

**REPUBLIC OF TURKEY
HACETTEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES**

**DEVELOPMENT OF NON-INVASIVE
VACCINE CARRIERS**

Dr. Burcu SAYIN

**Program of Pharmaceutical Technology
Ph.D. THESIS**

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**SUPERVISOR: Prof. Dr. Sevda ŞENEL
CO-SUPERVISOR: Prof. Dr. H. Oya ALPAR**

**Ankara
2007**

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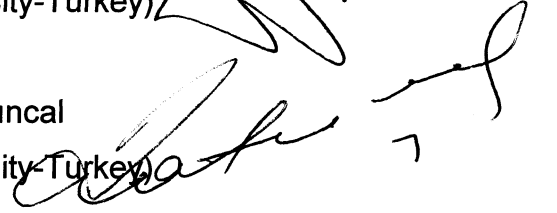
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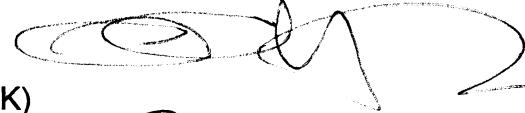
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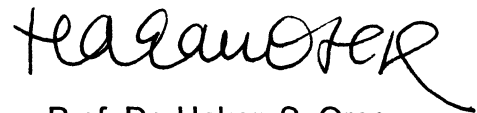


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Bu çalışma jürimiz tarafından Farmasötik Teknoloji Programı'nda DOKTORA TEZİ olarak kabul edilmiştir.

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ONAY:

Bu tez, Sağlık Bilimleri Enstitüsü Yönetim Kurulu'nca belirlenen yukarıdaki jüri üyeleri tarafından uygun görülmüştür ve Yönetim Kurulu'nun kararıyla kabul edilmiştir.



Prof. Dr. Hakan S. Orer

Enstitü Müdürü

ACKNOWLEDGEMENTS

The greatest thanks first belong to my supervisor **Prof. Dr. Sevda Şenel** for giving me the opportunity to enter the field of vaccines, for her guidance and support. She guided me how to become an objective scientist and I appreciate her sharp and useful comments on my studies.

I would like to thank **Prof. Dr. H. Oya Alpar** for her co-supervision on my thesis, her hospitality, sense of humor and the fruitful discussions we had in our meetings. I was lucky to have an opportunity to study in her laboratory.

Special acknowledgements have to be given to **Assist. Prof. Dr. Erdal Cevher**. I never forget his warm welcome to London and also generous support during this period in London.

I would like to express my gratitude thanks to **Dr. Satyanarayana Somavarapu** for providing his knowledge and experience in the design of experiments and his assistance in in vivo studies. I am thankful to my other collaborators **Dr. Xiong Wei Li** and **Dr. Maya Thanou** for their valuable assistance for my Ph. D. thesis.

Special thanks to **Assoc. Prof. Dr. Erem Bilensoy** for her friendship, encouragement and support. My hearty thanks go to **Tuğba Kökçak, Pharm. Selcan Türker (M.Sc.), Pharm. Tuğba Gülsün, Pharm. Mine Silindir, Eren R. Ayata** and **Merve Aytekin** for sharing my fun, stress and happy and sad moments and their tolerance.

Finally, my greatest thanks to my family, my mother, my father and my sister who always loved, believed, motivated, supported and encouraged. They are the meaning of love and trust.

I would also like to acknowledge the financial support provided by the British Council Turkey and Hacettepe University Scientific Research Foundation (H.Ü.B.A.B. 07.T09.102.003).

ABSTRACT

Sayin, B. Development of non-invasive vaccine carriers. Hacettepe University Health Science Institute Pharmaceutical Technology Programme, Ph. D. Thesis, Ankara, 2007. Mucosal application of a vaccine can effectively induce both systemic and mucosal immune responses. In general, mucosal applications of antigens result in poor immune responses. Therefore, adjuvant/delivery systems are required to enhance the immune response. Chitosan (CS) is a cationic biopolymer which exerts advantages as a vaccine carrier due to its adjuvant and bioadhesive properties as well as permeation and cellular uptake enhancement, antigen protection, immune stimulating activity. Similar effects are also shown by chitosan derivatives. In this study, chitosan, its polycationic derivative, N-trimethyl chitosan (TMC), and polyampholytic derivative, mono-N-carboxymethyl chitosan (MCC), were used to prepare nanoparticulate delivery systems for mucosal immunization. The cytotoxicity studies (MTT assay) on Chinese hamster ovary (CHO-K1) cell lines showed that cell viability was in the order of MCC, TMC/MCC complex, CS and TMC. CS, TMC, MCC and TMC/MCC complex nanoparticles were prepared using ionic gelation method and loaded with tetanus toxoid (TT). This was the first time that MCC and TMC/MCC complex nanoparticles were developed in this study and evaluated as antigen delivery system. Nanoparticles with high loading efficacy (>90%), particle size within the range of 40-400 nm, with a negative surface charge for MCC and positively surface charge for TMC, TMC/MCC complex and CS were obtained. The structural integrity of the TT in the nanoparticles was confirmed by SDS-PAGE electrophoresis analysis. The effective uptake of the FITC-BSA loaded nanoparticles into the cells was demonstrated by cellular uptake studies using J774A.1 cells. Immune response of the formulations loaded with TT was studied in vivo in Balb/c mice. CS and TMC nanoparticles which have positively charged surfaces induced higher immune responses when compared to those prepared with MCC which is negatively charged and has smaller particle size. TMC/MCC complex nanoparticles were found to induce the highest mucosal and systemic immunity response compared to CS, TMC or MCC alone. As a conclusion; chitosan and derivatives are promising polymers for mucosal vaccine delivery both as an adjuvant and a delivery system with their biocompatibility, high loading capacity and appropriate particle size

Keywords: Nanoparticles, chitosan, chitosan derivatives, nasal immunization, vaccine carrier

Supporting Institutions; Hacettepe University Research Foundation, The British Council

ÖZET

Sayın, B. Non-invazif aşı taşıyıcı sistemlerin geliştirilmesi. Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü Farmasötik Teknoloji Programı Doktora Tezi, Ankara, 2007. Mukozal yolla uygulanan aşı hem sistemik hemde mukozal immün cevap oluşturabilmektedir. Genellikle, antijenlerin mukozal yoldan uygulaması zayıf immün cevap oluşturur. Bu nedenle, immün cevabın artırılması için, adjuvant/ taşıyıcı sistemler gereklidir. Kitosan (CS), permeasyon ve hücreden alımı artırıcı, antijen koruyucu, immün stimülatör aktivitesinin yanı sıra, adjuvant ve biyoadhezif özellikleri nedeniyle de aşı taşıyıcı olarak üstünlük taşır. Benzer etkiler kitosan türevleri için de gösterilmektedir. Bu çalışmada, kitosan, polikasyonik türevi N-trimetil kitosan (TMC), ve poliamfolitik türevi mono-N-karboksimetil kitosan (MCC), mukozal immunizasyon için nanopartiküler taşıyıcı sistem hazırlanması için kullanılmıştır. Sitotoksikite çalışmaları (MTT tayini), çin hamster yumurtalık (CHO-K1) hücre dizisi üzerinde gerçekleştirilmiş olup, hücre yaşayabilirliği, en yüksekten en düşüğe olacak şekilde MCC, TMC/MCC kompleks, CS ve TMC sırasında gerçekleşmiştir. CS, TMC, MCC ve TMC/MCC kompleks nanopartikülleri iyonik jelasyon yöntemiyle hazırlanmış ve tetanoz toksoidi (TT) yüklenmiştir. Bu çalışmada, ilk kez MCC ve TMC/MCC kompleks nanopartikülleri geliştirilmiş ve antijen taşıyıcı sistem olarak değerlendirilmiştir. Yüksek yükleme etkinliğine sahip(>%90), partikül büyüklüğü 40-400 nm arasında değişen, MCC için negatif yüzey yükü, TMC, TMC/MCC kompleks ve CS için pozitif yüzey yüküne sahip nanopartiküller elde edilmiştir. Nanopartiküllerdeki TT'nin yapısal bütünlüğü SDS-PAGE elektroforez analizi ile konfirme edilmiştir. FITC-BSA yüklü nanopartiküllerin hücrelerden etkin alımı J774A.1 hücreleri kullanılarak gösterilmiştir. TT yüklü formülasyonların immün yanıtı Balb/c fareleri üzerinde incelenmiştir. Pozitif yüklü CS ve TMC nanopartikülleri, negatif yüklü ve daha küçük partikül büyüklüğüne sahip MCC nanopartiküllerinden daha yüksek immün cevap oluşturmuştur. TMC/MCC kompleks nanopartiküllerinin, CS, TMC ve MCC nanopartiküllerine göre en yüksek immün cevabı oluşturduğu bulunmuştur. Kitosan ve türevleri ile hazırlanan biyoparçalanır nanopartiküller, antijenin partikül formunda sunumu, antijenin enzimatik degradasyondan koruması, nazal mukozadan alımını sağlamıştır ve sonuç olarak, nazal immünizasyon için umut verici adjuvan/taşıyıcı sistemler olarak önerilmektedirler.

Anahtar Kelimeler: Nanopartikül, kitosan, kitosan türevleri, nazal immünizasyon, aşı taşıyıcı

Destekleyen Kurumlar; Hacettepe Üniversitesi Bilimsel Araştırmalar Birimi, The British Council

CONTENTS

	Page
CONFIRMATION PAGE (English)	iii
CONFIRMATION PAGE (Turkish)	iv
ACKNOWLEDGEMENTS	v
ABSTRACT (English)	vi
ABSTRACT (Turkish)	vii
CONTENTS	viii
SYMBOLS AND ABBREVIATIONS	xi
FIGURES	xiii
TABLES	xv
1. THEORETICAL PART	1
1. 1. INTRODUCTION	1
1.2. Types of Vaccines	3
1.3. Adjuvants	5
1.4. Mucosal Immunization	8
1.5. Mucosal routes for immunization	16
1.6. Antigen delivery systems for mucosal and systemic immunization	20
1.7. Tetanus Toxoid	35
2. MATERIALS and METHODS	38
2.1. Materials and Equipment	38
2.1.1. Chemical Reagents	38
2.1.2. Equipment	40
2.2. Methods	41
2.2.1. Synthesis of TMC	41
2.2.2. Synthesis of MCC	42
2.2.3. Characterization of the chitosan derivatives	42
2.2.4. In vitro cellular toxicity studies	42
2.2.5. Preparation of Nanoparticles	43
2.2.5.1. Chitosan and TMC Nanoparticles	44

2.2.5.2. MCC Nanoparticles	44
2.2.5.3. TMC/ MCC Nanoparticles	45
2.2.6. Characterization of Nanoparticles	45
2.2.6.1. Protein Loading Capacity of Nanoparticles	45
2.2.6.2. Particle Size Distribution	46
2.2.6.3. Zeta Potential Measurement	46
2.2.6.4. Microscopic Evaluation	46
2.2.7. Determination of the protein integrity	46
2.2.8. Stability of Nanoparticles	48
2.2.9. Cellular uptake studies	48
2.2.10. In vivo Studies	49
2.2.11. Enzyme-linked immunoabsorbent assay (ELISA)	51
2.2.12. Statistical analysis	52
3. RESULTS	53
3.1. Synthesis and Physicochemical Characterization of TMC	53
3.2. In vitro cellular toxicity studies	55
3.3. Preparation of Nanoparticles	57
3.4. Characterization of Nanoparticles	58
3.5. Determination of the protein integrity	62
3.6. Stability of Nanoparticles	63
3.7. Cellular uptake studies	67
3.8. In vivo Studies	71
3.8.1. Antibody responses of the formulations prepared with TMC, MCC, CS or TMC/MCC complex	71
3.8.2. Comparison of the immune responses of the formulations	75
4. DISCUSSION	81
4.1. Synthesis and characterization of the chitosan derivatives	81
4.2. In vitro cellular toxicity studies	81
4.3. Preparation of Nanoparticles	82
4.4. Characterization of Nanoparticles	86
4.4.1. Protein Loading Capacity of Nanoparticles	86

4.4.2. Particle Size Distribution	88
4.4.3. Zeta Potential Measurements	89
4.5. Protein integrity	90
4.6. Stability of Nanoparticles	90
4.7. Cellular uptake studies	91
4.8. In vivo Studies	93
5. CONCLUSION	98
REFERENCES	100
CURRICULUM VITAE	

SYMBOLS AND ABBREVIATIONS

APC	antigen presenting cell
ATCC	American Type Culture Collection
BALT	bronchoepithelium and lower respiratory tract
BCA	Bichinchoninic acid
BCG	Bacille Calmette-Guérin
BSA	bovine serum albumin
CHO-K1	Chinese hamster ovary cell line
CNS	central nervous system
CS	Chitosan
CT	cholera toxin
DCs	dendritic cells
DMEM	Dulbecco's modified eagle's medium
ELISA	Enzyme-linked immunoabsorbent assay
FAE	follicle-associated epithelium
FBS	fetal bovine serum
FITC	Fluorescein-5-isothiocyanate
GALT	gut-associated lymphoid tissue
HIV	human immunodeficiency virus
IEL	Intraepithelial lymphocytes
Ig A	immunoglobulin A
ISCOMs	Immune stimulating complexes
ISS	immunostimulatory sequences
LE	loading efficiency
Lf	limit of flocculation
LT	<i>Escherichia coli</i> heat labile enterotoxin
MALT	Mucosa associated lymphoid tissue
MAP	Multipl peptide antigens attached to lysine core
MCC	mono-N-carboxymethyl chitosan
MHC	Major Histocompatibility complex
MTT	methlythiazolyltetrazolium

NALT	Nasal associated lymphoid tissue
nm	nanometer
NMR	nuclear magnetic resonance
OVA	Ovalbumin chicken egg
PCS	photon correlation spectroscopy
PEI	Polyethylenimine
PGA	poly(glycolic acid)
PLA	Poly(lactic acid)
PLGA	Poly (lactic-co-glycolic acid)
PLL	Poly-L-Lysine
PP	Peyer's patches
PSA	Pig serum albumin
SARS	severe acute respiratory syndrome
SD	Standart deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TEER	Transepithelial electrical resistance
TEM	Transmission Electron Microscope
TGS	Tris/Glycine/SDS buffer
TMC	N-trimethyl chitosan
TT	Tetanus toxoid
URT	upper respiratory tract
WHO	World Health Organization

FIGURES

	Page
Figure 1.1. Antigen sampling at mucosal surfaces	10
Figure 1.2. Structure of chitin and chitosan	27
Figure 1.3. Chemical structures of chitosan, TMC, MCC	32
Figure 1.4. Clostridium tetani	36
Figure 2.1. Preparation of the nanoparticles	44
Figure 3.1. Reaction scheme of chitosan trimethylation and counter ion replacement	53
Figure 3.2. Proton nuclear magnetic resonance spectroscopy spectra of TMC	54
Figure 3.3. Schematic representation of the synthesis of the mono-N-carboxymethyl chitosan (MCC)	54
Figure 3.4. Proton nuclear magnetic resonance spectroscopy spectra of MCC	55
Figure 3.5. Dose viability curves of chitosan and its derivatives	56
Figure 3.6. Effect of TMC:MCC ratio on particle size of nanoparticles	58
Figure 3.7. TEM micrographs of TT loaded CS nanoparticles(a), TMC nanoparticles (b)	60
Figure 3.8. TEM micrographs of MCC nanoparticles; Blank(a), and TT loaded (b)	61
Figure 3.9. TEM micrographs of TMC/MCC complex nanoparticles; blank (a), and TT loaded (b)	61
Figure 3.10. SDS-PAGE gel displaying the TT determined in TMC/MCC complex nanoparticles after loading process	62
Figure 3.11. SDS-PAGE gel displaying the TT determined in TMC and MCC nanoparticles after loading process	63
Figure 3.12. TEM micrographs of TT loaded (a) TMC, (b) MCC, (c) TMC/MCC, (d) CS nanoparticles after 6 months storage at 4°C	64

Figure 3.13.	SDS-PAGE gel displaying the TT determined in nanoparticles after 6 months storage at 4°C	66
Figure 3.14.	Mouse Balb/c monocyte macrophage J774A.1 cells	67
Figure 3.15.	Confocal laser scanning images of nanoparticles after 30 min incubation.	68
Figure 3.16.	Confocal laser scanning images of nanoparticles after 1 hour incubation.	69
Figure 3.17.	Confocal laser scanning images of nanoparticles after 2 hours incubation.	70
Figure 3.18.	TT specific serum IgG antibody titers after administration of TMC formulations	71
Figure 3.19.	TT specific serum IgG antibody titers after administration of MCC formulations	73
Figure 3.20.	TT specific serum IgG antibody titers after administration of CS formulations	74
Figure 3.21.	TT specific serum IgG antibody titers after administration of TMC/MCC complex formulations	75
Figure 3.22.	TT specific serum IgG antibody titers after administration of i.n. or s.c. delivery of TT in solutions	76
Figure 3.23.	TT specific serum IgG antibody titers of i.n. delivery of TT loaded nanoparticles	77
Figure 3.24.	TT specific serum IgG1 antibody titers after administration of (a) i.n. or s.c. delivery of TT in solutions, (b) i.n. delivery of TT loaded nanoparticles on day 56	79
Figure 3.25.	TT specific serum IgG2a antibody titers after administration of (a) i.n. or s.c. delivery of TT in solutions, (b) i.n. delivery of TT loaded nanoparticles on day 56	80

TABLES

	Page
Table 1.1. Types of vaccines	3
Table 1.2. Classes of modern vaccine adjuvants	7
Table 1.3. Formulation approaches for mucosal immunization	22
Table 2.1. Materials for the SDS-PAGE studies	47
Table 2.2. Formulations investigated in vivo	51
Table 3.1. Cell viability for chitosan and its derivatives	56
Table 3.2. Properties of the blank MCC nanoparticles	57
Table 3.3. Blank nanoparticle formulations	59
Table 3.4. TT loaded nanoparticle formulations	59
Table 3.5. Particle size and zeta potential results of the TT loaded nanoparticle formulations at initial and after 6 months storage at 4°C	65
Table 3.6. Particle size and zeta potential results of the TT loaded nanoparticle formulations after preparation and 1 month's storage at 25°C	66
Table 3.7. Test results of pairwise comparison between groups in in vivo studies	72

1. THEORETICAL PART

1. 1. INTRODUCTION

Infectious diseases are the major cause of morbidity and mortality, accounting for a third of the deaths which occur in the world each year. They are also highly expensive in terms of health-associated costs of infected patients and loss in productivity at work. Vaccination is the most cost-effective approach to prevent economic losses and morbidity caused by infectious diseases. The ultimate aim of any vaccine is the production of long-term protective immune responses against a pathogen. Vaccination is the only type of medical intervention that has eliminated a disease successfully and represents one of the most cost-effective public health tools to combat and sometimes eradicate infectious agents (1). Therapeutic treatment is usually far more expensive than prevention of disease. Although commercialized vaccines are generally effective, improvement in efficacy and safety, reduction in the numbers of administrations and the ease of administration are currently considered important challenges to be met for the next decades. Recent advances in biotechnology and in the understanding of the immune system have now made it possible to design new generation vaccines that may approach these goals.

A very important part of the future is the improvement of routes for immunization in favor of the patient safety and compliance. Most vaccines today are given by parenteral injection, which induces systemic immune responses expressed by B and T cells in the blood (2). This route of immunization stimulates the immune system to produce antibodies in the serum but fails to generate a mucosal antibody response. In contrast, vaccines that are delivered mucosally stimulate antibody response along the

mucosal surfaces. Mucosal immunization which is a non-invasive route is an attractive alternative to parenteral immunization and using the appropriate delivery system it is possible to stimulate both mucosal and systemic immune responses. Mucosal vaccination offers also several benefits over parenteral routes of vaccination, including ease of administration, reduced side effects, the possibility of self-administration and especially in developing countries, reduced risk of the unwanted spread of infectious agents via contaminated syringes. All these benefits result in lower costs and better patient compliance. Needle phobia is an important issue for both adults and children, and makes immunization stressful. In addition, accidental needle sticks are a serious problem in both developed and developing countries. An estimated 5 accidental needle-stick injuries occur per 100 injections worldwide, posing a considerable risk to health-care providers. An even greater shortcoming of injections is their improper and unsafe use. The World Health Organization (WHO) has estimated that as many as one-third of immunization injections are unsafe in four of its six geographical regions (3).

The delivery of antigens by the mucosal route is associated with two major problems: (i) antigens delivered by this route are generally poorly immunogenic, and (ii) some antigens need to be protected against degradation. In the mucosal milieu, encapsulation protects labile material from enzymatic and chemical destruction and improves uptake into M-cells. Within the nasal mucosae, microencapsulated antigen may be less efficiently removed by mucociliary clearance mechanisms compared with soluble protein due to particle bioadhesion. This may improve vaccine absorption into subepithelial regions by prolonging antigen contact time with mucosal surfaces. Additionally, there is evidence that particulate material may be translocated across respiratory epithelial barriers and thence become systemically disseminated via the lymphatic system or blood stream. Thus,

nasally administered vaccines have the potential to initiate both local, and systemic, immunological responses in concert.

1.2. Types of Vaccines

The antigens in vaccines can be live organisms, dead organisms or subunit of organisms. Types of vaccines and examples for these types were summarized in Table 1.1 (4).

Table 1.1. Types of vaccines

Types of Vaccines	Examples
Attenuated live or killed bacteria	Cholera, paratyphoid fever, BCG (Bacille Calmette-Guérin)
Attenuated live virus	Polio virus, rabies
Subunit	Tetanus toxoid, diphtheria toxoid
Recombinant vaccine	Hepatitis B, influenza, human immunodeficiency virus (HIV)
DNA vaccines	Clinical trials ongoing for several infections

Live Vaccines

Live vaccines were the first to be discovered and they are still the most effective vaccine type. Live vaccines use organisms that have been attenuated (i.e. weakened) so that, although they replicate in the vaccine, they do not cause disease (5). The virulence of the organism is decreased without removing their ability to stimulate an immune response with

attenuation. When a live vaccine is used, the replicating agent provides an immunogenic stimulus over many days.

Killed and Inactivated Vaccines

In general, killed vaccines are less successful than live vaccines although they are theoretically much safer (6). Killed vaccines consist either of suspensions of killed organisms or of products or fractions of the microorganisms. To produce the equivalent stimulus with inactivated vaccine would require a vast dose of antigen, with the risk of producing severe reactions. This problem is partly overcome by combining the vaccine with an adjuvant, a substance which enhances the immune response to the antigen (7).

Subunit Vaccines

The approach for the subunit vaccines is the isolation of polypeptide subunits from the infectious agent. Subunits are components of pathogens and induce predominantly antibody response (8). Toxoids are types of subunit vaccine. They are bacterial exotoxins that have been chemically altered to make them safe, although they retain their immunogenicity. Tetanus toxoid is a good example of this approach. Subunit and toxoid vaccines are generally of low immunogenicity compared with intact organisms and they may need adjuvants in order to work effectively (9).

Recombinant Vaccines

A number of microbial vectors have been evaluated according to the development of recombinant vaccines with single or multiple antigenic

determinants, representing single or multiple pathogens. These include such vectors as some hepatitis B, influenza, human immunodeficiency virus (HIV) (7).

DNA Vaccines

DNA from bacteria has potent immunostimulatory properties that result from short sequence motifs called CpG motifs or immunostimulatory sequences (ISS). This approach involves the administration of plasmid DNA encoding a protein targeted for a therapeutic or protective response. This vector is taken up into cells where it can be expressed and can induce immune responses. The encoded protein to be targeted can be a foreign protein from a virus, bacteria, or protozoa, for example as well as an endogenous protein such as a tumor-specific antigen (4).

1.3. Adjuvants

Unlike attenuated live vaccines, killed whole organism or subunit vaccines generally require the addition of an adjuvant to be effective especially for mucosal routes (10). Adjuvants are compounds that enhance the immune response against co-inoculated antigens with the word adjuvant coming from the latin word *adjuvare*, which means to help or to enhance (11). Adjuvants can be used for various purposes: (i) to enhance the immunogenicity of highly purified or recombinant antigens; (ii) to reduce the amount of antigen or the number of immunizations needed for protective immunity; (iii) to improve the efficacy of vaccines in newborns, the elderly or immuno-compromised persons; or (iv) as antigen delivery systems for the uptake of antigens by the mucosa (12). An ideal adjuvant does not induce autoimmunity and it must be non-mutagenic, non-carcinogenic, non-

teratogenic and non-pyrogenic. It must be stable under broad ranges of storage time, temperature and pH (13). Among the main concerns related to adjuvant incorporation into the formulations are local reactions, systemic reactions (general toxicity, pyrogenicity), autoimmune diseases, hypersensitivity reactions, carcinogenicity, teratogenicity. It has been demonstrated that, new adjuvants can be especially relevant for developing new vaccines against infectious agents causing pathological conditions characterized by immunodeficiency, low responders and high-risk groups (14). Most commonly used vaccine adjuvants are summarized in Table 1.2.

