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Preparation and Characterization of Particles from Chitosan with Different Molecular Weights and Their Trimethyl Chitosan Derivatives for Nasal Immunization

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Abstract

According to previous studies, molecular weights (MW) of chitosan presented an influence on the level of immune response to model antigen when administering with chitosan through nasal route. Moreover, trimethyl chitosan (TMC) with a degree of quaternization (DQ) of 40%, chitosan derivatives, was also shown to be the most potent nasal OVA delivery platforms compared to TMC with DQ of 20% and 60% in mice model. The aims of this study were to prepare particles from chitosan with difference MW and their TMC derivatives (DQ = 40%) and to characterize size, zeta potential, loading efficiency and release profile in order to be used as data for further studies as nasal adjuvant. The results showed that MW of chitosan in the studied range did not influence the particle characteristics. The particles prepared from TMC derivatives are of smaller size than those prepared from chitosan. However, they possessed higher zeta potential, loading efficiency and release profile than the particles prepared from chitosan.

Key words : Chitosan, degree of quaternization, molecular weight, nasal immunization, particle, trimethyl chitosan

Introduction

The nasal mucosa is an attractive site for the delivery of vaccines and has certain advantages over other sites. It is highly vascularized, has a relatively large absorptive surface and has low proteolytic activity.(1) Importantly, nasal immunization can induce both mucosal and systemic immune responses.(2) However, in spite of these attractive features, most vaccines are not well absorbed from the nasal cavity when administered as simple solutions. Major factors limiting the absorption of nasally administered vaccines are their poor ability to cross nasal barriers and the mucociliary clearance mechanism, which removes soluble antigens from the nasal cavity.(3)

Mucoadhesive polymers such as chitosan and trimethyl chitosan (TMC), a partially quaternized derivative of chitosan which has a high water solubility compared with chitosan, especially at neutral and basic pH values,(4) offer a significant potential for the development of mucosa administered antigens.(5) Besides their biodegradability, biocompatibility, and very low toxicity,(6, 7) it was reported that chitosan and TMC solution enhanced immune responses by opening tight junctions through an interaction of the polycationic polymers with the negatively charged sites in the tight junctions.(4) Moreover, the mucoadhesion property of chitosan and TMC is known to prolong the residence time of antigens in the nasal cavity.(5) This combination of paracellular transport effects through opening tight junctions and mucoadhesion property has led us to consider the use of chitosan for the delivery of vaccines via the nasal cavity. In addition, particulate carriers based on chitosan and TMC have received special interest to be nasal adjuvants.(2) Particles are mainly taken up by M-cells of the NALT which are capable of transporting antigen across the cell by transcytosis without degradation.(5) Hence, chitosan and TMC may be employed for nasal vaccine delivery in both solution and particulate forms. Our previous studies found that following nasal administration in BALB/c mice immune responses to ovalbumin (OVA) dissolved in TMC with DQ of 40% showed to be the most potent delivery platform and adjuvant compared to TMC with DQ of 20% and 60%.(8) Moreover, the molecular weight (MW) of

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chitosan also showed to have influences on the level of immune induction to OVA following nasal administration (Figure 1). However, there is not much information on the preparation and ability of particles of chitosan with difference MW and their TMC derivatives (DQ = 40%) in the immune induction *in vivo*. Therefore, the aims of this study were to prepare and to characterize particles prepared from chitosan with difference MW and their TMC derivatives (DQ = 40%) to be used for further studies as mucosal adjuvant for inducing immune responses to OVA via the nasal route.

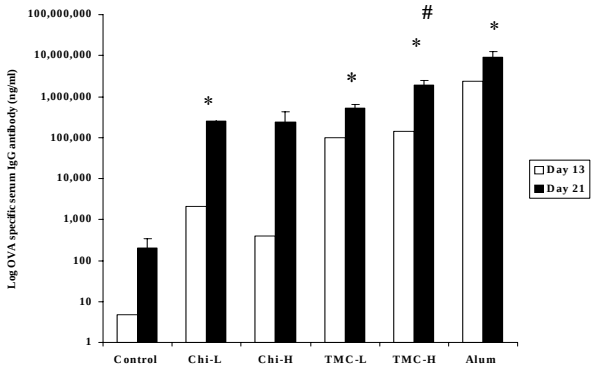


Figure 1. Serum IgG antibody titers were obtained from mice following intranasal immunization with OVA in saline solution (control group), Chi-L (MW 160 kDa) solution, Chi-H (MW 270 kDa) solution, TMC-L solution,and TMC-H solution. OVA in alum a dministered subcutaneously was used as a positive control. The IgG titers were expressed as mean ± S.E.M* Significant differences with control groups ($p<0.05$) and # significant differences with their starting chitosan ($p<0.05$).

