of chitosan nanoparticles. ***International journal of pharmaceutics,*** 250:215-226.

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