The only adjuvants approved in human are aluminium compounds with their safety, low cost and adjuvanticity with a variety of antigens (13). Aluminium adjuvants have certain limitations, such as occasional induction of local reactions at the site of injection, increased IgE production, ineffectiveness for some antigens and inability to augment cell-mediated immune responses, especially cytotoxic T-cell responses, allergenicity and potential neurotoxicity (14). In general, the more potent the adjuvant, the more inappropriate it is for human use.

Adjuvants that are effective parenterally are reported to be generally toxic or unstable when given mucosally (15). The most widely experimentally used mucosal adjuvants in animals are cholera toxin (CT) and the closely related *Escherichia coli* heat labile enterotoxin (LT) which promote strong mucosal IgA responses, systemic IgG responses and CTL to co-administered antigens. CT and LT consist of a homo-pentamer of cell-binding B subunits associated with a single toxic-active A subunit. The adjuvant activity of CT especially for the nasal route depends on its ability to increase the permeability of the nasal epithelium to antigen (16). The potential of the cytokines and chemokines as adjuvants has also been reported (17). Nano/microparticles also have a great potential as adjuvants and the aim is

to develop a candidate vaccine formulation based on a particulate antigen preparation with built-in adjuvanticity (18).

Table 1.2. Classes of modern vaccine adjuvants (12,14,19,20,21)

Vaccine Adjuvants	Examples
Mineral salts	Aluminum hydroxide Calcium phosphate
Surface active agents and particles	Microparticles Virosomes Saponin (QS-21) Immune stimulating complexes (ISCOMs)
Bacterial products	Monophosphoryl lipid A CpG oligodeoxynucleotides Muramyl dipeptides and tripeptides
Cytokines and hormones	Interleukin-2 Interferon- α
Unique antigen constructs	Multipl peptide antigens attached to lysine core (MAP)
Polyanions	Dextran
Polyacrylics	Polymethylmethacrylate
Miscellaneous	Transgenic plants Tripalmitoyl pentapeptide
Carriers	Cholera toxin B subunit CpG dinucleotides
Living vectors	Vaccinia virus Adenovirus
Vehicles	Water-in-oil emulsions (Freund's incomplete) Liposomes Biodegradable polymer microspheres

1.4. Mucosal Immunization

Mucosal immunization has recently attracted much interest as a means for eliciting protective immunity against infectious diseases. Some infections occur at or take their departure from a mucosal surface and in these infections topical application of a vaccine is usually required to induce a protective immune response. Mucosal immune responses are most efficiently induced by the administration of vaccines onto mucosal surfaces, whereas injected vaccines are generally poor inducers of mucosal immunity and are therefore less effective against infection at mucosal surfaces (22). Mucosally delivered vaccines offer a number of potential advantages over traditional vaccines including the potential to confer mucosal as well as systemic immunity, increased stability, increased shelf-life, elimination of needles and the need for specially trained healthcare specialists to administer vaccines (10). The major advantage is induction of mucosal as well as systemic immunity (23).

The major pathogens of man gain access to the body via the mucosal routes, either orally, nasally or genitally. These mucosal pathogens (bacteria, viruses, and parasites) adhere to mucosal surfaces of the respiratory, gastrointestinal and urogenital tracts to initiate infections. Pathogenic bacteria most often bind to receptors on surfaces of mucosal cells as a result of one or more adhesin molecules on their surfaces, each with a distinct specificity for a particular surface receptor. As a result of these distinct and varied adhesin phenotypes, a bacteria increases the tissues it can colonize while concurrently increasing antigenic variability, thus enhancing its ability to evade the immune response of the host (24). After colonization, some microorganisms generate disease by releasing toxic factors, others penetrate into host tissues and give systemic or organ-specific diseases. Thus,

stimulation of mucosal defenses is very important for controlling infection and preventing disease (25).

B and T cells that are activated in organized mucosal lymphoid tissues upregulate the expression of tissue-specific adhesion molecules and chemokine receptors that function as 'homing receptors' to guide the lymphocytes back to the mucosa through recognition of endothelial counter-receptors in the mucosal vasculature. Some of these receptors and chemokines are broadly expressed or exhibit redundancy. For example, IgA secreting B cells that are activated in mucosa-associated organized lymphoid tissues express CCR10, the receptor for CCL28, which is secreted by epithelial cells throughout the small and large intestines, salivary glands, tonsils, respiratory tract and lactating mammary glands. Therefore, CCR10+IgA+ B cells can be attracted to all of these tissues. This broad recognition system explains why mucosal immunization at one site can result in the secretion of specific IgA antibodies in other mucosal or glandular tissues; this is referred to as the 'common mucosal immune system' (26).

By contrast, systemic immunization is generally ineffective for the induction of mucosal IgA antibody responses because expression of CCR10, $\alpha 4\beta 7$ -integrin and other mucosal homing receptors is not induced on B cells that are activated in the peripheral lymph nodes.

Essential criteria for mucosal immunization

Although, mucosal vaccination offers several benefits over parenteral routes of vaccination, mucosal vaccines have to overcome several barriers. Therefore, vaccination through mucosal membranes requires potent adjuvants to enhance immunogenicity, as well as delivery systems to

decrease the rate degradation and to target the vaccine to the site of immune function (27).

For successful initiation of mucosal immunization, the following essential criteria have been identified (28):

1. Effective delivery of antigen to the mucosal immune induction site (e.g. the epithelial cells of the Peyer's patches (PP);
2. Enhancement of mucosal immune responses by the use of live attenuated vaccine strains, improved antigen delivery systems, or mucosal immunomodulators such as bacterial enterotoxins and cytokines;
3. Choice of a regime and route of immunization to induce protective responses at the desired mucosal site and preferably systemically as well.

Induction of mucosal immune responses

The induction of mucosal immune responses against foreign antigens, microorganisms and vaccines requires the presence of organized lymphoid tissue, either within the mucosa or in draining lymph nodes (Figure 1.1).

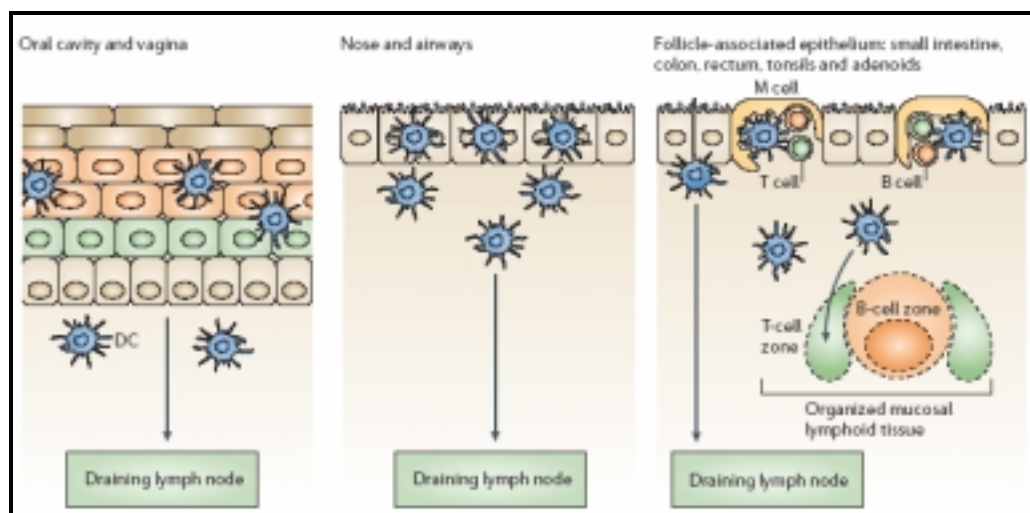


Figure 1.1. Antigen sampling at mucosal surfaces (29)

Antigen sampling strategies are adapted to the diverse epithelial barriers that cover mucosal surfaces throughout the body, but all involve collaboration with dendritic cells (DCs). DCs might congregate immediately under epithelia, migrate into the epithelial layer, and even extend dendrites into the lumen to capture antigens. DCs from any mucosal surface might travel to the nearest draining lymph node to present antigen to T cells. At sites of organized mucosal lymphoid tissues, specialized microfold (M) cells in the lymphoid follicle-associated epithelium deliver antigens across the epithelial barrier directly to subepithelial DCs that then present antigen locally in adjacent mucosal T-cell areas (29).

The mucosal immune system not only covers a large surface area ($> 400\text{m}^2$), but it also consists of separate sites, one where antigens/vaccines are encountered, processed and initial B- and T-cell triggering occurs (inductive sites), as well as separate areas where immune cells actually function (effector sites). The immunologic network operating on external mucosal surfaces consists of gut-associated lymphoid tissue (GALT), the lymphoid structures associated with bronchoepithelium and lower respiratory tract (BALT), ocular tissue, upper airway, salivary glands, tonsils and nasopharynx (NALT), larynx (LALT), middle ear cavity, male and female genital tracts, mammary glands, and the products of lactation (30). The organized lymphoid follicles in the GALT and NALT are considered the principal inductive sites of mucosal immune response. They are involved in the uptake, processing and presentation of antigens and comprise the MALT (16, 31).

Gut associated lymphoid tissue (GALT)

In addition to its functions in digestion, nutrient transport, water and electrolyte exchange, and endocrine and paracrine hormone production, the intestinal epithelium has a role in defining the barrier between the host and the external environment (32). GALT is critical to the induction of mucosal immunity in the intestine and consists of the tonsils and the adenoids, the PP of the small intestine, the appendix, and solitary lymphoid follicles of the large intestine and rectum (33). To induce a mucosal immune response by the oral route, an antigen must be taken up from the gut lumen and delivered to lymphoid cells in the GALT. The major inductive sites in the GALT for secreted immunoglobulin A (Ig A) responses are generally believed to be the organized lymphoid tissue and in particular the PP, which contain a dome region enriched in B and T lymphocytes and antigen presenting cell (APC) (34).

The direct interaction of particulates transcytosed by M cells with the underlying GALT makes these cells the primary target for oral vaccine delivery, e.g. by biodegradable vehicles (35). Targeting of drugs and delivery systems to specific M cells in the gastrointestinal tract requires the identification and exploitation of a unique feature of the site. Uptake of particulate antigen carrier systems by specialized M-cells of the gut-associated lymphoid tissue is still a limiting step in inducing efficient immune responses after oral vaccination. Once across the M cell, the antigen is processed and presented to T lymphocytes that proliferate in place and stimulate local B lymphocytes. These then migrate to distant sites. The preponderance of cells producing the secretory IgA molecules characteristic of human mucosal immunity (36,37). The uptake efficiency is mainly

dependent on the size of delivery systems: particles larger than 10 μ m are not internalised by the M-cells (38).

Peyer's patches (PP)

Peyer's patches are the organized lymphoid tissue in the lamina propria of the small intestine in which immune responses to ingested antigens may be initiated. Lymphocytes are found in large numbers in PP. Histologically; PP can be included four regions: germinal center, follicular area, parafollicular area and dome area (39). PP are composed mostly of B cells with smaller numbers of T cells and accessory cells, all arranged in follicles similar to those found in lymph nodes (40). The most obvious component of PP is the follicle, consisting of a proliferating germinal centre surrounded by B-lymphocytes. A mixture of lymphocytes, macrophages and dendritic cells are found on top of the follicle towards the gut lumen (41).

M Cells

Specialized antigen-sampling cells (M cells) that are located in the follicle-associated epithelium (FAE) of the organized mucosa associated lymphoid tissues (26). M cells use multiple endocytotic mechanisms for uptake of macromolecules, particles and microorganisms. M cells deliver antigens by transepithelial vesicular transport from the aerodigestive lumen directly to the subepithelial lymphoid tissues of nasopharynx-associated lymphoid tissue and PP. Soluble proteins, particles and live microorganisms transverse the M cell by transcytosis and are delivered into a "pocket" on the basolateral side of the cell that is packed with T and B lymphocytes, macrophages and dendritic cells. Owing to their transcytotic capacity, M cells

may represent an efficient potential portal for oral and nasal vaccine delivery (40,42).

Intraepithelial lymphocytes

Macrophages and T cells, both of which participate in cell-mediated responses, have been found in significant numbers in the lamina propria, Peyer's patches and other organized lymphoid follicles in the intestine (43). Intraepithelial lymphocytes (IEL) are located above the basement membrane between intestinal absorptive enterocytes. Absorptive enterocytes do not readily transport biodegradable microspheres but can take up and present soluble antigens, in conjunction with MHC I (Major Histocompatibility complex) and MHC II, to intraepithelial lymphocytes.

Antibodies

Antibodies or immunoglobulins are a family of structurally related glycoproteins produced in membrane-bound or secreted form by B lymphocytes. Membrane-bound antibodies serve as receptors that mediate the antigen triggered activation of B cells. The effector functions of antibodies are initiated only by antibodies that have bound antigens and not by free Ig. Changes in the isotypes of antibodies during humoral immun responses influence how and where the responses work to eradicate antigen (4). The most important protection mechanism is based on immune exclusion. Second mechanism is called immune complex transcytosis.

Immunoglobulin A (Ig A)

IgA is the major class of antibody that is produced in the mucosal immune system. An important characteristic of the mucosal adaptive immune response is the local production and secretion of IgA antibodies that, unlike other antibody isotypes, are resistant to degradation in the protease-rich external environments of mucosal surfaces (26). Evidence obtained from animal models shows that vaccines given via the mucosa are able to elicit neutralizing serum antibodies and cell-mediated immune responses as well as mucosal secretory IgA antibodies. IgA is produced by plasma cells in the lamina propria of mucosal tissue, binds to the poly-Ig receptor at the base of an epithelial cell and transported through epithelial cells into the intestinal lumen by this receptor (4). The existence of a common mucosal immune system permits immunization on one mucosal surface to induce secretion of antigen-specific S-IgA at distant sites.

Local IgG synthesis also can occur in the mucosal tissues following the administration of antigen or vaccine to mucosal surfaces, but, IgG concentrations are generally lower than those of sIgA. Nevertheless, intact IgG in mucosal tissues, whether locally produced or from serum, can potentially neutralize pathogens that enter the mucosa and prevent systemic spread (26).

Nasal associated lymphoid tissue (NALT)

The nasal mucosa is a common portal of entry of infectious agents; a topical application of vaccine may be the most efficient way of inducing a protective immune response (16). NALT is an abundance of intraepithelial and lamina propria lymphocytes. These functions make it an extensive and

extremely complex tissue. In NALT, morphologically and functionally similar cells have been described with GALT (16). NALT contains all of the immunocompetent cells that are required for the induction of antigen-specific immune responses. Because of these cells NALT is likely to have a central role in the development of a nasal vaccine (44). Inhaled particulate impacting on the mucus layer of the nasal mucosa may selectively be delivered to the organized NALT structures via the overlying specialized lymphoepithelium and induce an immune response, although it may be cleared rapidly by ciliary motion (45). NALT and PP are thought to have similar immunological characteristics and biological functions, as well as to contain the same types of resident immunocompetent cell (46). However, it was demonstrated that NALT is more a T-cell organ, while PP are B cell organs (16). In the upper respiratory tract (URT), the pharyngeal rings are conceived to be an equivalent to PP. So, similar to oral immunization, nasal immunization can stimulate antigen-specific cell-mediated responses and IgA responses in distant mucosal effector tissues. However, in general, NALT-targeted immunization effectively induces antigen-specific immunity in the respiratory and reproductive tissues (47,48).

1.5. Mucosal routes for immunization

Oral Route

Oral delivery of vaccines is an attractive mode of immunization because of its acceptability and its ease of administration (15). Moreover oral vaccines are generally inexpensive to produce, store and transport. However that the features of immune responses are similar in all mucosal lymphoid tissues; most of our knowledge of mucosal immunity is based on studies of the gastrointestinal tract. The two major disadvantages of oral vaccination

are: the state of immunological tolerance which normally occurs in response to food antigens, but which can also occur in responses to antigens derived from oral vaccines (15,49) and the degradation of antigen preparations in the digestive tract (46). Particle dilution in the intestinal tract, variable transit time and difficulties in scaling up formulations are the other limitations of oral immunization (50).

The first mucosal immunization was performed almost 50 years ago with the oral polio vaccine, which is still being applied, contains a live attenuated poliovirus. Several other needle-free vaccines (oral typhoid fever, oral cholera, oral rotavirus and nasal influenza), which also contain live attenuated pathogens, are now available (51).

Vaginal Route

In the absence of organized mucosal lymphoid tissues, antigens and pathogens might also be sampled on mucosal surfaces through another type of epithelial–DC collaboration. At mucosal locations such as the female genital tract, where there are no organized lymphoid follicles and the epithelium lacks M cells. Intraepithelial and subepithelial DCs that have captured pathogens could potentially interact with local lymphocytes to stimulate memory responses or immune tolerance, or they could exit the mucosa through lymphatics to present antigens to naive T cells in organized lymphoid tissues of draining lymph nodes (26). In general, vaginal and rectal immunization with non-living vaccines have limited success, owing to delivery and adjuvanticity issues (51).

Nasal Route

Intranasal immunization is a promising route by which local and systemic immunity can be achieved (52). First, the location, morphology, histology and cell phenotype of NALT support the notion that these lymphoid tissues are major inductive sites of the upper respiratory tract involved in the generation of immune responses. Second, due to low proteolytic activity of the nasal cavity and to its restricted area, the uptake of antigens can be less problematic than in the gut. Third, immunization studies in rodents and humans have also shown the mucosal and systemic immunity are achievable by intranasal administration, particularly with adjuvants. Fourth, the method used to deliver antigens to the nasal immune system is crucial to the outcome of the immune response. Delivery systems, immunostimulants or live vectors have been widely used to enhance mucosal and systemic immune responses after intranasal administration. Besides of all advantages, the manufacture and quality control of nasal vaccines would be less expensive than that of parenteral vaccines and the administration would be easier and avoid injection (47,53). Nasal immunization studies in humans and mice produced greater systemic antibody responses than other mucosal immunization routes (26,54). The nature of the interaction between antigen, the nasal mucosa and NALT depends on the composition, dose and frequency of administration of the antigen, and on the integrity of the epithelium (16). Another attractive feature of nasal immunization is that it requires a much smaller dose of antigen than does oral vaccination for the induction of antigen-specific mucosal and systemic immune responses, because the antigens are not exposed to degradation by digestive enzymes (16, 47).

Although vaccine degradation is not as drastic as in the gut, also for this route a carrier system is needed. Since the half time of clearance in the human nasal cavity is only about 15 min and protein-like agents are hardly transported over the epithelial barrier, vaccine encapsulation in microparticles may enhance the uptake by M-cells or co-administering the antigen with an absorption enhancer may increase the induction of immune responses after nasal application (55). Antigens are known to be more immunogenic in particulate form than in solution form, but they are vulnerable to antigen-degrading enzymes and acids that are associated with the mucosal environment. To overcome these obstacles, much effort has been focused on the creation of novel non-toxic and non-immunogenic vaccine vehicles that can effectively deliver even the soluble form of antigen to the organized mucosal inductive tissue. Such vehicles need to protect vaccine components from degradation, enhance their uptake from mucosal surfaces and perhaps function as an adjuvant (47).

For nasal vaccine delivery, various formulation strategies have been employed (52). These strategies can be divided into two major categories: solutions and particulate systems.

Hopkins et al. suggested that vaccines consisting of either attenuated bacteria or viruses applied via the nose are likely to be taken up and initiate immune responses in the NALT (46). Their results demonstrated that fluorescent *Salmonella typhimurium* could be detected in the NALT 24 hours after nasal immunization.

In recent years, on the basis of promising clinical trials showing the induction of protective immunity, an intranasally administered cold-adapted influenza vaccine, known as 'FluMist', has been made available to healthy Americans of ages 5 to 49 (1). It should be noted that the concept of cold-

adapted influenza virus was reported in 1967 (56), so more than 35 years were required for this discovery to be translated into an intranasal vaccine against infection with influenza virus. More recently, the concept of nasal immunization was adopted for the development of a vaccine against severe acute respiratory syndrome (SARS). An experimental nasally administered vaccine against SARS was able to induce protective immunity in African green monkeys (57).

1.6. Antigen delivery systems for mucosal and systemic immunization

The identification of promising new antigens, and an increased capacity to generate these antigens through chemical and biotechnology methods have led to many new vaccine opportunities. At this point; inappropriate antigen exposure, however, can result in unwanted outcomes, such as incomplete protection, allergic reactions, autoimmunity, infection or even tolerization. Thus, proper antigen delivery is critical for achieving the desired outcome.

Traditionally, vaccines come in several forms: live attenuated, replicating pathogens and non-replicating, inactivated pathogens or their subunits. The latter, non-viable category is the safest one, but because of poor or no immunogenicity, it often requires adjuvants to elicit an adequate immune response. In the absence of adjuvant, a lack of responsiveness may occur, and naive antigen-specific T cells may recognize the antigen but are not sufficiently activated or they even become tolerated (58).

Induction of immune reactivity depends on antigen reaching and being available in lymphoid organs in a dose and time dependent manner, and antigen that does not reach lymphoid organs is ignored by immune cells (59).

Thus, facilitation of effective antigen delivery to draining lymph nodes may be a desirable facet of candidate vaccine particulate delivery systems. Particulate adjuvants (e.g. emulsions, microparticles, ISCOMs, liposomes) have comparable dimensions to the pathogens that the immune system has evolved to combat. Immunostimulatory adjuvants may also be included in delivery systems to enhance the level of response, or to focus the response through a desired pathway (20, 60).

Microencapsulation of vaccine antigens offers a number of advantages over traditional vaccine formulations, including stability without refrigeration and the potential for utilizing less invasive routes of administration. Besides, the combination of antigens with biodegradable polymer delivery systems offers potential for a reduction in the number of administered doses (61).

Microparticles and Nanoparticles

Microencapsulation is a promising strategy for the development of the effective preventive vaccine delivery systems via mucosal, as well as parenteral routes (62). Delivery systems consisting of biodegradable nano/microparticles can potentially deliver either antigens or adjuvants to the desired location and durations to generate an optimal immune response. The carrier may also protect the vaccine from degradation until it is released. Adsorption or encapsulation of antigens to micro/nanoparticles resulted in the induction of significantly enhanced immune responses in comparison with alternative approaches. Polymeric particles with encapsulated antigens have become well-established in the last decade as potent antigen delivery systems and adjuvants, with experience being reported from many groups (63-65). Some examples are summarized in Table 1.3.

Table 1.3. Formulation approaches for mucosal immunization

Formulation	Antigen	Route	Human/ Animal model	Ref
Chitosan Solution	Inactivated subunit influenza	nasal	human	69
Chitosan Solution	Diphtheria toxoid	nasal	human	70
Chitosan Microparticle	Diphtheria toxoid	Nasal/ oral	mice	67
Chitosan Nanoparticle	Tetanus toxoid	Nasal	mice	71
Chitosan Microparticle	group C meningococcal conjugated vaccine	Nasal	mice	72
Chitosan Microparticle	OVA	oral	mice	73
Chitosan Microparticle	OVA / CT	nasal	rat	74
PLA microparticle	plague	nasal	mice	75
PEG / CpG solution	SARS-associated coronavirus	nasal	mice	76
Starch microparticle	purified flagellin from Salmonella	Oral / nasal	mice	77
Alginate microparticle	Pig serum albumin (PSA)	nasal	calves	78
Chitin microparticle	Influenza virus	nasal	mice	79
Hyaluronic acid microspheres	Influenza virus	nasal	mice/ rabbits/ pigs	80
PLA microparticle	Yersinia pestis subunits	nasal	mice	81
PLA microparticle	Tetanus toxoid	nasal	mice	82
PLG / polycaprolactone microparticles	<i>Schistosoma mansoni</i> antigen	nasal / oral	mice	83
Chitosan solution	Diphtheria toxoid	nasal	human	84
Alginate microparticle	Tetanus toxoid	nasal	rabbit	85
TMC nanoparticle	Influenza subunit vaccine	nasal	mice	86
TMC/ chitosan microparticle	meningococcal C conjugate vaccine	nasal	mice	87

The uptake of microspheres especially in the intestinal tract is a size dependent phenomenon (66). Microparticles smaller than 10 μm migrate to local lymph nodes and the spleen, enhancing induction of the systemic immunity besides mucosal immunity (67). Moreover, limited absorption of the microparticles in the GI tract may be improved through modification of their surface characteristics (68). Particulates for mucosal vaccination (microspheres or liposomes) should possess some degree of mucoadhesiveness to resist a premature clearance and increase the residence time. This ensures that sampling cells for particulate materials and antigen-presenting cells have the chance to recognize the particles and ingest them when administered especially by the nasal or pulmonary routes (62).

Besides of all advantages, polymer microspheres have certain limitations. Few polymers are approved by the regulatory authorities (for example polylactide, polylactide co-glycolide is approved by FDA). The loading of antigen into the microspheres can be very low (less than 1% w/w) and the antigen can be degraded during processing by strong shear forces or after loading when the polymer breaks down and releases. Using organic solvents during producing microparticles can harm the antigen encapsulated (88). Besides, during microencapsulation, for example during the spray-drying process, the protein is exposed to physical stresses (organic solvents, aqueous/organic interface and relatively adverse temperature and emulsion conditions) that may produce structural changes of the protein and thus may result in partial or total loss of enzymatic activity or antigenicity (87). Destabilisation and loss of immunogenicity can occur and accentuate during manufacture, storage and application, including lyophilization, freezing and drying, rehydration and incubation under physiological conditions. The

necessity to understand causes of destabilisation and developing routes to ensure maximum stability of the delivered antigen is even more critical (90). The most commonly observed degradation reactions are deamidation, oxidation and aggregation. These reactions are highly antigen-dependent, and may or may not have a prominent influence on the immunogenicity of a particular antigen (68).

Nanoparticles as well as microparticles are used as drug carriers or as adjuvants for vaccines. The historical development of nanoparticles were starting in the late 1960s and early 1970s. In 1950s and the 1960s, retarded and controlled release became a major focus of attention. The strategy for retarded and controlled release was a development of miniaturised delivery systems. At first investigated polyacrylic beads for oral administration was developed, then focussed on microcapsules and the first nanoparticles for drug delivery purposes and for vaccines (91). Nanoparticles for pharmaceutical and medical application are around now for over 35 years.

The main advantages of micro/nanosystems lie in their capacity to cross biological barriers, to protect macromolecules, such as peptides, proteins, oligonucleotides, and genes from degradation in biological media, and to deliver drugs or macromolecules to a target site with following controlled release (92). Nanosystems, due to their colloidal size, are able to cross and, hence, transport the associated drug through the mucosal barrier, thus acting as transmucosal macromolecular nanocarriers.

Polymers for antigen delivery

Development of effective delivery systems for presentation of antigens to mucosal surfaces is critical to the success of the vaccines. Widely used

polymers as vaccine delivery systems to encapsulate antigens are summarized in this section.

Alginate

Alginates are a family of linear unbranched polysaccharides (93). Alginate polymers with biodegradable, biocompatible and mucoadhesive properties have been widely used in numerous biomedical applications, including drug delivery systems and biotechnology industry (94). This bioadhesive polymer can be used to encapsulate a wide variety of antigens under mild conditions free of organic solvents (93). Alginate was thought to be an attractive polymer as antigen delivery system and have been used in several oral and nasal studies (66,78,95,96). First, the alginate matrix could protect the antigen from hostile environments. Second, alginate possesses mucoadhesive properties which could increase the contact time between alginate microspheres and the absorptive epithelium and the MALT M cells and therefore could enhance the uptake of encapsulated antigen. Third, biodegradable alginate microspheres may show variable release kinetics. Fourth, the low toxicity and low immunogenicity of alginate make this polymer a safe matrix. Fifth, alginate is readily available and inexpensive.

Poly (lactic-co-glycolic acid) (PLGA)

Poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and their copolymers (PLGA) which are nonimmunogenic have been extensively investigated because of their biocompatibility and biodegradability. The choice of PLA and PLGA as the matrix for vaccine formulations is based on its long-term safety in humans, its biodegradability, and the commercial availability of a variety of polymers of different molecular weights and monomer ratios (97). PLGA

microspheres are extremely flexible delivery systems capable of encapsulating a wide range of antigens. Besides, PLGA degrades to molecules normally found in the body as metabolic by-products and hence have no unwanted side effects (98). Studies with model antigens using PLGA as a delivery system showed that microparticles were capable of inducing potent serum and secretory antibody responses to entrapped antigens, following mucosal administration. In addition, it was shown that antigens entrapped in microparticles were capable of inducing protective immunity following mucosal administration (63,99).

Chitosan (CS)

Chitosan [$\alpha(1-4)$ 2-amino 2-deoxy β -D glucan] is a natural polysaccharide composed of β -1,4-linked D-glucosamine and it is obtained by deacetylation of chitin, a naturally abundantly available polymer (e.g. in crustaceans) (Figure 1.2). Acetamide group of chitin can be converted into amino group to give CS with deacetylation. Deacetylation can be done by alkaline treatment or by enzymatic reaction. The alkaline deacetylation is carried out by treating chitin with NaOH at high temperature. The degree of deacetylation increases with increasing temperature or NaOH concentration (100). In recent years, the production of chitosan from fungi, using fermentation methods is also gaining much interest (94).

Chitosan is biocompatible with living tissues since it does not cause allergic reactions and rejection. It can be naturally degraded by general lysozyme. Lysozyme in the body degrades chitosan into a common amino sugar, N-acetyl glucosamine, which is subsequently excreted as carbondioxide (101). Its chemical properties are determined by the molecular weight, degree of deacetylation and viscosity. It is positively charged and soluble in a weak acidic environment. Recent years the pharmaceutical and

food applications of chitosan increased remarkably because of its biodegradability, biocompatibility, its low production costs, having low toxicity (102). Also, chitosan has the property of transmucosal drug absorption enhancement. On the other hand, when compared to the effects of known absorption enhancers, chitosan's effect on the integrity of the epithelium or the cell membranes are minimal (103). Chitosan was found to enhance the permeability of intestinal and nasal epithelia by opening the tight junctions between cells, thereby favouring paracellular drug transport (104). It has also good adhesion, coagulating ability and immunostimulating activity (100).

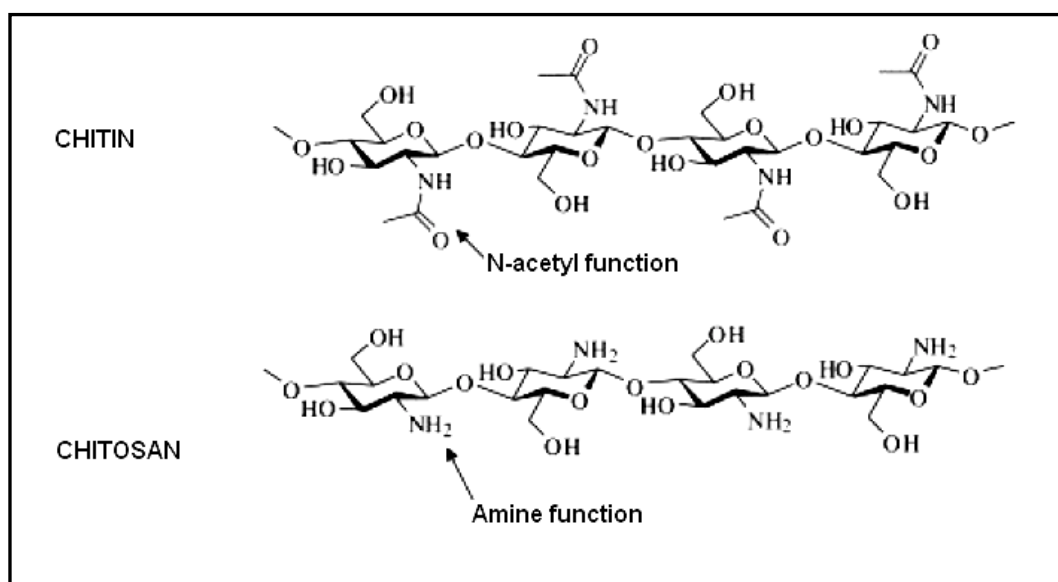


Figure 1.2. Structure of chitin and chitosan

Chitosan exhibits a variety of favorable physicochemical and biological properties, therefore it is receiving worldwide interest for its industrial uses as antimicrobials, biomedical materials, cosmetics, food additives, separators, sewage disposal, agricultural materials, and so on and it has found numerous applications as a biomaterial and as a pharmaceutical excipient in pharmaceutical technology for drug delivery (105,106).

Chitosan delivery systems for mucosal immunization

A very interesting and recent approach used for protein or vaccine delivery with biodegradable polymers carrying cationic or anionic groups, such as chitosan and its different charged derivatives (102). To improve the delivery of macromolecular drugs, chitosan has been used in the form of simple solutions and powders as well as in more sophisticated particulate formulations (100).

Unlike chitin, chitosan is easily soluble in aqueous solutions of many inorganic and organic acids. In addition, chemical and biochemical reactivity of chitosan are higher than those of chitin since chitosan has free primary amino groups distributed regularly in its molecular chain (107). The primary amino groups render special properties that make chitosan very useful for pharmaceutical applications. The interaction of the protonated amine groups of chitosan with the cell membrane results in a reversible structural reorganization of tight junction-associated proteins which is followed by opening of the tight junctions.

In order to obtain high immune response via mucosal route, it is necessary to apply an adjuvant/delivery system. The benefits of incorporating adjuvants into vaccine formulations to enhance immunogenicity must be weighed against the risk that these agents will induce adverse reactions. Chitosan has been incorporated with different vaccines and was proven to be an effective and safe adjuvant (72). Besides the adjuvant activity, as a mucosal drug delivery system, chitosan increases the bioavailability of the drug due to adhesion to the mucosal surfaces and absorption enhancing effects. Chitosan also enhances the contact interaction of macrophages with thymocytes (108). When administrated by nasal route, because of its

bioadhesive property, chitosan slows down the clearance of formulations from the nasal cavity (88).

Microparticles made from such polyelectrolytes exposed surface charges, which were used to adsorb oppositely charged protein antigens or DNA onto the polymers. The great advantage of this approach is the mild conditions that prevail for protein or DNA loading (109-111).

Chitosan easily forms microparticles and nanoparticles and has many advantages, particularly for developing micro/nanoparticles. These include: its ability to control the release of active agents; it avoids the use of hazardous organic solvents while fabricating particles, it is a linear polyamine containing a number of free amine groups that are readily available for crosslinking and it has mucoadhesive character, which increases residual time at the site of absorption. Moreover, various sterilization methods such as ionizing radiation, heat, steam and chemical methods can be suitably adopted for sterilization of CS for clinical applications (100).

Chitosan nano/microparticles encapsulate large amounts of antigens. It has been shown that ovalbumin loaded chitosan microparticles are taken up by the Peyer's patches of the GALT. Additionally, after co-administering chitosan with antigens in nasal vaccination studies, a strong enhancement of both mucosal and systemic immune responses is observed. This makes chitosan very suitable for nasal vaccine delivery. Large amounts of the protein BSA (bovine serum albumin) or vaccine tetanus toxoid (TT) can be incorporated in chitosan microparticles (109).

Different methods have been used to prepare CS particulate systems. Selection of any of these methods depends upon factors such as particle size requirement, thermal and chemical stability of the active agent, reproducibility of the release kinetic profiles, stability of the final product and residual toxicity

associated with the final product. Methods using to prepare CS nano/microparticles are listed below (100):

- Emulsion crosslinking
- Coacervation/precipitation
- Spray-drying
- Emulsion-droplet coalescence method
- Ionic gelation
- Reverse micellar method
- Sieving method

It has been demonstrated that CS micro and nanoparticles are able to entrap large quantities of antigen and are also able to transport the associated protein to the Peyer's patches. It was shown using with confocal laser scanning microscopy that the model antigen was not only associated to the surface, but also incorporated within the microparticles (67).

The influence of chitosan microparticles and of the mucosal adjuvant LTK63, a nontoxic *Escherichia coli* enterotoxin (LT) mutant on the quality of the immune response after intranasal administration of mice with the group C meningococcal conjugated vaccine was evaluated. The results demonstrated that especially if nontoxic LTK63 and chitosan microparticles are formulated together, the immunogenicity and efficacy of intranasally delivered vaccines significantly enhance (72).

In another study, 15 or 7.5 µg of the standard inactivated trivalent influenza vaccine were administered with chitosan nasally or 15 µg of the same vaccine intramuscularly and chitosan was found a potentially successful and easily administered nasal delivery system for vaccination against inactivated, subunit influenza vaccine (69).

Vila et al. investigated the effect of chitosan molecular weight (23, 38 and 70 kDa) on the ability of these nanoparticles to elicit significant immune responses. For this purpose, they entrapped the tetanus toxoid in chitosan nanoparticles by an ionic cross-linking technique. Nanoparticles were then administered intranasally to mice in order to study their feasibility as vaccine carriers. In conclusion, it has been shown that nanoparticles made of low molecular weight chitosan are promising carriers for nasal vaccine delivery (71).

A number of in vitro and in vivo studies showed that chitosan is a suitable material for efficient non-viral gene and DNA vaccine delivery. The major advantage of chitosan as a gene transfection system is the safety properties (110,112). Another advantage of chitosan is the mucosal adhesive property that can facilitate non-parenteral administration for the delivery of DNA. Chitosan forms polyelectrolyte complexes with DNA (113). It has been showed that chitosan effectively bind DNA in saline or acetic acid solution and partially protect DNA from nuclease degradation. Chitosan is positively charged polysaccharide that through ionic interaction with a negatively charged plasmid DNA can complex and form nanoparticles (111). The stable coupling of DNA to chitosan is an advantage for gene delivery because unstable interaction between DNA and carriers will result in short expression and reduction of the transfection efficiency.

Chitosan Derivatives

Chitosan is a weak base, soluble and active in its protonated form at low pH. Even though the amino groups of chitosan are predominately primary amine, they have a pKa of 6.5, therefore, at neutral pH, chitosan hardly carries charge, has a low solubility and as a result essentially inactive (114).

Its solubility problem limits its applications. To overcome this problem, the chitosan derivatives have been synthesized. Chemical modifications of chitosan are important to get the desired physicochemical properties such as solubility, hydrophilicity, etc.

Chitosan carries functional groups (amine, hydroxyl) therefore, various chitosan derivatives have been synthesized by chemical modifications in order to increase its solubility and study for different applications (115-117). The aspecific adjuvant activity of chitosan derivatives for mucosal immunization seems to be dependent on both the degree of deacetylation and the type of formulation (102). Chitosan derivatives like as chitosan also may represent potentially safe and efficient cationic carriers for gene delivery.

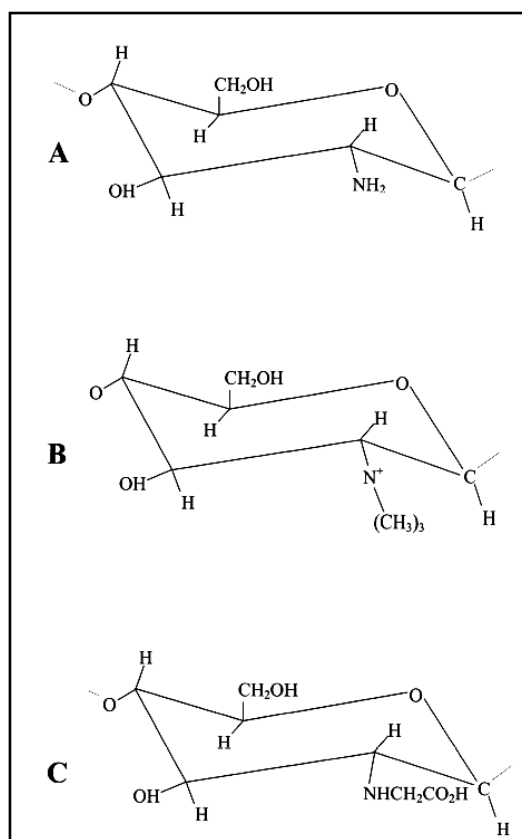


Figure 1.3. Chemical structures of chitosan (A), N-trimethyl chitosan (TMC) (B) and N-monocarboxymethyl chitosan (MCC) (C)

Amongst the derivatives of chitosan, trimethylated chitosan (TMC) and mono-N-carboxymethyl chitosan (MCC) are the most commonly investigated ones whereas with MCC, there are studies only as solution for intestinal absorption (118) or intraocular penetration (116) enhancer purposes. Chemical structures of chitosan and its derivatives are shown in Figure 1.3.

Trimethylated chitosan (TMC)

TMC is a partially quaternized chitosan derivative and is freely soluble over a wide pH range as compared to other chitosan salts and derivatives. 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 result improved solubility (115). At all degrees of quaternisation, TMC bears positive charges, independently of the environmental pH. Therefore, it can be predicted that TMC will also bind similarly to the cell surfaces as chitosan. The mechanism by which TMC is enhancing the intestinal permeability is similar to that of protonated chitosan (103). Because of its positive charge TMC is only suitable for improved delivery and absorption of hydrophilic, macromolecular drugs with neutral or basic properties (102).

TMC polymers proved to increase substantially the intestinal absorption and bioavailability of peptide analogs in both rats and pigs compared with administrations without the polymer. TMC showed no indication of epithelial damage or cytotoxicity, whereas its effect on the tight junction's integrity appears to be reversible (103). In another study, Florea et al. investigated the potential use of chitosan and TMC with different quaternisation degrees as pulmonary absorption enhancers and

demonstrated safely enhance peptide drug permeation across the bronchial barrier in vitro and in vivo (119).

Soluble TMC has mucoadhesive properties and excellent absorption enhancing effects even at neutral pH. Because of these properties, TMC is an attractive alternative to chitosan for the design of protein-loaded particles by ionic gelation method. At present only a few studies have reported the use of TMC solutions and TMC microparticles in nasal vaccine delivery (86,120-122).

Amidi et al. showed that TMC nanoparticles have interesting features suitable for nasal antigen delivery, such as high protein loading capacity, low cytotoxicity, and in vivo uptake by the nasal epithelium and NALT (121). In another study by the same group, the potential of TMC nanoparticles loaded with influenza antigen to elicit local and systemic immune responses after intranasal administration in mice investigated (86). Results showed that TMC nanoparticles enhanced significantly the systemic and local immune response, compared to i.m. or i.n. administration of soluble (conventional) influenza vaccine. Baudner et al. used LTK63 adjuvant together with chitosan or TMC delivery systems to enhance the immunogenicity and efficacy of intranasally delivered vaccines and results showed that this strategy of vaccination may lead to a more efficacious, protective response against meningococci intranasally. All these results makes the TMC nanoparticles a promising vehicle for nasal delivery of different antigens.

Mono-N-carboxymethyl chitosan (MCC)

MCC is a polyampholytic polymer, able to form visco-elastic gels in aqueous environments or with anionic macromolecules at neutral pH values. MCC appears to be less potent compared to the quaternized derivative (123).

Whereas chitosan forms precipitates with polyanions, MCC is compatible with anionic compounds. HPLC determinations of glyoxylic and glycolic acids, in conjunction with NMR analysis, permitted identification of the structure of the product, which is partly N-mono-carboxymethylated, N,N-dicarboxymethylated and N-acetylated depending on the level of deacetylation of the starting chitosan (124). MCC was claimed to behave as an intestinal absorption enhancer for macromolecular therapeutics (118). Maya et al. showed that both low and high viscosity MCCs managed to significantly decreased of Transepithelial electrical resistance (TEER) of Caco-2 cell monolayers when it was applied apically at concentrations of 3-5% (m/v). Moreover the same study indicated that, MCC was used to enhance the permeation of low molecular weight heparin in rats after oral administration and did not show any cytotoxicity.

1.7. Tetanus Toxoid

Tetanus is an acute, often fatal bacterial disease in which the clinical manifestations are not due to invasive infection, but to the release of potent toxins (125). Tetanus is unique among the vaccine-preventable diseases in that it is not communicable. Instead, tetanus is acquired through environmental exposure. The clinical presentation results from the actions of this toxin on the central nervous system (CNS). Prevention of tetanus can be achieved by use of a chemically inactivated toxin ('toxoid') that induces production of neutralizing antibodies (antitoxin). It can also be prevented by the introduction of exogeneous antibody (126).

The toxin is one of the most potent known poisons on a weight basis. As little as 1ng/kg may kill a mouse, and 0.3 ng/kg will kill a guinea pig. The estimated minimum lethal dose is less than 2.5 ng/kg (127).

The potent neurotoxin 'tetanus toxoid' is produced intracellularly by *Clostridium tetani* as a single polypeptide chain of 150,500 Da and numerous attempts are made to purify the toxoid (125). *Clostridium tetani* is a gram-positive, spore-forming, motile, anaerobic bacillus (128) (Figure 1.4).

Preparations should be stored at 2 to 8 °C and generally have a 2-year expiration date. Higher ambient temperatures for short periods, generally 7 days or less, do not reduce the potency of the toxoid except at temperatures of 45 °C or greater. Freezing, particularly repeated freezing can reduce potency (127).

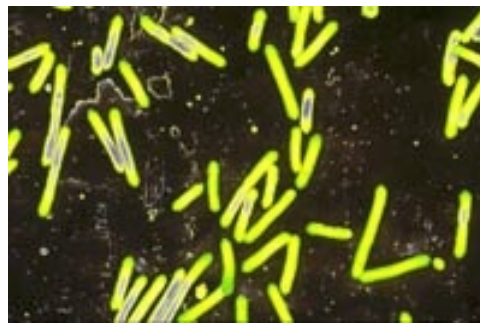


Figure 1.4. *Clostridium tetani*

Available Preparations of Tetanus Toxoid and Future Trends

Tetanus and diphtheria toxoids and pertussis vaccines are combinations universal administered parenterally in infants and children younger than 7 years. High-dose toxoid has been under investigation to reduce the number of doses needed for primary immunization (127). One strategy for this purpose is encapsulation of high doses to time-released microparticles. It was thought that microencapsulation allows gradual or pulsed antigen release in parenteral vaccination and also permits the eventual possibility of vaccination by the mucosal route (129). The

development of an effective non-parenteral vaccine include tetanus toxoid could be readily distributed and potentially self-administered but purified tetanus toxoid is poor immunogenic following nasal delivery. Significant immunological responses following intranasal application of these antigens is realized with an adjuvant or appropriate delivery system.

The World Health Organization has indicated that the development of improved immunization strategies for tetanus, as well as diphtheria, is a priority (World Health Organization (WHO) World Health Report 1999).

2. MATERIALS and METHODS

2.1. Materials and Equipment

2.1.1. Chemical Reagents

Chitosan, low viscosity (DD: 97%, Mol Wt: 150kDa)	Fluka, Germany
Chitosan glutamate (CS) (Protosan™ UP G 113), (DD: >75-90%, Mol Wt: 150kDa)	Novamatrix, Norway
Tetanus toxoid, MW 150 kDa, 5000 Lf/ml	Division of Bacteriology, NIBSC, UK
Ovalbumin chicken egg (OVA) Grade V, 45 kDa	Sigma-Aldrich, USA
Goat Anti Mouse Ig G	Sigma, USA
Goat Anti Mouse Ig A:HRP	AbD Serotec , UK
Goat Anti Mouse Ig G1:HRP	AbD Serotec , UK
Goat Anti Mouse Ig G2a:HRP	AbD Serotec , UK
Sodium iodide	Aldrich, Germany
1-methyl-2-pyrrolidinone	Aldrich, Germany
Iodomethane 99%	Sigma, Germany
Sodium chloride	BDH, UK
Potassium chloride	Sigma, USA
di-sodium hydrogen orthophosphate	BDH, UK
Potassium dihydrogen orthophosphate	BDH, UK
Glyoxylic acid monohydrate	Aldrich, Germany
Sodium hydroxide	Fisher, UK
Sodium borohydride	Sigma-Aldrich, Germany
Sodium tripolyphosphate penta basic	Sigma-Aldrich, USA

Calcium chloride	Sigma, USA
Citric acid	Fisher, UK
Tween 80	Sigma, USA
Tween 20	Sigma, USA
2,2 Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt tablets (ABTS)	Sigma, USA
Albumin from bovine serum, fraction V	Fluka, Germany
Bicinchonic acid solution	Sigma-Aldrich Co., USA
Coomassie Brilliant Blue	Bio-Rad Labs, USA
Ammonium persulfate	BioRad, Germany
10xTGS (Tris/Glycine/SDS buffer)	BioRad, Germany
Acrylamide/Bis solution, 30%	BioRad, Germany
Laemmli Sample Buffer	BioRad, Germany
2-mercaptoethanol	BioRad, Germany
Simply Blue Safestain	Invitrogen
Polyethylenimine (PEI)	Sigma, USA
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide	Sigma, USA
FITC-BSA	Sigma, USA

Cell Lines

Chinese hamster ovary (CHO-K1)	ATCC, USA
Mouse Balb/c monocyte macrophage J774A.1	ATCC, USA

Cell Culture Medium

Ham's F12-K	Sigma, USA
Dulbecco's modified eagle's medium (DMEM)	GIBCO, UK

2.1.2. Equipment

Micro BCA protein assay kit	Perbio Science UK Ltd, UK
Confocal Microscopy	Zeiss, Germany
Transmission Electron Microscope	FEI CM 120 BioTwin, Philips, Netherlands
Magnetic stirrer	IKA-WERKE RT, Germany
Ultracentrifuge	Sorvall Combi-Plus Dupont, USA
Laboratory centrifuge	Heraeus Sepatech, Germany
Sub-micron Particle Analyser	Malvern System 4700c, Malvern, UK
Zeta Potential Analyser	Malvern Zetamaster 3000, UK
Microplate Absorbance Reader	Dynex Technologies, USA
SDS-PAGE Gel Imaging	Gene Genius Bio Imaging System, USA
Vivaspin column (20 ml, 100,000 MW)	Vivascience, Sartorius, Germany

2.2. Methods

2.2.1. Synthesis of TMC

TMC was synthesized by reductive methylation of chitosan using methyl iodide (CH_3I) in the presence of a strong base (NaOH) at 60°C . The method of synthesis used in the present study was adapted from that previously reported by Thanou et al. (130). Chitosan (MW 150 kDa, Fluka) and sodium iodide were dissolved in 1-methyl-2-pyrrolidinone on a water bath at 60°C with stirring. Following dissolution of chitosan, aqueous sodium hydroxide solution and methyl iodide were added and both stirring. The product was precipitated using ethanol and subsequently isolated by centrifugation. After washing with ethanol and ether, the product was filtered and vacuum dried. In a second step *N*-trimethyl chitosan iodide initial product was dissolved in 1-methyl-2-pyrrolidinone on a water bath at 60°C with stirring. Sodium iodide, aqueous sodium hydroxide solution and methyl iodide were added with rapid stirring and the mixture was stirring on a water bath at 60°C . Afterwards, methyl iodide and sodium hydroxide pellets were added and stirring was continued for 1 h. Ethanol was added slowly and the mixture was stirred for 5 minutes and waited for the precipitation, filtered and vacuum dried. The product was dissolved in 10% (w/v) sodium chloride aqueous solution. The product was precipitated again with ethanol, isolated by centrifugation, filtered and dried. Then, the product was dissolved in water. Afterwards, ethanol was added, product was precipitated and after the centrifugation, it was filtered and washed with ether, filtered again and dried.

2.2.2. Synthesis of MCC

MCC was synthesized with the carboxymethylation of chitosan by reacting the free amine groups of chitosan with glyoxylic acid to produce a soluble, gel-forming imine which is then reduced (116). Chitosan (MW 150 kDa, Fluka) was dissolved in an aqueous acetic acid solution. Then, glyoxylic acid was added and the mixture was stirred at room temperature for 1 h. Following formation of imine, the pH was elevated to 4.5 by addition of 1M sodium hydroxide. Subsequently, an aqueous solution of 5% (m/v) sodium borohydride was added dropwise to reduce the formed imine. The mixture was stirred for 1 h and the product was isolated by precipitation with addition of ethanol. The product was washed on a glass filter under vacuum by ethanol/water aliquots. This product underwent a second step of reaction of carboxymethylation with the same amount of glyoxylic acid, same reaction time and isolation steps as described above.

2.2.3. Characterization of the chitosan derivatives

TMC and MCC were characterized by nuclear magnetic resonance (NMR) spectroscopy, and the degree of substitution was determined by ^1H -NMR on a 600 MHz spectrometer (Bruker, Karlsruhe Germany).

2.2.4. In vitro cellular toxicity studies

In order to evaluate the safety of the chitosan derivatives, a cytotoxicity experiment on CHO-K1 (ATCC, CCL61) cell lines was carried out by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Sterile, flat bottomed 96-well plates were seeded with CHO-K1 cells to a density of 10^4 cells/well and incubated at 37 °C and 5% CO_2 for 24 h. Polymer solutions were diluted with the cell culture media F-12 (Hams) from a 20 mg/ml stock to a series of solutions with concentrations of 0, 0.01, 0.02,

0.05, 0.1, 0.5, 1, 2, 5 mg/ml in sterilised water. Each concentration was prepared in triplicates. Following incubation for 4 h, the media was removed. The cells were washed with 50 μ l of PBS. Fresh culture media of 200 μ l was added into each well. The cells were then incubated for another 24 h. MTT (20 μ l of 5 mg/ml solution in PBS) was added to each well and the plates were subject to a further incubation at 37 °C in 5% CO₂ for 4h. The media was aspirated and the plates were gently tipped. 100 μ l DMSO was then added to each well and the plates were incubated at 37 °C for 30 min to dissolve the crystals. The plates were read at 570 nm using a Wallac Victor II microplate reader (1420 Multilabel Counter, Wallac Oy, Turku, Finland). Cells incubated in cell culture medium alone were used as a control to assay 100% viability. Viability of cells exposed to test materials was expressed as a percentage of the viability seen in control untreated cells. Polyethylenimine (PEI) was used as a control with its toxic structure (PEI was found to be the polymer with its high cytotoxicity). The IC₅₀ (i.e. concentration of chemical resulting in 50% inhibition of cell growth or reducing cell survival by 50%) was calculated as polymer concentration which inhibits growth of 50% of cells relative to non-treated control cells.

2.2.5. Preparation of Nanoparticles

Nanoparticles were prepared using ionic gelation method in the presence of Tween 80 (0.5 % m/v) as a resuspending agent to prevent particle aggregation (69,129,130). TT (tetanus toxoid) was added in the polymer solution during preparation of the nanoparticles (120 limit of flocculation (Lf) of antigen to 10 mg polymer). Preparation of nanoparticles is shown in Figure 2.1.

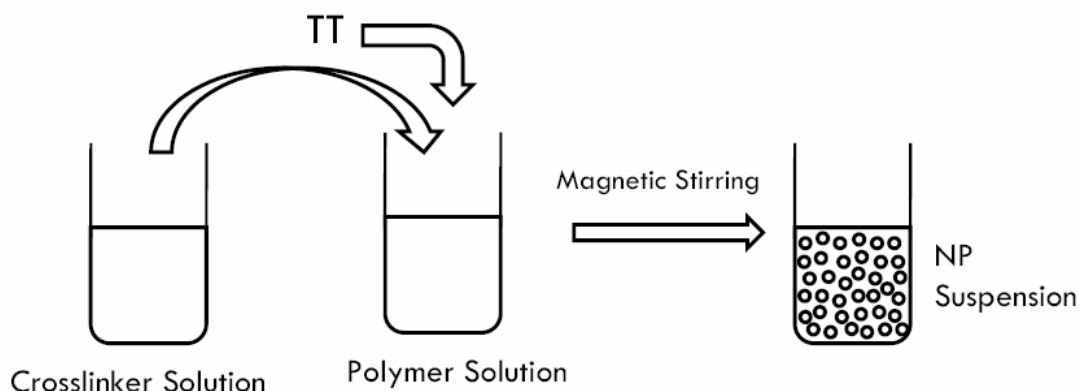


Figure 2.1. Preparation of the nanoparticles

2.2.5.1. Chitosan and TMC Nanoparticles

CS (Protasan™ UPG 113) and TMC nanoparticles were prepared with the same procedure using polyanionic tripolyphosphate (TPP) as a crosslinker at different concentrations (TMC:TPP ratios (m/m) between 3-6 and CS:TPP ratios (m/m) between 8-14). 2 mg/ml CS or TMC was dissolved in distilled water. Then TPP solution was added as a CS:TPP ratio of 12 and as a TMC:TPP ratio of 5.5 dropwise to this solution under constant stirring.

2.2.5.2. MCC Nanoparticles

MCC nanoparticles were prepared using different concentrations (2 and 4mg/ml) of MCC. Calcium chloride (CaCl_2) was used as the crosslinker. MCC was dissolved in distilled water and CaCl_2 solutions at different concentrations (MCC: CaCl_2 ratios (m/m) of 3,5,6,7.5 or 10) were added dropwise to this solution under constant stirring.

2.2.5.3. TMC/ MCC Nanoparticles

Nanoparticles were prepared using MCC as a crosslinker at different concentrations (TMC:MCC ratios (m/m) between 3-7). 2 mg/ml TMC was dissolved in distilled water. Then MCC solution was added dropwise to this solution under constant stirring.

2.2.6. Characterization of Nanoparticles

2.2.6.1. Protein Loading Capacity of Nanoparticles

The micro-bichinchoninic acid protein assay (microBCA, Pierce, Rockford, USA) and QuantiPro™ BCA assay kit (Sigma,USA) were used for the determination of TT. Briefly, 50 µl samples or standards (0–50 µg/ml TT) were added to each well in 96-well-plates. Bicinchoninic acid (solution A) and copper ions (solution B) were mixed (1:50) immediately before use and 200 µl was added to each vial. The plates were kept at 60°C for 1 h, and absorbance was read at 570 nm wavelength with an ELISA plate reader (Behring ELISA processor II; Dade Behring).

The amount of tetanus toxoid (TT) loaded to the particles, (loading efficiencies, LE) was calculated from the difference between the initial amount and the amount present in the supernatant after centrifugation(Eq 1):

$$LE = [(total\ TT)-(free\ TT)] / total\ TT \quad (Eq.1)$$

The concentration of total protein was determined from standard curves after plotting absorbance vs. the concentration, by linear regression analysis.

2.2.6.2. Particle Size Distribution

Particle size distribution of the nanoparticles was examined as mean diameter and polydispersity index by photon correlation spectroscopy at 90° angle and at 25°C (PCS, Malvern Instruments, UK). Samples were diluted before measurements.

2.2.6.3. Zeta Potential Measurement

Zeta potential of nanoparticles was measured by using Zetasizer (Malvern Instruments, UK) following dilution of nanoparticle samples in 0.001M KCl solution. Measurements were performed at 25°C.

2.2.6.4. Microscopic Evaluation

The morphological and surface characteristics of the nanoparticles were examined by means of transmission electron microscopy (TEM, FEI CM 120 BioTwin, Philips, Netherlands).

2.2.7. Determination of the protein integrity

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to evaluate the effect of the preparation process on protein integrity and antigenity. Materials used for the SDS-PAGE studies are presented in Table 2.1.

Table 2.1. Materials for the SDS-PAGE studies

Separating gel	Mini gel volumes (ml) 10%
Solution A	2.5
Solution C	3.325
Distilled water	4.0
10% (w/v) SDS	0.1
10% (w/v) APS	0.1
Overlay	Mini gel volumes (ml) 10%
Solution A	1.0
Distilled water	3.0
10% (w/v) SDS	0.040
Stacking gel	Mini gel volumes (ml) 10%
Solution B	0.95
Solution C	1.25
Distilled water	5.0
10% (w/v) SDS	0.075
10% (w/v) APS	0.075
Sample buffer	Mini gel volumes (ml) 10%
Distilled water	1.0
1 M Tris	0.2
Glycerol	0.1
10% (w/v) SDS	0.4
Bromophenol blue	0.1
β -mercaptoethanol	0.2
Solution A	
Reagent	Amount
Tris	36.3 g
1 M HCl	48 ml
TEMED	0.23 ml
Distilled water	Up to 100 ml
Solution B	
Reagent	Amount
Tris	5.98 g
1 M HCl	4.8 ml
TEMED	0.46 ml
Distilled water	Up to 100 ml
Solution C	
Reagent	Amount
Acrylamide	30
Bis-acrylamide	0.8
TGS buffer	
Reagent	Amount
Tris	15.1 g
Glycine	94 g
10% SDS (w/v)	50.0 ml
Distilled water	Up to 1000 ml

Nanoparticles were destabilized by adding 1 ml of 10% (m/v) NaCl to 4.5 ml of nanoparticle suspension. The protein sample was prepared in electrophoresis loading buffer (sample buffer) and heated for 5 min. at 95°C. Samples were loaded onto a vertical slab gel and subjected to electrophoresis at 200mV, using Biorad Power Pac 1000 (Biorad, Hemel Hempstead, UK) in TGS buffer solution. Gels were subsequently fixed and stained with Coomassie brilliant blue staining solution (50 ml, 0.1% (m/v)) (Sigma) for 1 h. Gels were then destained until protein bands were distinct. After destaining, gels were analyzed using Gene Genius Bio Imaging System (Syngene, USA). Materials used for the SDS-PAGE studies are presented in Table 2.1.

2.2.8. Stability of Nanoparticles

Nanoparticles were kept at 4°C and 25°C as aqueous suspensions, and particle size, zeta potential, surface morphology and protein integrity were examined after 1 and 6 months.

2.2.9. Cellular uptake studies

Cellular uptake studies were performed using FITC-BSA loaded nanoparticles. FITC-BSA was incorporated into nanoparticles during preparation.

Mouse Balb/c monocyte macrophage J774A.1 (ECACC, 91051511) was cultured with Dulbecco's Modified Eagle's Medium (DMEM) supplied with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin in an incubator at 37 °C and 5 % CO₂ atmosphere with 95% humidity. Poly-L-Lysine (PLL, 1 mg/ml) solution was sterilised by passing

through a 0.22 μm filter and 20 times diluted as a working solution (50 $\mu\text{g/ml}$). The coverslips were sterilised in 95% ethanol and dried before coating and then were placed in a single layer in a sterile 6-well plate containing the working solution and incubated for 1 h at 37 °C. PLL solution was removed and the surfaces of coverslips were allowed to dry in air. Before placing the particles, macrophages were washed with PBS and then resuspended by scrapping. After collection, 4×10^5 cells in 2 ml of DMEM were added in each well of the 6-well plate with coverslips. Cells were incubated for 18 h and then washed for 2 times with DMEM-L-glutamine to remove non-adherent cells and FCS. Subsequently, 2 ml of DMEM L-glutamine medium containing 200 μg of nanoparticles loaded with FITC-labelled BSA were added to each well and incubated to allow phagocytosis of the particles. To investigate time-dependent uptake, cells were incubated for 30, 60, and 120 min at 37°C. After incubation, cells were fixed with cold methanol for 5 min. Then the fixed cells were washed with PBS. The coverslips with fixed cells were mounted on microscope slides and sealed using nail polish. Cell uptake (phagocytosis) was observed using a Zeiss LSM 510 Meta laser scanning confocal microscope. The channels used for the observation were set at a multiple channel function using T1: 488 nm, T2: 633 nm. Pixel time set at 32 μs . The objective was Plan-apochromat 63x/1.4 oil (DIC: differential interference contrast).

2.2.10. In vivo Studies

For *in vivo* studies, TT loaded TMC, MCC, TMC/MCC complex and CS nanoparticles (Table 2.2) were administered intranasally (i.n) to Balb/c mice (n=5) aged up to 6-8 weeks and weighing about 25 g. Animals were maintained on a normal mouse diet through-out the study. All procedures were performed in accordance with Animal Scientific Procedures Act 1986.

For comparison, immune response of the solutions of TMC, MCC, TMC/MCC or CS were investigated following i.n. or subcutaneous (s.c.) administration. Nanoparticle suspensions were centrifugated with vivaspin column (20 ml, 100.000 MW) and concentrated to the volume include the needed dose. Solutions were prepared at 0.2 % m/v polymer concentration, containing 5Lf TT/ mice dose.

Mice were immunized twice either intranasally or by subcutaneous injection (priming dose on day 0 and boosting on day 22). Mice were lightly anesthetized with fluorethane before i.n. application and 25µl formulation was applied drop wise into the nares (12.5µl/nostril) with a micropipette. 200 µl of subcutaneous injections were applied to un-anesthetized animals. To assess immune responses, serum tail vein blood samples were collected on day 20 and, postboosting titres were obtained on days 42 and 56. The blood samples were centrifuged for 10 min at 10000 rpm in an eppendorf centrifuge, the serum was collected and stored at -70°C until analysis for IgG, IgG1 and IgG2a titres. Vaginal washes were also obtained by introducing 100µl PBS (pH7.4) into vagina with 200 µl pipettor and flushing it four times. The washes were vortexed and centrifuged for 10 min at 10000 rpm at 4°C. The fluid samples were stored after adding sodium azide as a bacteriostatic agent at - 70°C after centrifugation until being assayed. At day 56, mice were sacrificed and serum and mucosal washes (nasal and vaginal) were collected.

Table 2.2. Formulations investigated in vivo

Formulation	Formulation Code
TT in PBS solution	Free-TT (Control)
Chitosan nanoparticles	CS-np
TMC nanoparticles	TMC-np
TMC/MCC complex nanoparticles	TMC-MCC-np
MCC nanoparticles (MCC: Ca ₂ Cl ratio = 7.5)	MCC- np-1
MCC nanoparticles (MCC: Ca ₂ Cl ratio = 6.0)	MCC- np-2
Chitosan solution (0.2%)	CS-Sol
TMC solution (0.2%)	TMC-Sol
TMC/MCC solution (0.2%)	TMC/MCC Sol
MCC solution (0.2%)	MCC Sol

2.2.11. Enzyme-linked immunoabsorbent assay (ELISA)

Titration of TT-specific IgG, its subunits and IgA antibody in serum samples and mucosal washes collected from immunized animals was achieved with ELISA (133). Briefly, 96-well microtiter plates (Nunc Maxisorp; Fisher) were coated, in each well with 100 µl of 0.5 limit of flocculation (Lf) TT in PBS and incubated overnight at 4°C. The plates were washed with PBS containing 0.05% Tween 20 (PBS-T) and were blocked with 2% BSA solution for 1 h at 37°C. After washing with PBS-T, serial 2-fold dilutions of an in-house reference mouse serum (initially diluted to 0.02 IU/ml) and test mouse serum in PBS were titrated across the plate, to a final volume of 100 µl. Plates were incubated for 1 h at 37°C. At the end of the incubation period,

plates were washed with PBS-T and were incubated with 100 µl of goat anti-mouse Ig G (Sigma) or 100 µl of goat anti-mouse Ig A (Star) diluted 1:2000 with PBS/well, for 1 h at 37°C. Unbound conjugate was removed by washing with PBS-T and enzyme activity was determined by adding a tablet of 2,2 Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABST) (Sigma) and 5 µl of hydrogen peroxide solution (30% m/v) in 0.05 mol citric acid buffer/l. All ELISA procedures were performed at room temperature. The absorbance was read at 410 nm on an ELISA reader (Dynex Technologies, MRXII Micro plate Absorbance Reader, USA).

The concentration of active TT was determined from standard curves after plotting absorbance vs. the concentration, by regression analysis.

For Ig G1 and Ig G2a antibody subclasses, a similar protocol which is described above was followed. Reference goat anti-mouse Ig G1 or goat anti-mouse Ig G2a subclasses (Star) were diluted 1:4000 with PBS.

2.2.12. Statistical analysis

Kruskal Wallis variance analysis was used to analyze the differences between and within- in vivo groups for all data. $p < 0.01$ was taken as significant.

3. RESULTS

3.1. Synthesis and Physicochemical Characterization of TMC

TMC was synthesized from chitosan via a two-step methylation procedure described in section 2.2. After the first methylation step, TMC underwent a second methylation step and yielded TMC with a degree of substitution of 57% that was called TMC 57.

The schematic representation of the derivatization procedure for TMC is shown in Figure 3.1.

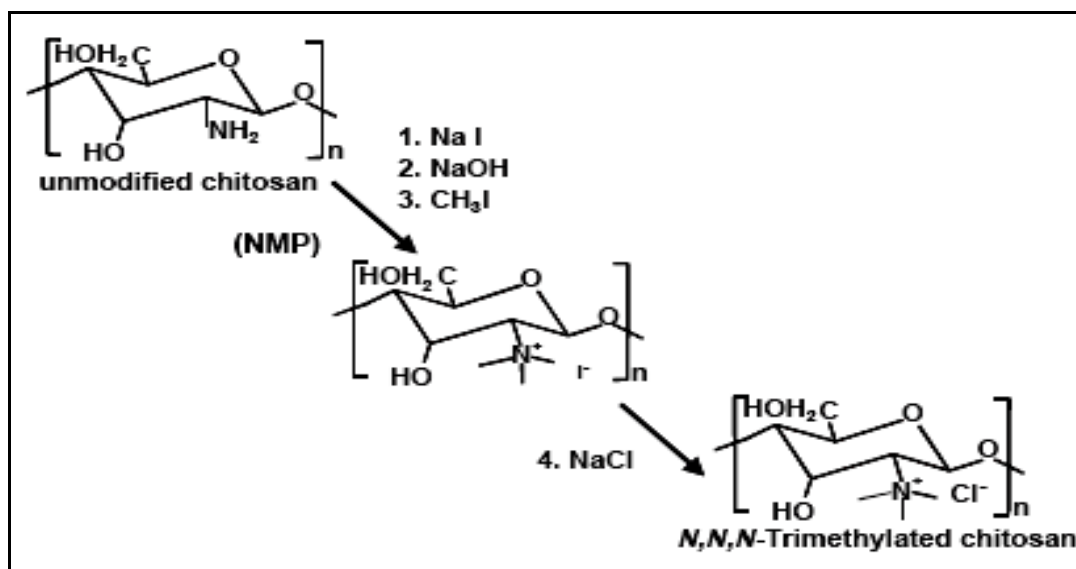


Figure 3.1. Reaction scheme of chitosan trimethylation and counter ion replacement

The ¹H NMR spectra revealed quaternization degree or methylation of TMC as 57% (Figure 3.2).

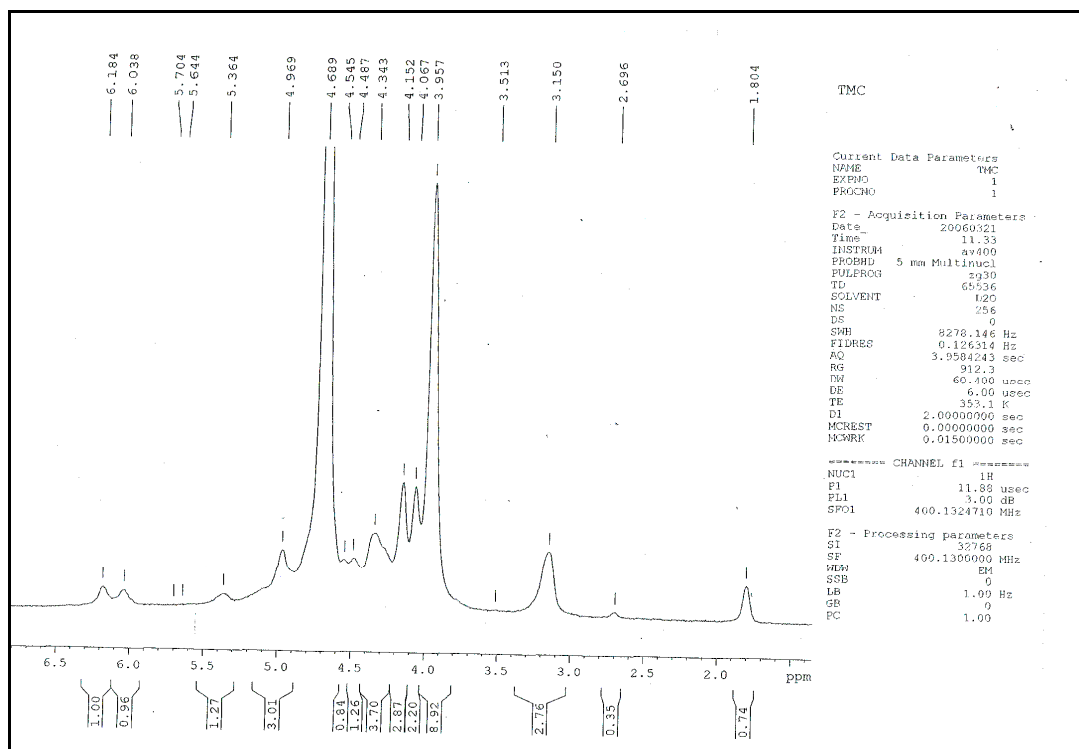


Figure 3.2. Proton nuclear magnetic resonance spectroscopy (^1H NMR) spectra of TMC

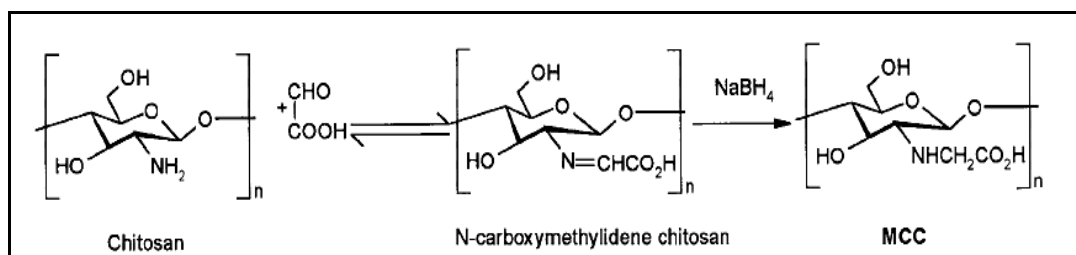


Figure 3.3. Schematic representation of the synthesis of the mono-N-carboxymethyl chitosan (MCC)

MCC was synthesized with the two carboxymethylation step of chitosan by reacting the free amine groups of chitosan with glyoxylic acid to produce a soluble, gel-forming imine which is then reduced. The schematic representation of the derivatization procedure for MCC is shown in Figure

3.3. The ^1H NMR spectra revealed the substitution of MCC (carboxymethylation on the respective polymer as 70%) (Figure 3.4).

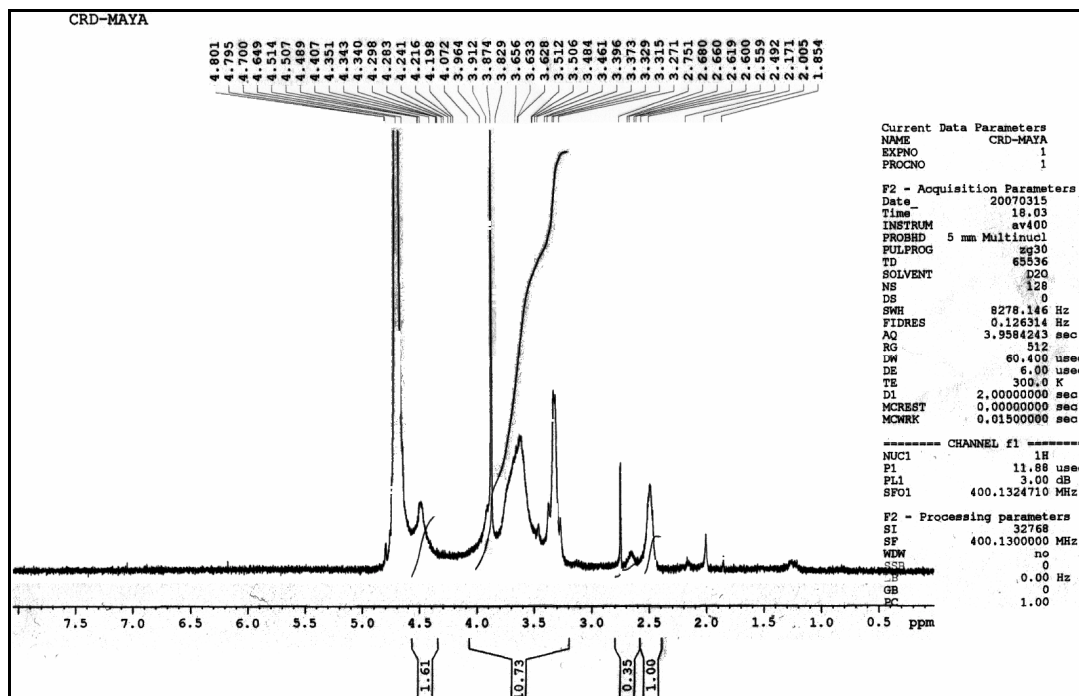


Figure 3.4. Proton nuclear magnetic resonance spectroscopy (^1H NMR) spectra of MCC

3.2. In vitro cellular toxicity studies

Chitosan and its derivatives were applied to the cells at concentrations ranging from 0-5 mg/ml and the effect on cell viability was measured. Results are shown in Figure 3.5, and IC_{50} results are summarized in Table 3.1.

For chitosan and its derivatives, cellular viability was found to be significantly higher when compared to that of PEI. Cell viability was found in the order of MCC, TMC/MCC complex, CS and TMC. Cell viability of chitosan and MCC solutions were higher than TMC solution. Cellular viability with TMC was found to be highly reduced above 0.5mg/ml concentrations. At

higher polymer concentrations (2-5 mg/ml), cellular viability was reduced with chitosan. IC_{50} of MCC and TMC/MCC complex were found to be higher than 5 mg/ml.

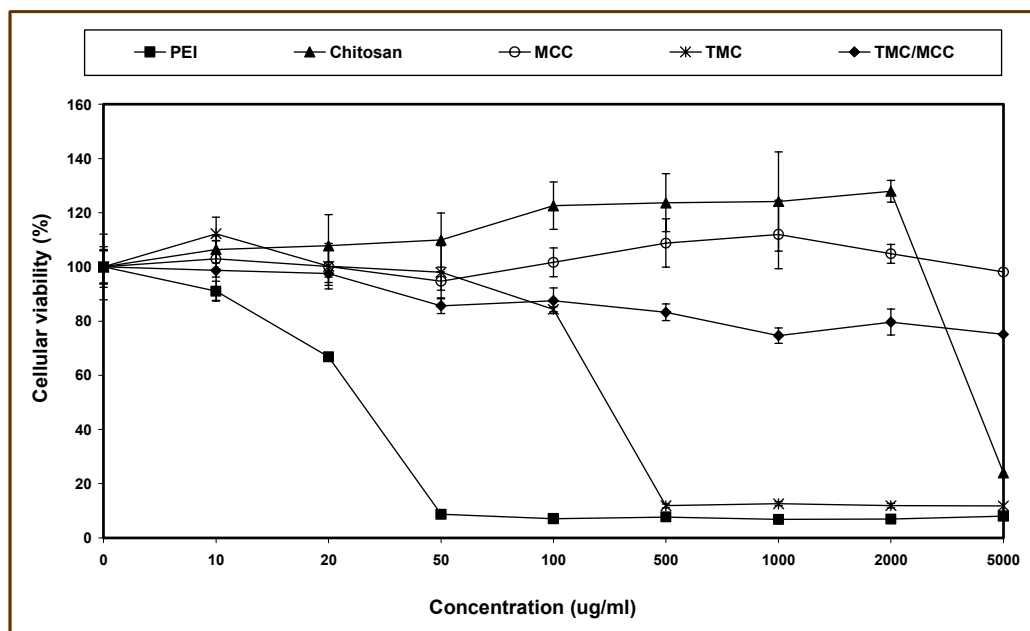


Figure 3.5. Dose viability curves of chitosan and its derivatives ($n=3 \pm S.D.$)

Table 3.1. Cell viability for chitosan and its derivatives

Polymer	IC_{50} ($\mu g/ml$)
CS	> 2000
TMC	> 500
MCC	> 5000
TMC/MCC complex	> 5000
PEI	> 20

* IC_{50} : concentration of chemical resulting in 50% inhibition of cell growth or reducing cell survival by 50%

3.3. Preparation of Nanoparticles

Different initial concentrations of chitosan derivatives (TMC or MCC) and also different crosslinkers (TPP, CaCl_2 or MCC) at various concentrations were used to establish preparation conditions at which nanoparticles were formed. Following the preparation of the nanoparticles, three different systems were identified: clear solution, opalescent suspension and aggregates. The zone of opalescent suspension, which corresponded to a suspension of very small particles was further investigated. These were the formulations prepared using 2 mg/ml MCC and at MCC: CaCl_2 ratios (m/m) of 6 and 7.5 for MCC nanoparticles (Table 3.2) , 2 mg/ml TMC and at a TMC:TPP ratio (m/m) of 5.5 for TMC nanoparticles and for TMC/MCC complex nanoparticles, 2 mg/ml TMC and TMC:MCC ratio (m/m) of 5.0. Aggregates were observed at higher TMC or MCC concentrations and low MCC: CaCl_2 or TMC:TPP ratios (m/m).

Table 3.2. Properties of the blank MCC nanoparticles

	MCC Concn (mg/ml)	MCC: CaCl_2 Ratio (m/m)	z Average (nm) $\pm$ S.D.	Polydispersity Index	ζ - potential (mV)
Trial 1	4	5	Aggregates		
Trial 2	4	3	Aggregates		
Trial 3	2	10	39.6 $\pm$ 2.2	0.360	-14.45
Trial 4 (MCC np1)	2	7.5	37.2 $\pm$ 2.9	0.334	- 26.05
Trial 5	2	5	150.6 $\pm$ 4.6	0.246	- 26.57
Trial 6 (MCC np2)	2	6	86.0 $\pm$ 3.4	0.294	- 22.20

ζ : zeta; z Average: particle size ; np : nanoparticle

With TMC/MCC complex nanoparticles, the effect of TMC:MCC ratio (m/m) on the particle size was also investigated. Different MCC concentrations with a constant concentration of TMC were used to establish preparation conditions at which TMC/MCC complex nanoparticles were formed. Results are shown in Figure 3.6. No significant differences in particle size was observed with increasing TMC:MCC ratio (m/m). The ratio TMC:MCC, which corresponded to a suspension of very small particles without any aggregation, was further investigated. This was the formulation prepared using 2 mg/ml TMC and at TMC:MCC ratio (m/m) of 5.

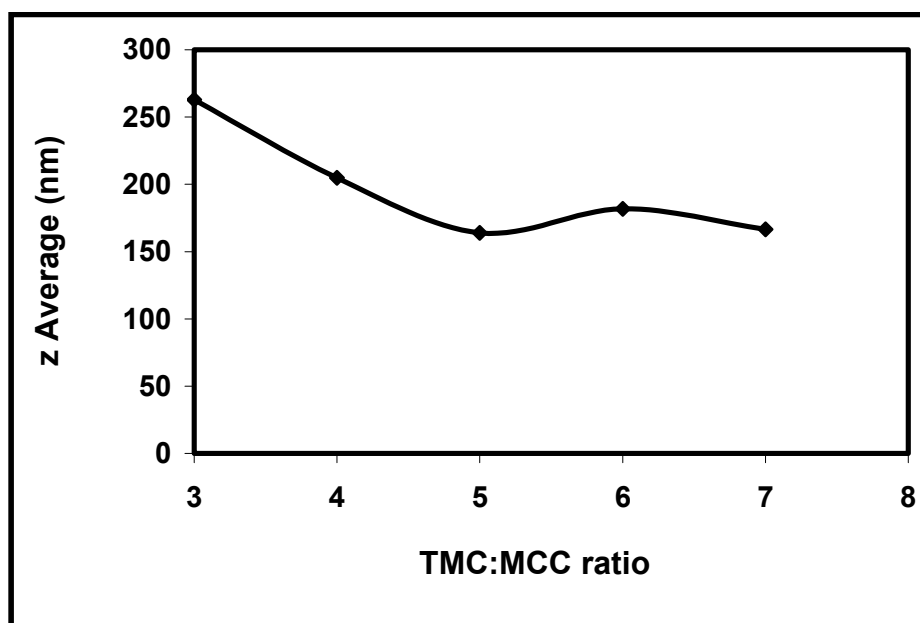


Figure 3.6. Effect of TMC:MCC ratio (m/m) on particle size of nanoparticles

3.4. Characterization of Nanoparticles

The particle size, zeta potential and polydispersity index of the blank nanoparticles are shown in Table 3.3. and for the TT loaded nanoparticles with the loading efficiency results are shown in Table 3.4.

Table 3.3. Blank nanoparticle formulations

Formulations	ζ Potential (mV) $\pm$ S.D.	z Average (nm) $\pm$ S.D.	Polydispersity index $\pm$ S.D.
CS-np	55.4 $\pm$ 0.3	186.0 $\pm$ 12.7	0.287 $\pm$ 0.015
TMC-np	16.6 $\pm$ 0.4	109.5 $\pm$ 0.1	0.208 $\pm$ 0.061
TMC-MCC-np	55.7 $\pm$ 0.2	163.1 $\pm$ 1.7	0.174 $\pm$ 0.004
MCC- np 1	-26.5 $\pm$ 0.6	37.2 $\pm$ 2.9	0.334 $\pm$ 0.045
MCC- np 2	-22.2 $\pm$ 0.4	86.0 $\pm$ 3.4	0.294 $\pm$ 0.038

ζ : zeta; z Average: particle size ; np : nanoparticle

Table 3.4. TT loaded nanoparticle formulations

Formulations	ζ Potential (mV) $\pm$ S.D.	z Average (nm) $\pm$ S.D.	Polydispersity Index $\pm$ S.D.	Loading Efficiency %
CS-np (CS:TPP ratio = 12)	53.8 $\pm$ 0.8	397.8 $\pm$ 7.8	0.302 $\pm$ 0.028	78 $\pm$ 4
TMC-np (TMC:TPP ratio = 5.5)	18.52 $\pm$ 1.2	306.0 $\pm$ 2.4	0.182 $\pm$ 0.039	93 $\pm$ 3
MCC-np 1 (MCC:CaCl ₂ ratio=7.5)	- 29.0 $\pm$ 2.5	43.7 $\pm$ 1.3	0.438 $\pm$ 0.021	95 $\pm$ 2
MCC-np 2 (MCC:CaCl ₂ ratio = 6)	- 28.8 $\pm$ 2.2	90.9 $\pm$ 0.8	0.280 $\pm$ 0.013	97 $\pm$ 2
TMC-MCC np (TMC:MCC ratio = 5)	43.0 $\pm$ 7.5	283.5 $\pm$ 2.5	0.193 $\pm$ 0.049	95 $\pm$ 3

MCC: mono-N-carboxymethyl chitosan ; TMC: N-trimethyl chitosan ; CS: chitosan; TPP: tripolyphosphate ; S.D.: standart deviation; ζ : zeta; z Average: particle size, np: nanoparticle

For CS and TMC (Table 3.4), the TT loaded nanoparticles were found to have similar sizes (300–400 nm), and a positive surface charge of +53 mV and +18 mV, respectively. It was possible to incorporate large amounts of TT into the nanoparticles prepared with chitosan and its derivatives with the loading efficiency of 78% for CS, and 93% for TMC (Table 3.4). Due to the positive charge of chitosan and TMC, and negative charge of TT, the antigen loading is expected to be provided by electrostatic interactions. For MCC nanoparticles, the particle size was found to be between 40-90 nm with a negative surface charge of -29 mV and a high loading efficiency (95-97%) (Tables 3.3 and 3.4). TEM images confirmed the particle size results and showed non-aggregated nanoparticles (Figures 3.7, 3.8, 3.9).

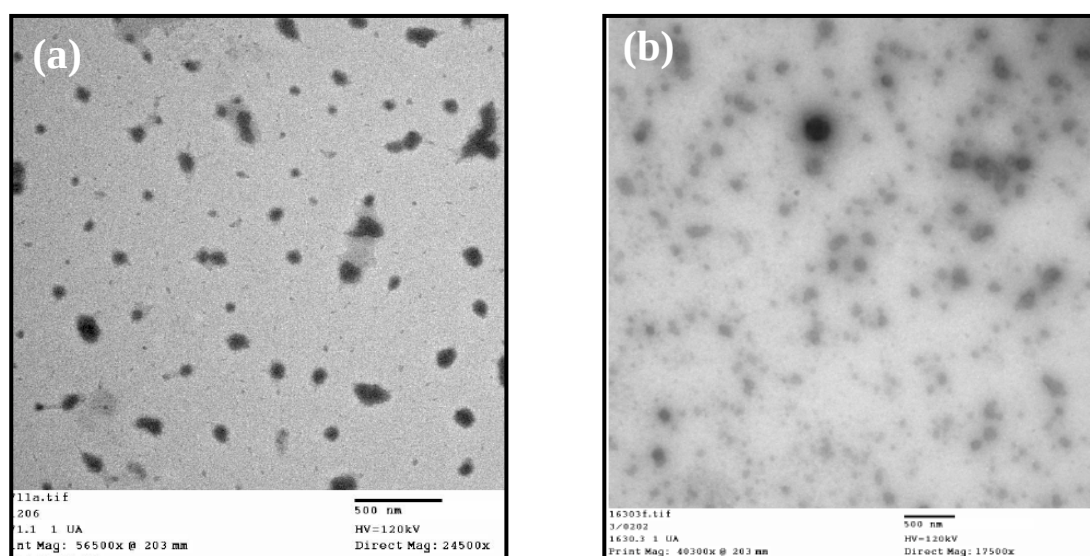


Figure 3.7. TEM micrographs of TT loaded CS nanoparticles(a), TMC nanoparticles (b)

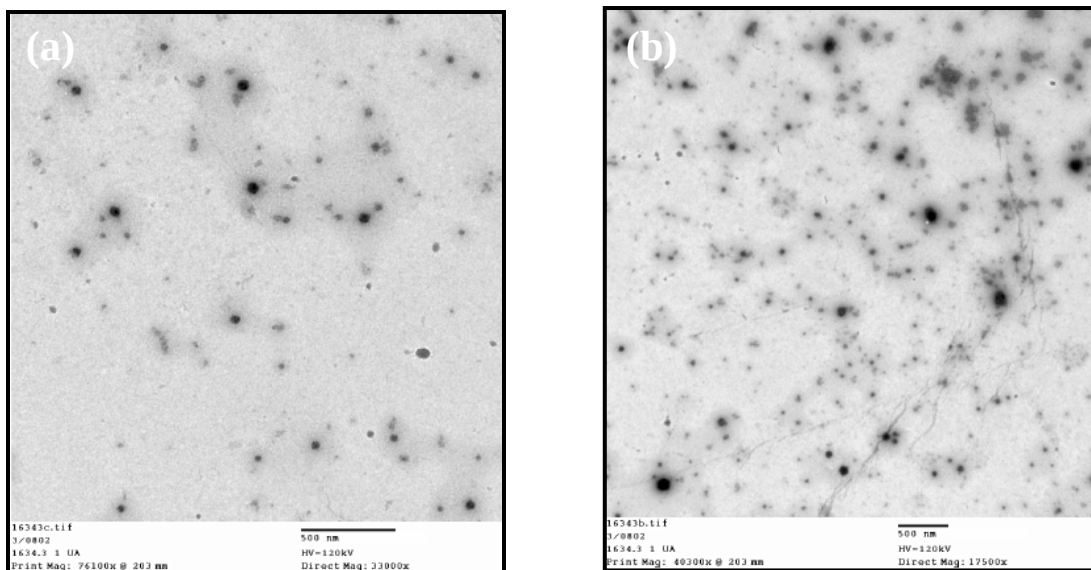


Figure 3.8. TEM micrographs of MCC nanoparticles; Blank(a), and TT loaded (b) (MCC concentration: 2 mg/ml; MCC:CaCl₂ ratio (m/m):7.5)

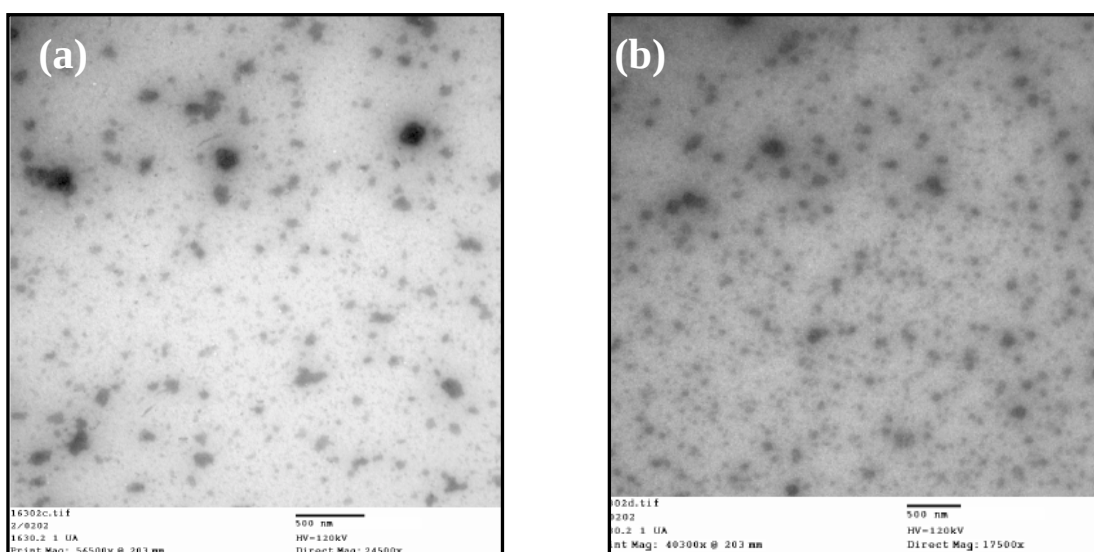


Figure 3.9. TEM micrographs of TMC/MCC complex nanoparticles; blank (a), and TT loaded (b)

For TMC/MCC complex nanoparticles, positively charged nanoparticles with a narrow particle size distribution was obtained (Figure 3.9, Table 3.4). The average particle size of the nanoparticles was found to

be 163 nm for blank, and 283 nm for TT loaded and a positive surface charge (Tables 3.3 and 3.4). Large amounts of TT (95%) could be incorporated into the TMC/MCC nanoparticles (Table 3.4).

3.5. Determination of the protein integrity

SDS-PAGE studies confirmed that the integrity of the antigen remains unaltered during encapsulation procedure (Figures 3.10 and 3.11). With blank nanoparticles, no protein staining in the lane was observed. The TT antigen was seen as a broad band at around 150 kDa. No additional bands that would indicate the presence of aggregates or fragments were visible.

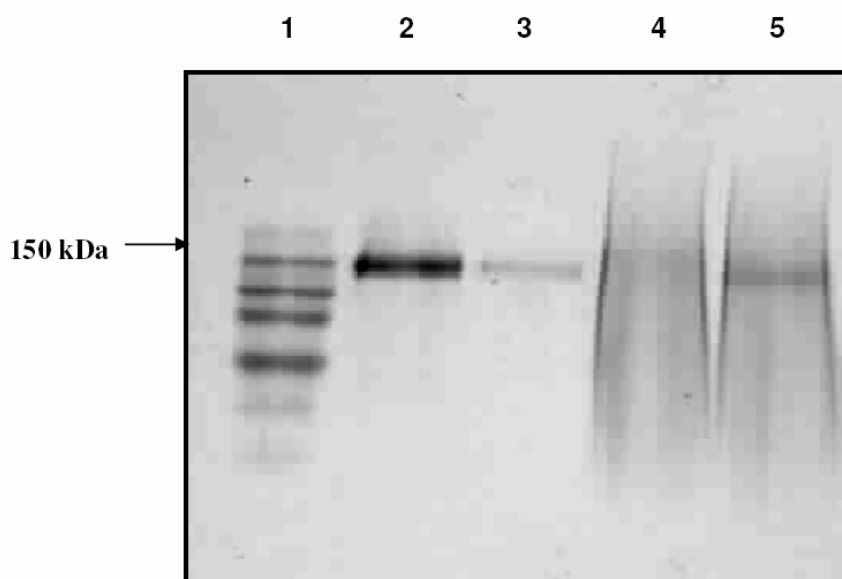


Figure 3.10. SDS-PAGE gel displaying the TT determined in TMC/MCC complex nanoparticles after loading process.

Loading order: lane1: molecular weight marker; lanes 2 and 3: TT standard; lane 4: blank TMC/MCC nanoparticles; lane 5: TT loaded TMC/MCC nanoparticles

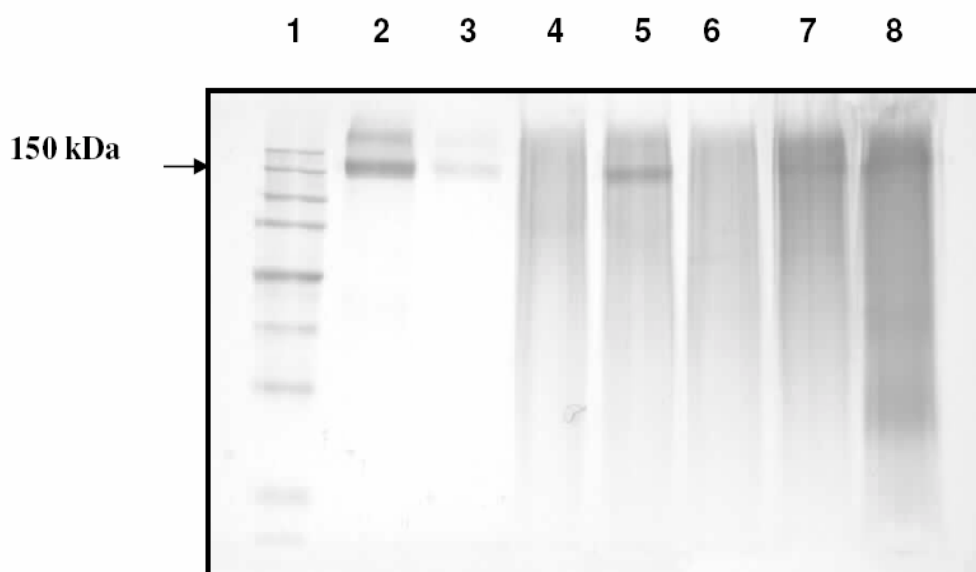


Figure 3.11. SDS-PAGE gel displaying the TT determined in TMC and MCC nanoparticles after loading process.

Loading order: lane1: molecular weight marker; lanes 2 and 3: TT standard; lane 4: blank TMC nanoparticles; lane 5: TT loaded TMC nanoparticles; lane 6: blank MCC nanoparticles; lane 7: TT loaded MCC nanoparticles 1 and 8: TT loaded MCC nanoparticles 2. (Mol. Wt of TT : 150 kDa)

3.6. Stability of Nanoparticles

Stability experiments demonstrated that TT-loaded CS, TMC, TMC/MCC nanoparticles did not show any morphological alterations when stored at 4°C for 6 months (Figure 3.12). In addition, no significant differences in size or zeta potential were observed over this period for TMC, CS and TMC/MCC complex nanoparticles (Table 3.5).

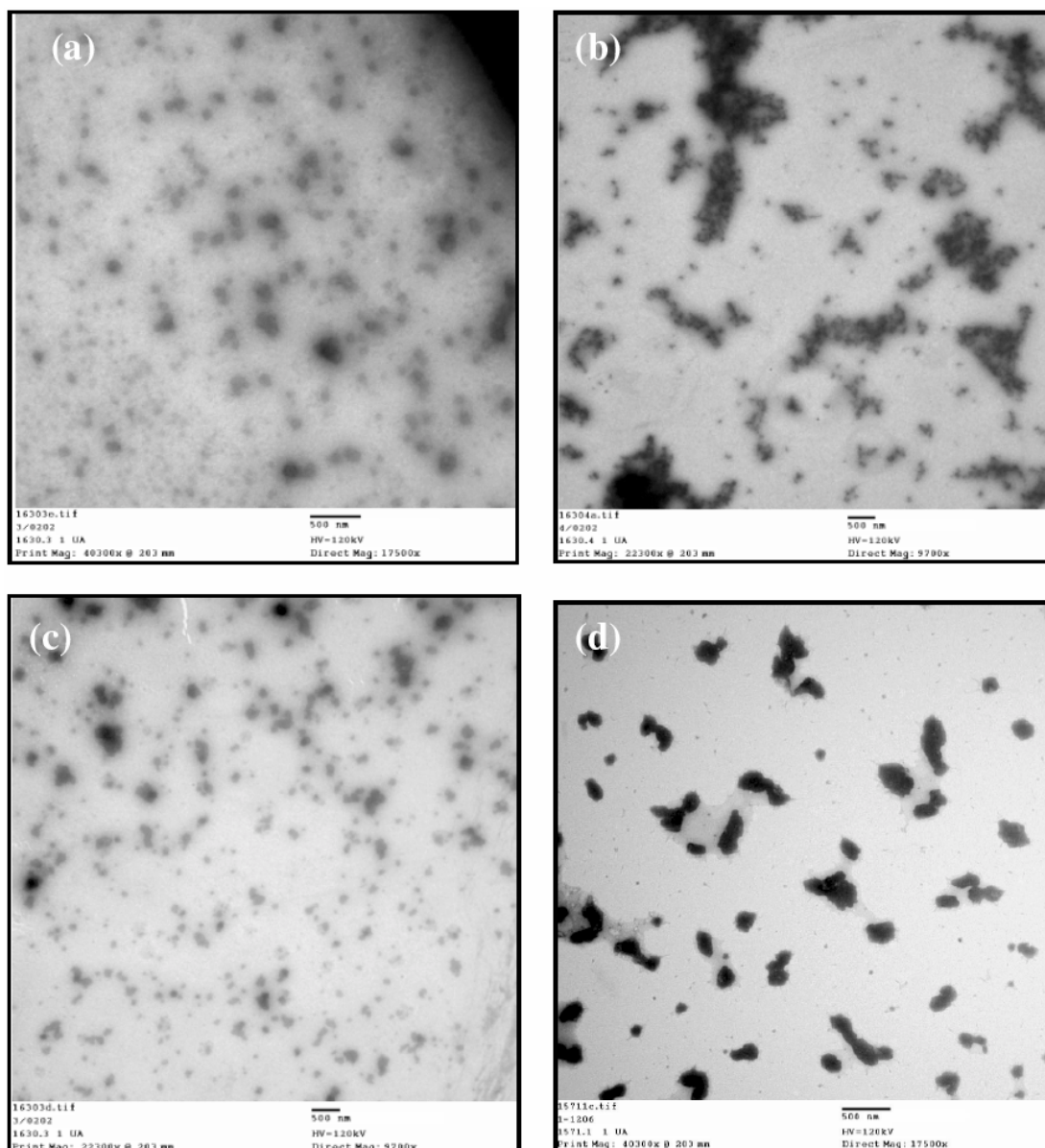


Figure 3.12. TEM micrographs of TT loaded (a) TMC, (b) MCC, (c) TMC/MCC, (d) CS nanoparticles after 6 months storage at 4°C

The integrity of TT encapsulated in the nanoparticles for 6 months at 4°C was confirmed by SDS-PAGE (Figure 3.13).

Aggregates were observed in the TEM micrographs of MCC nanoparticles after 6 months (Figure 3.12.b), although SDS-PAGE results did not show any degradation of TT.

Table 3.5. Particle size and zeta potential results of the TT loaded nanoparticle formulations at initial and after 6 months storage at 4°C.

Formulations	ζ Potential (mV)		z Average (nm)		Polydispersity Index	
	Initial	6months	Initial	6months	Initial	6months
CS	53.8 ± 0.8	53.2 ± 1.2	397.8 ± 7.8	453.2 ± 10.2	0.302 ± 0.028	0.282 ± 0.014
TMC	18.52 ± 1.2	20.1 ± 0.7	306.0 ± 2.4	260.7 ± 5.2	0.182 ± 0.039	0.205 ± 0.010
TMC-MCC	43.0 ± 7.5	38.0 ± 1.0	283.5 ± 2.5	366.1 ± 6.1	0.193 ± 0.049	0.243 ± 0.008
MCC	-28.8 ± 2.2	-19.7 ± 0.4	90.9 ± 0.8	302.0 ±146.9	0.280 ± 0.013	0.732 ± 0.232

ζ : zeta; z Average: particle size

The stability results observed after 6 months storage at 25°C showed that nanoparticles are not stable at room temperature. 1 month's stability results of nanoparticles at 4°C were shown in Table 3.6.

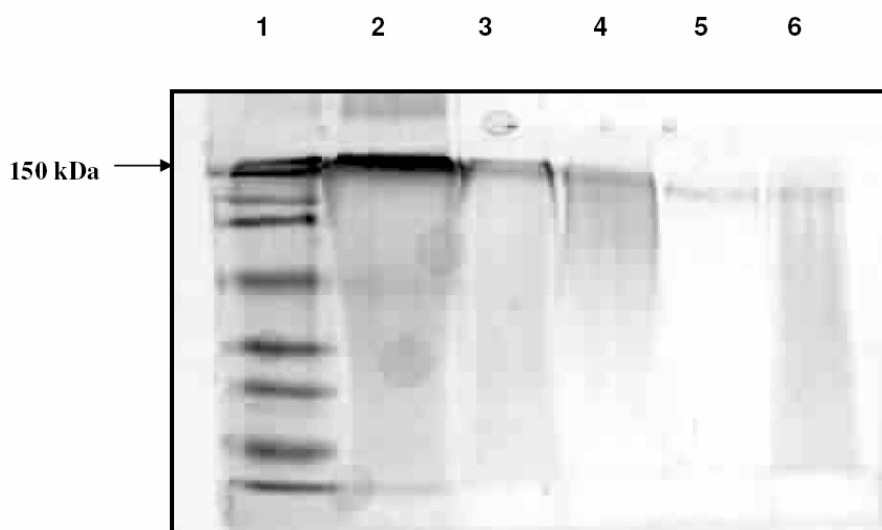


Figure 3.13. SDS-PAGE gel displaying the TT determined in nanoparticles after 6 months storage at 4°C.

Loading order: lane1: molecular weight marker; lane 2: TT standard; lane 3: TMC nanoparticles; lane 4: MCC-2 nanoparticles; lane 5: CS nanoparticles; lane 6: TMC/MCC complex nanoparticles

Table 3.6. Particle size and zeta potential results of the TT loaded nanoparticle formulations after preparation and 1 month's storage at 25°C

Formulations	ζ Potential (mV)		z Average (nm)		Polydispersity Index	
	Initial	1 months	Initial	1months	Initial	1months
CS	53.8 ± 0.8	52.8 ± 4.2	397.8 ± 7.8	505.6 ± 10.3	0.302 ± 0.028	0.574 ± 0.017
TMC	18.52 ± 1.2	20.4 ± 1.9	306.0 ± 2.4	314.6 ± 2.4	0.182 ± 0.039	0.181 ± 0.018
TMC-MCC	43.0 ± 7.5	40.0 ± 5.8	283.5 ± 2.5	284.4 ± 4.3	0.193 ± 0.049	0.243 ± 0.008
MCC	-28.8 ± 2.2	Aggregates	90.9 ± 0.8	Aggregates	0.280 ± 0.013	Aggregates

ζ: zeta; z Average: particle size

3.7. Cellular uptake studies

All nanoparticles were taken up by the murine macrophage J774A.1 cells (ATCC) (Figure 3.14). The uptake of the positively charged chitosan, TMC and TMC/MCC complex nanoparticles were found to be higher than the negatively charged MCC nanoparticles.

The uptake was also found to be time-related, that means the amount of the particles taken up increased with the incubation time (Figures 3.15, 3.16, 3.17).

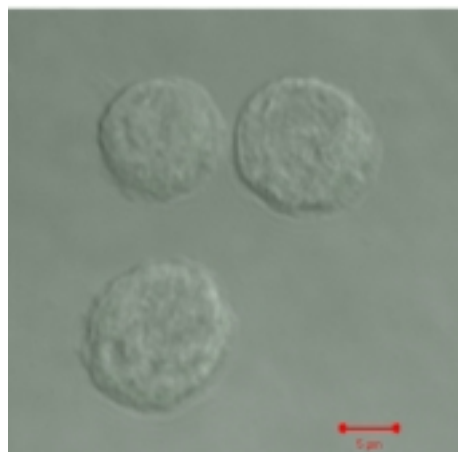


Figure 3.14. Mouse Balb/c monocyte macrophage J774A.1 cells (ECACC, 91051511)

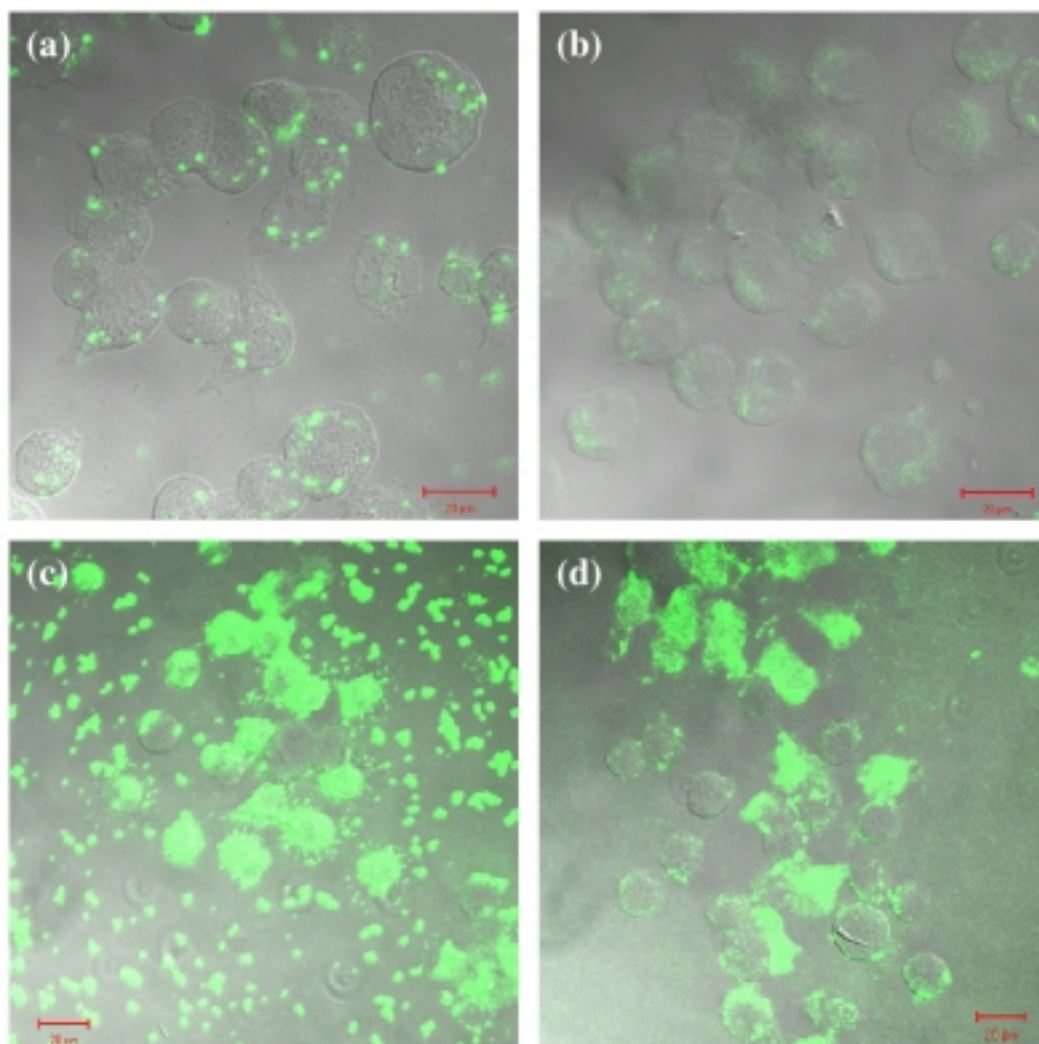


Figure 3.15. Confocal laser scanning images of nanoparticles after 30 min incubation. (a) TMC (b) MCC (c) TMC/MCC (d) CS

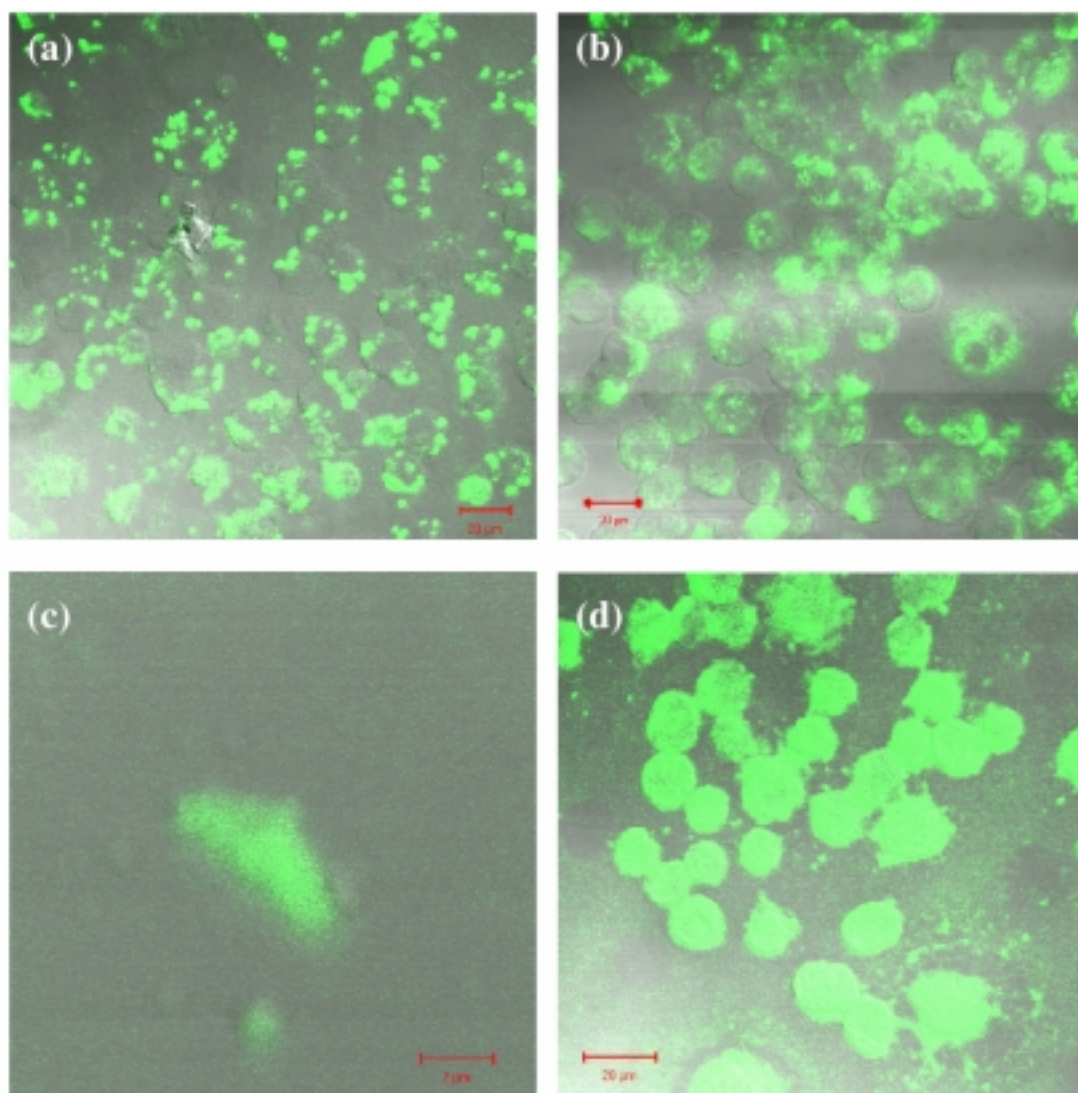


Figure 3.16. Confocal laser scanning images of nanoparticles after 1 hour incubation. (a) TMC (b) MCC (c) TMC/MCC (d) CS

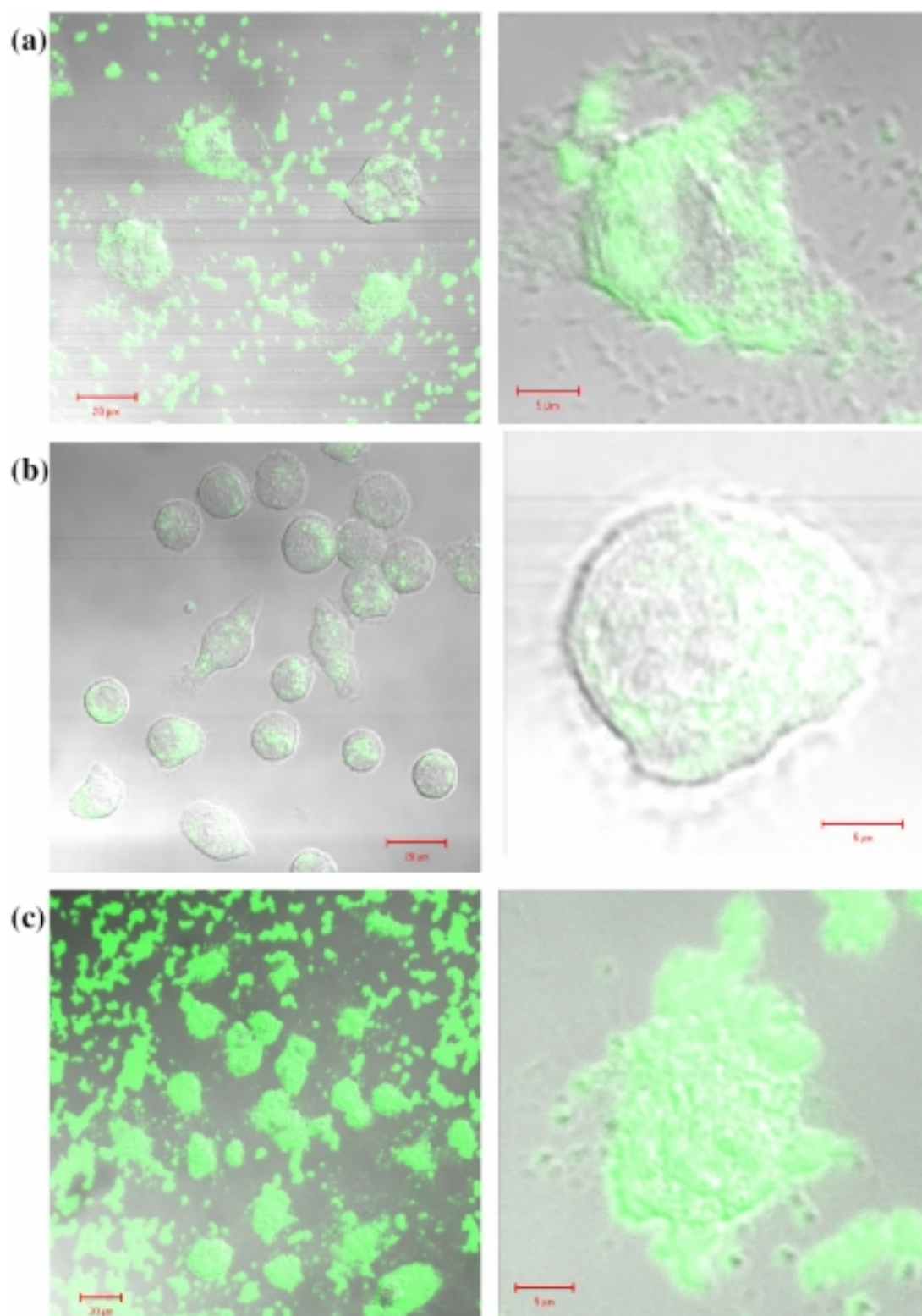


Figure 3.17. Confocal laser scanning images of nanoparticles after 2 hours incubation. (a) TMC; (b)MCC; (c) TMC/MCC

3.8. In vivo Studies

3.8.1. Antibody responses of the formulations prepared with TMC, MCC, CS or TMC/MCC complex

All formulations prepared with CS and its derivatives induced higher immune responses compared to the control group. Test results of pairwise comparison between groups in in vivo studies are summarized in Table 3.7.

TMC nanoparticles and solution induced significantly higher ($p < 0.01$) immune responses compared to intranasally administered TT in PBS solution (control) (Figure 3.18). Nasally administered TMC solution generated significantly higher ($p < 0.01$) immune responses than subcutaneously administered TMC solution and nanoparticle formulation.

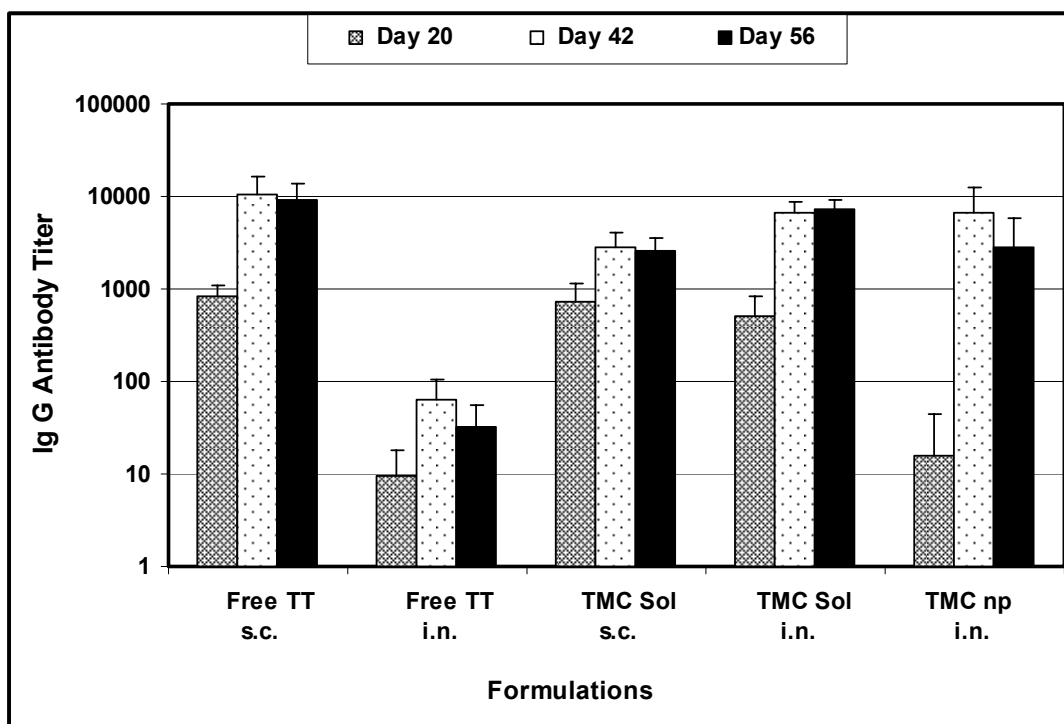


Figure 3.18. TT specific serum IgG antibody titers after administration of TMC formulations (n=5, Mean $\pm$ SD)

Table 3.7. Test results of pairwise comparison between groups in in vivo studies

Formulations	Application Route					
Solutions versus Solutions	i.n.			s.c.		
	Day 20	Day 42	Day 56	Day 20	Day 42	Day 56
Control –TMC	+	+	+		+	+
Control – MCC 0.2 %		+	+		+	+
Control - MCC 1.0 %						
Control – CS	+	+	+		+	+
Control - TMC/MCC					+	+
TMC – MCC 0.2 %	+	+	+			
TMC – CS			+			
TMC – TMC/MCC				+	+	+
MCC 0.2 % - CS	+	+	+			
MCC 0.2 % – TMC/MCC				+	+	+
MCC0.2 %-MCC1.0%			+			
CS – TMC/MCC				+	+	+
Nanoparticles versus Solutions	i.n. – Solutions			s.c. - Solutions		
	Day 20	Day 42	Day 56	Day 20	Day 42	Day 56
TMC np – TMC	+		+	+	+	
MCC np – MCC				+	+	+
CS np – CS						
TMC/MCC np- TMC/MCC				+	+	+
Nanoparticles versus Nanoparticles/Control	i.n.					
	Day 20	Day 42	Day 56			
TMC – MCC 2		+	+			
TMC – CS		+	+			
TMC – TMC/MCC	+	+	+			
MCC 1- MCC 2			+			
MCC 2- CS	+	+	+			
MCC 2– TMC/MCC	+	+	+			
CS – TMC/MCC	+	+	+			
Control –TMC		+	+			
Control – MCC 1						
Control - MCC 2						
Control – CS	+	+	+			
Control - TMC/MCC	+	+	+			

(+) indicates significant difference (p<0.01), i.n.:intranasal, s.c.:subcutaneous administration

MCC solutions and nanoparticles administered nasally were found to induce significantly ($p<0.01$) lower systemic immune responses than the groups subcutaneously vaccinated with MCC solution (Figure 3.19). Results showed that increasing the MCC concentration did not effect the immune response, as solutions with lower MCC concentration (0.2%) induced significantly ($p<0.01$) higher immune responses compare to MCC solution of 1.0% concentration. The effect of the particle size was investigated, and significantly ($p<0.01$) higher immune responses were obtained with large (90nm) particle size nanoparticles compared to that of small (44 nm) particle size nanoparticles on Day 56.

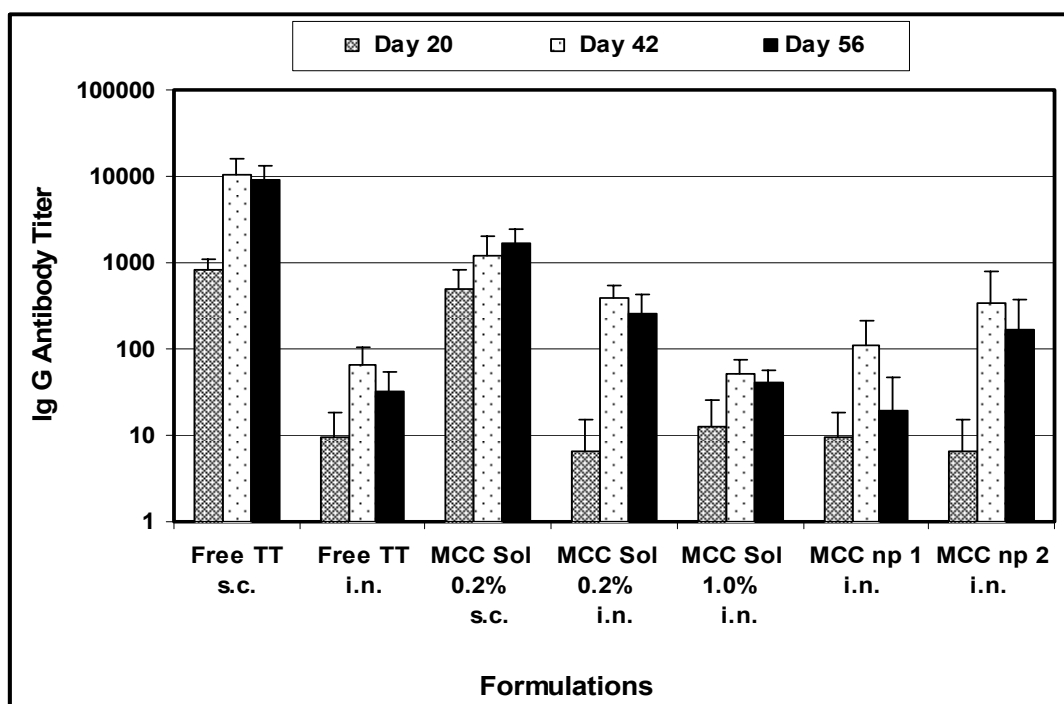


Figure 3.19. TT specific serum IgG antibody titers after administration of MCC formulations (n=5, Mean $\pm$ SD)

For CS formulations, no significant differences ($p<0.01$) in immune responses were observed between intranasal and subcutaneous

immunizations (Figure 3.20). All CS formulations induced significantly higher immune responses compared to nasally administered TT (control group).

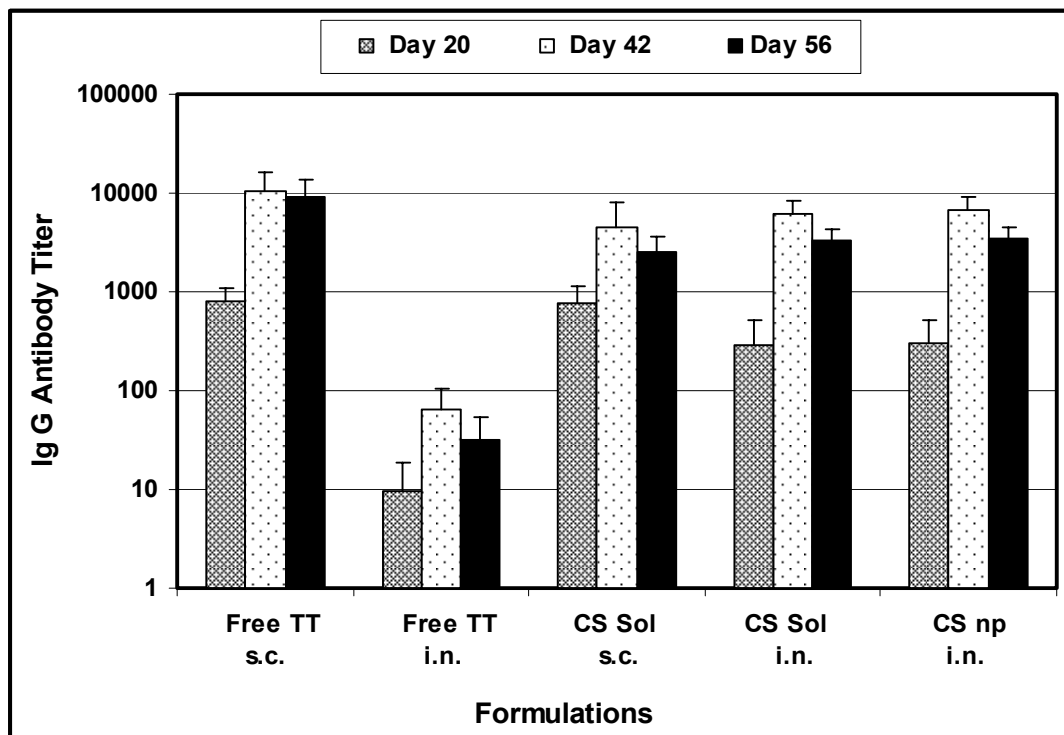


Figure 3.20. TT specific serum IgG antibody titers after administration of CS formulations (n=5, Mean $\pm$ SD)

For TMC/MCC complex formulations, following boosting, the immune responses were found to be increased (Figure 3.21). Increased IgG anti-TT titers in mice immunized intranasally were obtained beginning with minimal increases at Day 20 and progressing to a stronger response by days 42 and 56. Significantly higher ($p < 0.01$) immune responses were obtained with TMC/MCC formulations compared to that of subcutaneously or intranasally administered TT in PBS solution (control groups). Significant difference ($p < 0.01$) in immune response was observed between intranasally administered TMC/MCC nanoparticles and subcutaneously administered TMC/MCC solution.

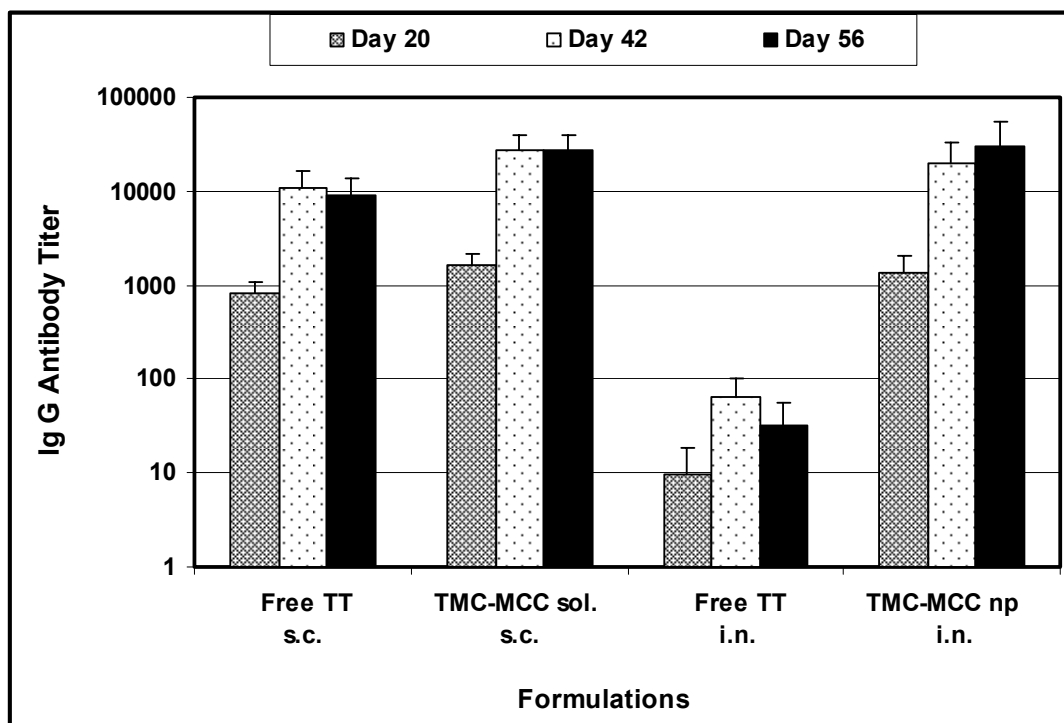


Figure 3.21. TT specific serum IgG antibody titers after administration of TMC/MCC complex formulations (n=5. Mean $\pm$ SD)

3.8.2. Comparison of the immune responses of the formulations

IgG anti-TT titers in mice immunized intranasally or subcutaneously with TT loaded formulations was increased beginning at Day 20 and progressing to a strong response after boosting (Day 42) (Figure 3.22, 3.23). With intranasal administration of TT, significantly higher ($p < 0.01$) immune responses were obtained with TMC, MCC or CS solutions compared to that of control (TT in PBS solution) (Figure 3.22). When administered subcutaneously, no significant difference was observed between the immune responses of the solutions prepared using TMC, MCC or CS (Figure 3.22).

Following nasal administration, TT loaded TMC and CS nanoparticles resulted in significantly higher ($p < 0.01$) levels of serum IgG titer than those obtained with MCC formulations (Figure 3.23). When TMC and CS nanoparticle formulations are compared, higher immune responses were obtained with CS nanoparticles ($p < 0.01$). Highest immune response was obtained with TMC/MCC nanoparticles compared to all other formulations.

The formulations prepared through TMC/MCC complexation induced the highest immune responses ($p < 0.01$) when administered either subcutaneously or intranasally (Figures 3.22, 3.23).

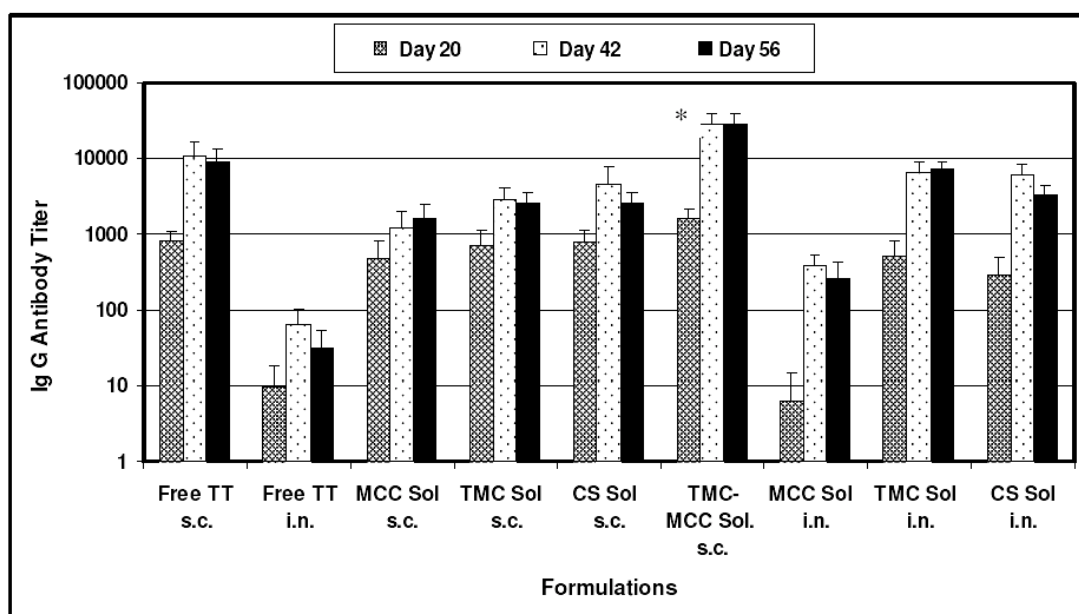


Figure 3.22. TT specific serum IgG antibody titers after administration of i.n. or s.c. delivery of TT in solutions ($n=5$, Mean $\pm$ SD)

i.n. application: 5 Lf TT/ mice dose was administered in 25 μ l; s.c. applications : 5 Lf TT / mice dose was administered in 200 μ l *Significantly higher than all formulations at all timepoints (Days 20,42 and 56)

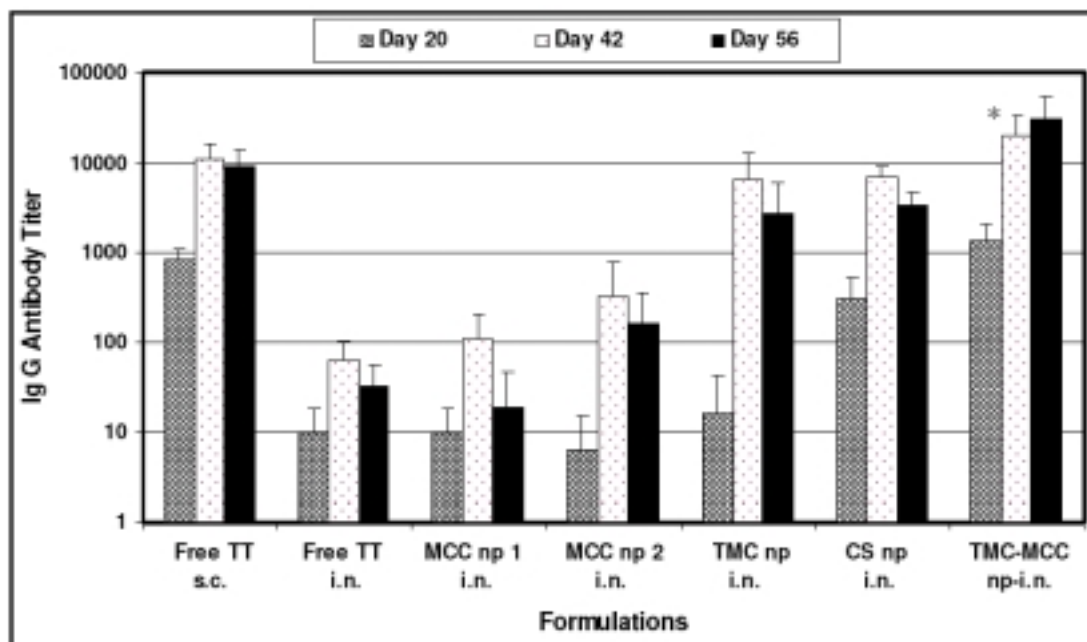


Figure 3.23. TT specific serum IgG antibody titers of i.n. delivery of TT loaded nanoparticles (n=5, Mean $\pm$ SD)

i.n. application: 5 Lf TT/ mice dose was administered in 25 μ l; s.c. applications : 5 Lf TT / mice dose was administered in 200 μ l *Significantly higher than all formulations at all timepoints (Days 20,42 and 56)

IgG subclasses were also determined with ELISA. The IgG subtype (IgG1/IgG2a) profile of TT-specific antibodies were determined and for nanoparticle and solution formulations, lower immune responses were obtained compared to IgG responses. (Figure 3.24 and 3.25). On day 56 serum IgG1 anti TT titers indicated that both TMC, CS and TMC/MCC complex solutions following i.n. or s.c. administration induced significantly ($p < 0.01$) higher immune responses when compared to control group (Fig 3.24.a). Immune responses of the MCC solutions were significantly lower than that of solutions ($p < 0.01$). Subcutaneous administration of MCC solution induced significantly ($p < 0.01$) higher immune response than intranasal group. For nanoparticle formulations (Fig 3.24.b), no significant

differences were found in IgG1 anti-TT responses for MCC whereas with TMC, CS and TMC/MCC complex, the immune responses were significantly enhanced ($p < 0.01$).

No significant Ig G2a titres were observed between control group, MCC and TMC nanoparticle formulations following nasal administration (Figure 3.25.b), however for solution formulations, IgG2a immune responses (Figure 3.25.a). Significantly higher IgG2a anti-TT responses were obtained with TMC/MCC complex solution and nanoparticle formulations. Generally, IgG1 responses remained much stronger than the IgG2a responses.

Local Ig A antibody titres were analysed in vaginal and day 56 nasal secretions and there were found to be not significantly high.

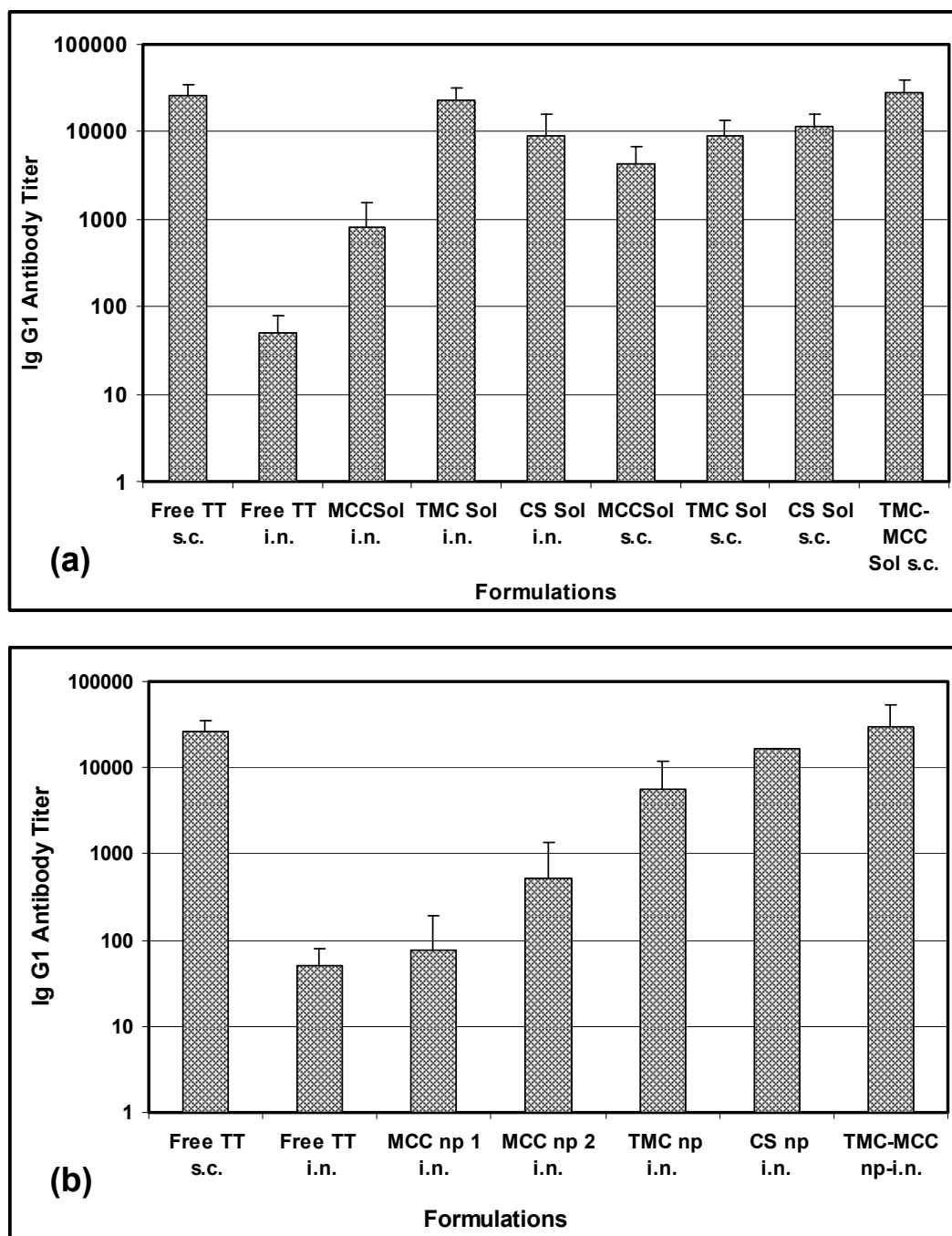


Figure 3.24. TT specific serum IgG1 antibody titers after administration of **(a)** i.n. or s.c. delivery of TT in solutions, **(b)** i.n. delivery of TT loaded nanoparticles on day 56 (n=5.Mean±SD)

i.n. application : 5 Lf TT/ mice dose was administered in 25 µl; s.c. applications : 5 Lf TT / mice dose was administered in 200 µl (n=5)

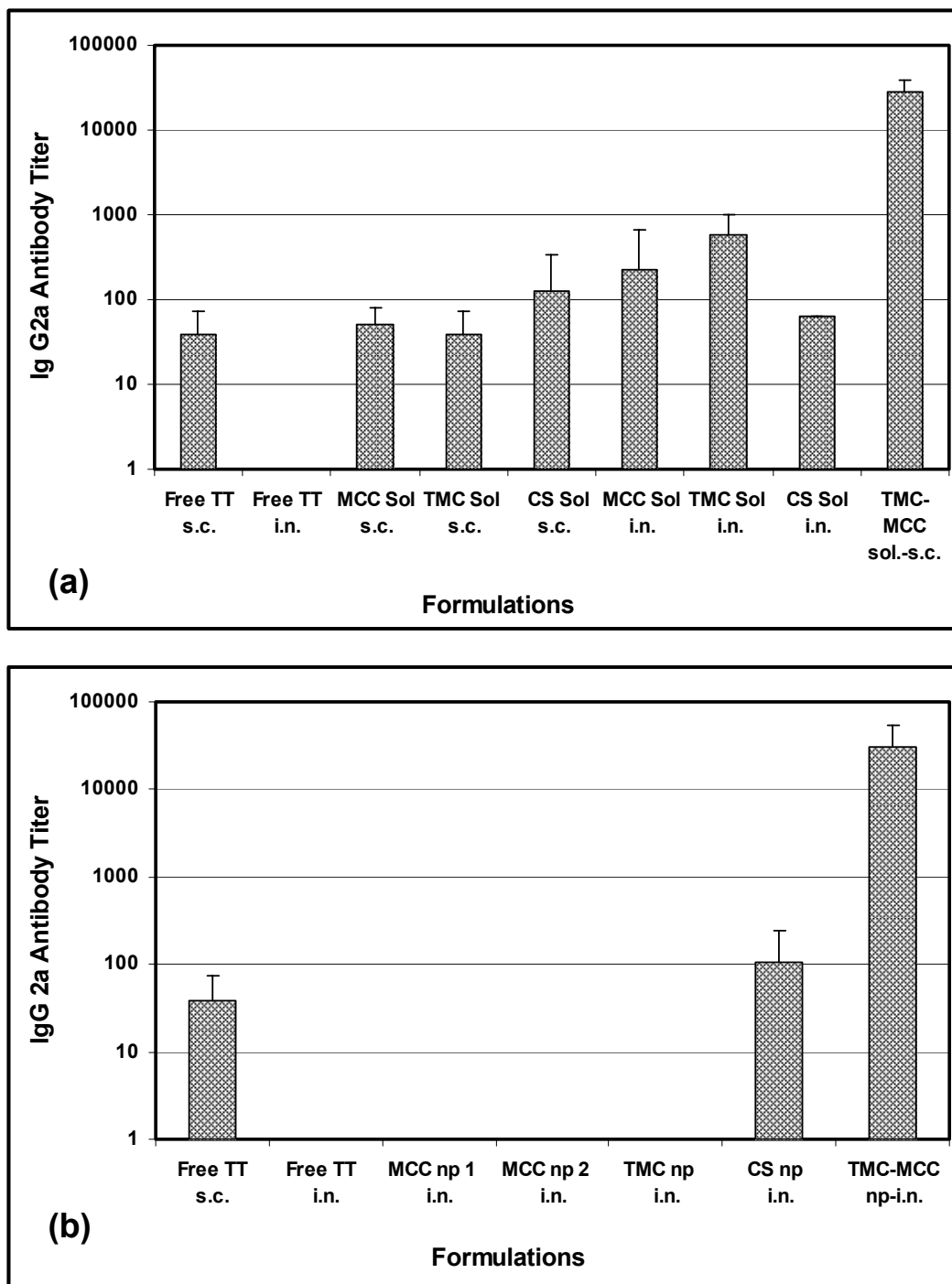


Figure 3.25. TT specific serum IgG2a antibody titers after administration of (a) i.n. or s.c. delivery of TT in solutions, (b) i.n. delivery of TT loaded nanoparticles on day 56 (n=5. Mean $\pm$ SD)

i.n. application : 5 Lf TT/ mice dose was administered in 25 μ l; s.c. applications : 5 Lf TT / mice dose was administered in 200 μ l (n=5)

4. DISCUSSION

4.1. Synthesis and characterization of the chitosan derivatives

TMC is a derivative of chitosan with enhanced cationic properties by the induction of the N-trimethyl groups. TMC is synthesized by reductive methylation of chitosan in an alkaline environment at elevated temperature whereas MCC is synthesized by the carboxymethylation of chitosan.

The charge density of chitosan derivatives was defined by the degree of quaternization for TMC, and by carboxymethylation of MCC. Increasing the number of positive or negative charges on the polymer chain causes the polymeric molecule to expand in solution due to repelling force between the functional groups, this resulting in higher solubility (123). However, especially for the positively charged chitosan derivatives (TMC), increasing toxicity with increasing degree of trimethylation was shown (117). On the other hand, it was observed that, TMC with higher derivatization degree (60%) showed higher transport enhancement ratios than TMC with lower derivatization degree (TMC40) in all concentrations tested (0.05-1.0%w/v) (134). Based on these previous results, TMC with trimethylation degree of 57% and MCC with carboxymethylation of 70% were synthesized.

4.2. In vitro cellular toxicity studies

Previous studies reported that especially cationic polymers displayed concentration-dependent toxicity towards cells (135,136). In regard to this, in vitro cellular toxicity of the chitosan and its derivatives has been investigated.

Our experimental results showed that the cellular viability with chitosan was higher than that with TMC. Kean et. al also showed that cell viability was found to decrease with increasing degree of trimethylation of chitosan in COS-7 and MCF-7 cells (117).

The cellular viability was decreased in the order of MCC, TMC/MCC complex, CS and TMC. Highest cellular viability with MCC can be explained with its negatively charged property due to the induction of negatively charged carboxymethyl groups and reduced amino groups. In general cationic materials would interact stronger with the anionic cellular surface and therefore results in the lysis of the cells. Many cationic polymers have been found to be toxic and that this toxicity was attributed the interactions with the plasma membrane (136). Other possible mechanisms can be the interaction with negatively charged cell components and proteins (135).

Our results also showed that TMC had a IC_{50} of about 0.5 mg/ml whereas the viability of TMC/MCC complex solution was still high over at 5 mg/ml. These results indicated that the application of MCC with TMC as a TMC/MCC complex decreased the cytotoxicity. This can be explained by decreased interaction with cell membranes. Thanou et al. showed that both with TMC and MCC, damaging effect on cell membranes, and the viability of intestinal epithelial cells was not altered (123).

4.3. Preparation of Nanoparticles

One of the major approaches to protect antigens from degradation during the mucosal applications is to incorporate them into particulate systems. Particulate systems have been investigated with narrow size distribution since many years as vaccine delivery systems (64,71,86,137).

Formulation of a suitable antigen carrier, which must be biodegradable and biocompatible, is a difficult task. For particulate systems, particle size and loading have to be adjusted carefully, and more important, protein stability during preparation must be ensured. There are various preparation methods available for nanoparticles and also employed to obtain chitosan nano/ microparticles (92,100). Chitosan particles can be obtained by ionic gelation, where micro or nanoparticles are formed by means of electrostatic interactions between the positively charged chitosan chains and polyanions employed as cross-linkers. Chitosan chains can also be chemically cross-linked leading to quite stable matrixes, where the strength of the covalent bonds stands out. Glutaraldehyde is broadly used as the cross-linking molecule. Due to the reported toxicity of glutaraldehyde and other organic molecules used in the preparation of gels covalently stabilized, ionic gelation method has been preferred in our study for preparation of the particulate systems.

The effect of the crosslinker on cytotoxicity is an important issue. Carreno-Gomez et al. investigated the effect of the crosslinker used to prepare CS delivery systems on the cytotoxicity and it was found that glutaraldehyde-crosslinked CS microspheres were cytotoxic at all concentrations used (138). By the same group, cytotoxicity of soluble chitosans was assessed using a murine melanoma cell line, B16 F10, in combination with the MTT assay and their ability to cause lysis of rat erythrocytes in vitro was also studied. In our study, we used TPP and calcium chloride (CaCl_2) as crosslinkers, both reported to exert no cytotoxicity.

Preparation conditions can play an important role for the structural integrity of the antigen, which can be denatured even by limited exposure to heat, organic solvents or high levels of energy (e.g. sonication, homogenization). which is applied to achieve sizes on the nanoscale range.

Stability problems have been reported for tetanus toxoid in acidic conditions during microparticle preparation (139).

Different polymers have been used to prepare vaccine delivery systems. A disadvantage of poly(lactide/glycolic acid) nano/microspheres which are frequently used for this purpose is that the vaccine is highly dispersed in the polymer and, consequently, directly exposed to deleterious factors such as the acidic microenvironment created inside the microspheres during polymer degradation and the reactivity of the protein with the polymer and/or its degradation products (140). Moreover the organic solvents which are needed to prepare particulate systems with PLGA polymer is considered to be harmful to proteins, leading to aggregation (141).

In our study, chitosan and its water-soluble derivatives which are all biocompatible, biodegradable polymers have been used for preparation of a delivery system. The formation of nanoparticles with chitosan and its derivatives by ionic gelation represents an easy method to prepare such systems for antigens in mild conditions avoiding heat and use of any organic solvents (71,142). Moreover, with water-soluble chitosan derivatives, there is no need for acidic solutions to dissolve the polymer. The preparation method involves first mixing the antigen with polymer solution. Crosslinking agent (sodium tripolyphosphate or calcium chloride) is then added to the suspension under constant stirring. Hence, this preparation method is very simple and straightforward. Due to the complexation between oppositely charged polymer and crosslinker, polymer undergoes ionic gelation and precipitates to form nanoparticles. There are two salts that have been generally used in the preparation of chitosan particles by the ionic gelation mechanism: sodium tripolyphosphate (TPP) (71,142) and sodium sulfate (Na_2SO_4) (143,144). We preferred TPP in the preparation of CS and TMC nanoparticles which is a strong basic salt and can be used low

concentrations and gives a narrow size distribution. TPP is a salt composed of strong acidic TPP ions and strong basic sodium ions. The anionic characteristic of the TPP is stable without going under change with the pH of the solution. CS and TMC particles, therefore, formed on the basis of the ionic gelation with TPP are expected to be much more stable than with the other strong basic salts (e.g. sodium citrate). When TPP is added into the CS or TMC solution, the stretched linear polymer molecules is expected to be coiled and aggregate into particles due to the interaction between NH_2H^+ groups on the chitosan chains and the multivalent anions (tripolyphosphate ions), which acts as cross-linking agents.

Our results showed that when a certain amount of TPP is added into the chitosan solution, the solution changes from a clear solution to an opalescent suspension. This indicates that the system is gelated and the particles are formed. The more TPP added, the more obvious is the white colour of the suspension. When the amount of the TPP reaches to a certain level, the suspension becomes aggregated and the aggregates precipitate out. The change of the appearance of the chitosan solution indicates that there has been a change of the physical states of the chitosan with the increase of TPP, from solution to nanoparticles, then microparticles and eventually aggregates. Homogenous nanoparticles of chitosan can be prepared by controlling the amount of TPP. The mechanism of the chitosan nanoparticle formation with TPP is based on the interaction between the cationic groups on the chitosan chains and the multivalent anions.

In this study, it was the first time to develop a nanoparticulate delivery system with MCC. CaCl_2 was used as a crosslinker for the preparation of MCC nanoparticles. Di- or polyvalent cations of calcium can induce the gelation by cross-linking of the guluronic acid units of alginate (66). Because

of the polyampholytic structure of MCC, it was possible to form a nanoparticulate system with CaCl_2 .

In our study, another novel approach was brought by using differently charged chitosan derivatives to form a nanoparticulate system by ionic complexation without using any crosslinker and organic solvent. The formation of TMC/MCC nanoparticles occurs spontaneously upon incorporation of the polyampholytic MCC into the polycationic TMC solution, so in this method MCC had the function as a crosslinker. In addition to the advantages such as the absence of the organic solvent and heat during preparation, for the preparation of TMC/MCC complex nanoparticles, there was no need for an additional crosslinker except than chitosan derivative MCC.

4.4. Characterization of Nanoparticles

4.4.1. Protein Loading Capacity of Nanoparticles

Various methods of loading have been developed to improve the efficiency of loading, which largely depends upon the method of preparation as well as physicochemical properties of the drug/antigen. In the previous studies by our research group, it was prepared CS microparticles and loaded with BSA by incubation of CS microparticles with BSA in phosphate buffer (pH 7.4) under mild shaking at 37°C for 180 min (145,146). Maximum drug loading can be achieved by incorporating the drug during the formation of particles, but it may get affected by the process parameters such as method of preparation, presence of additives, etc (100).

In our preparation method, TT was added to the polymer solution before adding of the crosslinker. Different approaches have been suggested

for incorporation of the antigen and crosslinker during nanoparticle preparation for ionic gelation method. In a study by Calvo et al. (147), BSA was incorporated into the medium in different ways: (i) dissolving in the CS solution (pH 5.00), (ii) dissolving in the TPP solution (pH 8.00), or (iii) dissolving in the nanoparticles suspension (after the incorporation of the TPP to CS solution). The high association efficiencies were obtained when BSA was dissolved in the TPP or CS solutions, whereas a minimum amount of BSA was entrapped following its incorporation into the nanoparticle suspension.

In our study, loading efficiency results for TT chosen as a model of a large antigen were found to be significantly high for all nanoparticle formulations. Due to the positive charge of chitosan and TMC, and negative charge of TT, the antigen loading is expected to be provided by electrostatic interactions. Large amounts of TT (95%) could be incorporated into the TMC/MCC nanoparticles as well (Table 3.4.). Because that TMC/MCC nanoparticles are also positively charged due to the presence of TMC, TT can easily be incorporated into the nanoparticles. TT loading of MCC nanoparticles were also found high in spite of the negative charge. These promising loading efficiency results for MCC nanoparticles can be attributed to the polyampholytic structure of the MCC, having positively and negatively charged groups on the polymer chain. There are also other studies reported using negatively charged polymers for antigen delivery (66,78,95,141). Jung et al. (141) studied on loading of TT to negatively charged nanoparticles containing poly (sulfobutyl-polyvinyl alcohol)-g-(lactide-co-glycolide) and their results suggested an exothermic adsorption process due to coulombic interactions between the negatively charged particle surface and positively charged protein residues. It was concluded that this negatively charged nanoparticles were suitable for adsorptive loading with TT and might have

potential for mucosal vaccination. Other frequently used polymer as an antigen delivery system is alginate (66,85,95). Tafaghodi et al. prepared tetanus toxoid (TT)-containing alginate microspheres and evaluated their efficiency, as nasal delivery system and adjuvant by nasal immunization in rabbits (85). They obtained almost 50% of TT loading and strong systemic IgG and mucosal IgA responses with this system.

Tween 80 has been used to prevent aggregation during preparation. No negative affect was observed on loading efficiency. Similar results were obtained by Amidi et al. for ovalbumin-loaded TMC nanoparticles (121).

4.4.2. Particle Size Distribution

In our study, we obtained nanoparticles smaller than 500nm particle size, and using MCC polymer, it was possible to obtain 40-90nm sized particles. For mucosal immunization, uptake of particulate have been reported to be size- dependent and it has been shown that the critical particle diameter should be less than 10 μm (64).

Polymers and crosslinkers were tried with various concentrations to obtain nanoparticle structure. For MCC preparation, with 4mg/ml polymer concentration and MCC:CaCl₂ ratio (m/m) of 3-5 caused aggregate formation. MCC:CaCl₂ ratio (m/m) of 7.5-10, we obtained MCC nanoparticles with smallest size. For CS and TMC nanoparticle preparation, different polymer:TPP ratio were also examined. It has been reported that the concentration of CS and TPP and the CS/TPP ratio in aqueous solution have a large influence on the size of the nanoparticles obtained: increase in the amount of TPP leads to a higher degree of CS cross-linking and a decrease in nanoparticle dimensions (131). TT loaded TMC nanoparticles showed a

significant increase in size, which is most probably due to slight aggregation of particles whereas the polydispersity index did not increase.

4.4.3. Zeta Potential Measurements

The electrical double layer plays an important role in stabilization. The electrical double layer may be developed by means of dissociation on surface particles, ion adsorption or both. The electrostatic forces act within longer distances than the Van der Waals forces, and thus the sticking of the particles is hindered. The magnitude of repulsion can be measured as zeta potential. This potential acts between the surfaces of the sticking and the moving charge layers. If the zeta potential is high enough, the charges form potential barrier, so the colloidal system turns stable. Conversely, if the particle zeta potential is relatively small, the colloidal system will agglomerate. Zeta potential is mostly influenced by the electrolyte concentration and the pH value of the medium.

The surface charge of CS nano/microparticles is dependent on the surrounding medium and the surface composition. The pH, concentration, and type of electrolyte present affect the zeta potential, which always remain positive and was higher at a low pH. The positive charges resulted from free amino groups. A reaction involving these functional groups would alter the zeta potential (148). Our results showed that the zeta potential of TMC nanoparticles (18 mV) was lower than that of CS nanoparticles (53 mV). It may be because of the reduction of the free amino groups of the CS structure and this result is in accordance with those reported by Amidi et al. (86,121). On the other hand, zeta potential for MCC nanoparticles was found around -30 mV which indicates a negative charged surface. N-substitution of chitosan with carboxyl groups, yield polyampholytic (zwitterionic) polymeric

structure for MCC. The higher zeta potential result for TMC/MCC complex nanoparticles (43 mV) compared to TMC nanoparticles indicated better stability.

The stability of the systems depends, to a great extent, on the electrical state of the nanoparticles. In the absence of steric stabilization mechanisms, information on the electrical properties of the system helps to explain aggregation features (92). The physicochemical properties not only affect the stability of the system but also govern absorption and release processes the interaction of the particles with different biological compounds (proteins or membranes) of the tissue where they are introduced.

4.5. Protein integrity

A critical point for antigens during preparation and storage is the preservation of their molecular weight (150 kDa for TT) which is an indicator of the integrity of the molecule and consequently their antigenicity. Our results did not show any differences in molecular weight therefore, it has been concluded that no substantial degradation of TT occurs during the loading process. SDS-PAGE gel analysis results confirmed the structural integrity of the TT in the nanoparticles indicating that the stability of TT has been maintained during preparation and also after 6 months storage at 4°C (Figures 3.10, 3.11 and 3.13)

4.6. Stability of Nanoparticles

Zeta potential and particle size of the positively charged nanoparticles were found to be between 20-53 mV and 250-450 nm respectively and did

not display significant changes upon storage for 6 months at 4°C in aqueous dispersion.

Vaccines are often very temperature sensitive. Higher temperatures can cause denaturation and degradation of the antigens. Temperatures below freezing can often produce ice crystal formation that may also denature the antigen, or alternatively alter the structure of the vaccine–adjuvant complex. Thus, to be effective, vaccines must be stored within a relatively narrow temperature range (149).

4.7. Cellular uptake studies

Investigation of the transport of soluble drugs over the epithelial barrier of the gut is studied in vitro generally by using the Caco-2 cell model, but this was not suitable for investigation of vaccine delivery due to the absence of M-cells (150). In this study, we used Mouse Balb/c monocyte macrophage J774A.1 cells.

FITC-labelled CS, TMC, MCC and TMC/MCC complex nanoparticles were all taken up by the murine macrophage J774A.1 cells (Figures 3.15, 3.16, 3.17) which indicates that the nanoparticles would be recognized by the cells related to the immune system. Moreover, the uptake of nanoparticles was found to be dependent on the surface charge of the particle. The uptake of the positively charged CS, TMC and TMC/MCC complex nanoparticles were found to be higher than the negatively charged MCC nanoparticles which indicate a stronger interaction between positively charged particles and negatively charged cell membrane. Since cell membranes are negatively charged, TMC and chitosan nanoparticles are expected to be more easily associated and subsequently taken up by the membranes than negatively

charged MCC nanoparticles. Compared to TMC nanoparticles, MCC nanoparticles were less concentrated but still located in the macrophages. CS nanoparticles were located within the whole cell and showed larger uptake amount by the macrophages. There are several studies in the literature confirming cellular uptake of CS particles due to its mucoadhesive structure. Behrens et al. (151) showed that CS nanoparticles had a higher association and internalization with gastrointestinal tissue cells due to electrostatic interactions compare to the polystyrene nanoparticles.

The uptake of the nanoparticles is affected by several factors, including particle-size (152), particle-hydrophobicity, and also by adjuvants associated onto the particles such as lectin and B-12 (153). Particle size is reported to be very important for uptake by the mucosal associated lymphoid tissues (MALTs) (35,36). The effect of particle size on immunogenicity is likely to be a consequence of enhanced uptake into antigen presenting cells (APCs). Nanoparticles are expected to cross the mucosal epithelium better than microparticles, since not only M cells overlaying the MALT but also the epithelial cells are involved in the transport of nanoparticles (33,52). Mc Clean et al. evaluated the binding, uptake and absorption of poly-DL-lactide (PLA) micro- and nanoparticles in Caco-2 monolayers and in ileal tissue and gut associated lymphoid tissue (GALT) of rats and rabbits, and nanoparticles were found to be taken up better than microparticles (154). In another study, PLGA micro/nanoparticles with mean diameters of 0.1 μ m, 1 μ m and 10 μ m containing bovine serum albumin as a model protein and 6-coumarin. It was shown that the uptake in Caco-2 cells was significantly depending on the diameter of the particle. The 0.1 μ m diameter particles had 2.5 fold greater uptake on the weight basis than the 1 μ m and 6 fold greater than the 10 μ m diameter microparticles (152). But we can not conclude that particle size

appears the main factor influencing the cellular uptake, because we did not observe better results with smallest MCC nanoparticles.

4.8. In vivo Studies

In this study nanoparticles were administered intranasally for mucosal immunization. The nasal route for immunization has been shown to be more efficient than oral immunization at inducing secretory (saliva, genital, intestinal and bronchial secretions) in addition to systemic antibody responses with different formulations (46,71,155,156). And also it was demonstrated that the intranasal route can be as effective as the intramuscular route in inducing a specific immune response depending on the antigen (157).

4.8.1. Immune responses to the formulations prepared using TMC, MCC, CS or TMC/MCC complex

With TMC nanoparticles, it was possible to obtain higher immune response compared to that of subcutaneously administered TMC solution. The highest immune response was obtained with intranasal administration of TMC solution (Figure 3.18). These results confirms the advantage of the intranasal administration with TMC formulations.

MCC formulations did not show high IgG antibody titers (Figure 3.19). The highest immune responses with MCC formulations were obtained with subcutaneously administered MCC solution (0.2 %) however this result was still not higher than the control group (s.c. administered TT in PBS solution). Increasing the concentration of the MCC solution from 0.2% to 1.0% did not have any increasing effect on immune response. The lower immune

response obtained by MCC formulations can be attributed to the negative charge and smaller particle size of the formulations.

Intranasally administered CS nanoparticles or solutions showed higher immune responses compared to the control whereas there was no significant difference between CS nanoparticle and solution formulations (Figure 3.20). Read et al. was administered inactivated trivalent influenza vaccine with CS solution intranasally. Results suggested that CS is a potentially successful nasal delivery system for vaccination against influenza (69). Zaharoff et al. (158) investigated CS solution as an adjuvant for subcutaneous vaccination in mice with a model antigen (β -galactosidase) in comparison to parenteral vaccine adjuvants, and chitosan was found to be equipotent to incomplete Freund's adjuvant (IFA) and superior to aluminium hydroxide. Their results demonstrated that chitosan was a promising and safe platform for subcutaneous vaccine delivery.

Formulations prepared using both TMC and MCC in the same formulation resulted highest immune responses following subcutaneous or intranasal administrations (Figure 3.21).

It has been demonstrated that the residence time in the nasal cavity is considerably increased with particulate systems when compared to the solutions (159). Soane et al. described the clearance characteristics of three bioadhesive nasal delivery systems; starch microspheres, CS microspheres and CS solution. The results have supported the hypothesis that CS delivery systems can reduce the rate of clearance from the nasal cavity, thereby increasing the contact time of the delivery system with the nasal mucosa. The slow clearance of the chitosan and starch systems were explained by the bioadhesive properties of both positively charged CS systems and the negatively charged starch microspheres (160). In the same study, it was

demonstrated that with the increasing of the temperature, solution viscosity was decreased. This was explained as a result of an increase in the flexibility of the CS chains and not CS degradation. This is important since at nasal cavity CS systems will possess increased flexibility, giving enhanced penetration of the mucin network. In our study, for subcutaneously administered solutions, no significant difference was observed between the immune responses with TMC, MCC or CS whereas with nanoparticles, CS and TMC nanoparticles resulted in significantly higher ($p < 0.01$) levels of both serum IgG and mucosal IgA than those obtained with MCC. With intranasal administration of MCC nanoparticles, a significant effect of particle size on immune response was observed on Day 56. The immune response was significantly higher ($p < 0.01$) with larger particle size (90 nm) than that of the smaller (45 nm). These differences can be explained both by the difference in particle size and surface charge of the nanoparticles which were found to be significantly smaller than TMC and CS nanoparticles (see Table 3.4). In a previous study by Gutierro et al. (161), significantly higher immune responses were reported following nasal administration of antigen in nanometer size particles (500 nm) than that of free antigen administration. It can be suggested that although smaller particle size is needed for enhanced immune response, this should not be go down after a certain particle size. Besides, it is possible to compensate the immune response with the electrostatic interactions between mucosal membrane and nanoparticles, because the negatively charged glycocalyx on the apical membrane of mucosal cells can be strongly interacting with positively charged chitosan and TMC nanoparticles whereas it can not be easier for MCC nanoparticles.

The difference we observed between the immune responses of positively charged polymers (CS and TMC), and negatively charged derivative MCC can be explained by a mechanism. The differences in

surface charge will affect the molecular interactions; such as the electrostatic, van der Waals and hydrogen bonding forces, between the interpenetrating bioadhesive polymer chains and the mucin network. Both negatively charged MCC and positively charged CS and TMC possess the electronegative groups associated with hydrogen bonding. However, the anionic MCC nanoparticles will also induce repulsive electrostatic forces with the negatively charged mucin network. These repulsive electrostatic forces could reduce the overall mucoadhesive strength. Charge differences, in conjunction with the differences in molecular contact and flexibility, can explain the differences observed between immune responses of the two different charged nanoparticle systems.

Especially for the intranasal applications, following boosting, the immune responses were found to be increased. Similarly, enhanced immune responses for various antigens were reported with TMC and CS nanoparticle formulations (72,87).

4.8.2. Comparison of the immune responses of the formulations prepared with TMC, MCC, CS and TMC/MCC complex

Generally, when we compare to the control group, immune responses were enhanced with CS and its derivatives following nasal administration whereas with the same formulations subcutaneous administration did not enhance the immune responses when compared to that of the control group (Figure 3.22 and 3.23). Due to the bioadhesive property which is able to decrease the clearance of formulations from the nasal cavity, nasally administered TT loaded formulations provided higher immune responses. On the other hand via subcutaneous route, chitosan's bioadhesive property did not effect the immune response.

The immune responses elicited by the antigen-loaded nanoparticles (Figure 3.23) would be based on cellular uptake of the nanoparticles in the nasal epithelium and NALT and subsequent access of the vaccine to submucosal lymphoid tissues.

We obtained the significantly the highest immune responses with TMC/MCC complex formulations. When serum IgG results of TMC, MCC and CS nanoparticles are compared to that of TMC/MCC complex nanoparticles (Figure 3.23), TMC/MCC complex nanoparticles showed significantly higher ($p < 0.01$) levels of IgG than those corresponding to TMC, MCC and CS nanoparticles. The nanoparticles prepared with chitosan and its derivatives is expected to enable the polymer chains to penetrate the mucin network and establish adhesive bonds. Adhesion between the polymer and the mucins should be rapid especially for TMC and MCC formulations, since they are already in a protonated form over a wide pH range. CS has been shown to enhance cell permeability by affecting paracellular pathways (71,162,163). This hypothesis was correlated with our results.

The use of chitosan derivatives (especially with TMC) as a strategy to improve the response to nasally administered antigens is likely to be governed mainly by its adjuvant effects and absorption enhancer effect aiding the contact of the antigen with intraepithelial and submucosal lymphocytes independent from pH.

We were not able to obtain significant IgA antibody titres in vaginal washes. Similarly, in a study performed by Van der Lubben et al. (67), following nasal application of DT loaded microparticles, the local antibody levels were not found to be high whilst enhanced systemic immune response was obtained.

5. CONCLUSION

The encapsulation of antigens in particulate systems is aimed to reduce antigen degradation and enhance mucosal antigen delivery. Moreover encapsulation of TT into the nanoparticles would bring advantages over solution form especially for cellular uptake at the nasal surface and also to prevent the loss of the antigen by ciliary clearance before reaching the target M-cells within the delivery system. Preparation of chitosan based nanoparticles does not need any organic solvent which makes it more attractive and safe compared to those prepared with polymers requiring utilization of an organic solvent. If we compare these delivery systems to other nanoparticles prepared with different polymers for example PLGA, chitosan or its derivatives are very promising because of the preparation method which do not need to use any protein stabilizers. Moreover due to the bioadhesive properties of chitosan and its derivatives, it is possible to extend the retention times of the formulations in the nasal cavity.

In this study, TT-loaded nanoparticulate systems with particle size within range of 40-400 nm containing chitosan or chitosan derivatives with different charges (TMC positively charged and MCC negatively charged) have been prepared and their potentials as adjuvant/delivery system for mucosal immunization has been demonstrated. This was the first time a negatively charged chitosan derivative (MCC) was used to prepare nanoparticles for this purpose. Furthermore, it was the first time a TMC-MCC complex nanoparticulate system was prepared without need of a crosslinker. Nanoparticles were prepared by ionic gelation method based on the complexation between oppositely charged chitosan derivatives (positively charged TMC and negatively charged MCC). The loading of TT to TMC/MCC

nanoparticles has been proven to be a very mild process resulting in a very high loading efficiency.

TMC, chitosan and TMC/MCC complex nanoparticles were found to be promising adjuvant/delivery systems for mucosal immunization. Our results emphasized that surface charge and particle size exert an important role in obtaining an enhanced immune response. Although MCC induced lower immune response for TT when compared to TMC and chitosan, it was shown that it is possible to prepare nanoparticles with small particle size, narrow size distribution and high loading capacity using MCC. With these promising features, MCC can be suggested as a potential delivery system for diverse range of drugs as well as a gene/protein delivery.

As a conclusion; chitosan and derivatives are promising polymers for mucosal vaccine delivery both as an adjuvant and a delivery system with their biocompatibility, high loading capacity and appropriate particle size.

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