Materials and Experimental Procedures

Materials

Low MW chitosan (Chi-L, MW = 160 kDa, degree of deacetylation (DD) = 96%) from Aqua Premier (Chonburi, Thailand) and high MW chitosan (Chi-H, MW = 270 kDa, DD = 93%) from Primex (Haugesund, Norway) were used for synthesizing the TMC (see below). Glacial acetic acid was purchased from J.T. Baker (New Jersey, USA). Diethyl ether was obtained from Labscan (Bangkok, Thailand). Methyl iodide, N-methyl-2-pyrrolidinone (NMP), sodium hydroxide, and sodium iodide were received from Merck (Darmstadt, Germany). Deuterium oxide (D_2O) was purchased from Sigma (Milwaukee, USA). OVA was purchased from Sigma Aldrich (St. Louis, USA). For the preparation of particles, Sodium tripolyphosphate pentabasic (TPP), practical grade, 90-95%, was purchased from Sigma (Missouri, USA). Phosphate buffer saline solution (PBS), pH 7.4, was prepared by combining sodium hydrogen orthophosphate (Ajax Finechem, New South Wales, Australia), potassium dihydrogen orthophosphate (Fisher Scientific, Leicestershire, England), potassium chloride (J.T. Baker, New Jersey, USA), sodium chloride (Merck, Darmstadt, Germany) and distilled water.

TMC Synthesis

TMC with a degree of quaternization of 40% were synthesized by reductive methylation of chitosan.[8] One gram of chitosan, 2.4 g of sodium iodide, and 5 ml of 20 % w/v aqueous sodium hydroxide were dissolved in 40 ml of NMP. The mixture was kept in a water bath with controlled temperature at 60ºC for 20 mins. Subsequently, 5 ml of methyl iodide were added to the mixture and the reaction was carried out in a Liebig condenser for 30 mins. Afterwards, 5 ml of methyl iodide and 5 ml of 20 %w/v aqueous sodium hydroxide were added. The reaction was further continued in a Liebig condenser at a controlled temperature of 60ºC for 30 mins. The product was precipitated by using 150 ml of diethyl ether three times. The products obtained were pooled and dissolved in 50 ml of 10%w/v sodium chloride solution to exchange the iodide ion with chloride. The final product was freeze-dried after dialysis and kept in light-protected desiccators.

Characterization by NMR Spectroscopy

TMC products synthesized were analyzed by ^1H – Nuclear Magnetic Resonance (^1H – NMR) spectroscopy (AV400, Bruker, Denmark). The NMR spectrum of TMC was obtained by dissolving 5 mg of each sample in 700 µl of D_2O at 80ºC with suppression of the water peak. DQ was calculated using data obtained from the ^1H – NMR spectra using the following Eq. (1): [9]

$$DQ(\%)=\{[(CH_3)_3]/[H]*1/9\}*100 \qquad (1)$$

where DQ (%) is the degree of quaternization as percentage, $[(CH_3)_3]$ is the integral of the chemical shift of the N-trimethyl amino group at 3.3 ppm attributed to the nine hydrogen atoms of the methyl groups pertaining to trimethylated amino groups. [H] is the integral of the ^1H peaks between 4.7 – 5.7 ppm (reference signals) representing the protons attached to the carbon of the glucosamine unit of the glucopyranose ring.

Determination of Molecular Weight

The molecular weights of the chitosan and synthesized TMC were measured using a gel permeation chromatography system (GPC) (Waters 600E, Waters Co., US).

In brief, the mobile phase used consisted of 0.5 M acetic acid and 0.5 M sodium acetate (acetate buffer pH 3). Two mg of samples were dissolved in 1 ml of acetate buffer and were filtered through 0.45 µm nylon 66 membrane filters before injection. Twenty microlitres were injected into the ultrahydrogel linear columns (MW resolving range = 1,000 – 20,000,000) at a flow rate of 0.6 ml/min and were kept at 30ºC during samples analysis. After that, samples solutions were analyzed with the refractive index detector.

Preparation of Chitosan and TMC Particles

Particles from chitosan with difference MW and their TMC derivatives were prepared by ionic gelation of chitosan with TPP as described by Boontha et al.[10] Briefly, 1% w/v chitosan and TMC were dissolved in 2% acetic acid aqueous solution and distilled water, respectively. After stirring overnight, 10 ml of TPP solution were added dropwise to 30 ml of either chitosan or TMC solution under stirring (300 rpm). The weight ratio (WR) of polymer to TPP used in this study was fixed at 1:1 except for the WR of TMC-H : TPP which was 1:0.25.[10] Following stirring overnight, the particle suspension was centrifuged at 4ºC at 18,000 rpm. The particles were washed and subjected to characterize zeta potential, particle size, and release profile. The supernatant was analyzed for loading efficiency. OVA-loaded chitosan or TMC particles were prepared as described above by dissolving OVA (0.1 mg/ml) in TPP solution before adding to chitosan or TMC solutions.

Physicochemical Characterization of Chitosan and TMC Particles

For measurement of zeta potential and size of chitosan and TMC particles, ZetaPALS (Zeta Potential Analyzer, Brookhaven Instrument Corporation, UK) was used which is based on photon correlation spectroscopy (PCS) techniques. The zeta potential of particles was measured by diluting chitosan or TMC particles in 1 mM KCL with appropriate concentration, while these particles were diluted with distilled water to measure particle size. These samples were analyzed and presented as mean value.

Determination of Loading Efficiency

The loading efficiency (LE) of OVA-loaded chitosan or TMC particles was determined by separating the particles from the amount of OVA, remaining in the supernatant following centrifugation at 18,000 rpm. The amount of free OVA was determined by using Micro Bicinchoninic Acid (BCA) assay (Pierce Biotechnology, Rockford, IL) at a wavelength of 540 nm. The supernatant of non-loaded chitosan or TMC particle was used as a blank. The LE of particles was calculated from Eq. (2) indicated below:

$$\%LE = \frac{\text{Total amount of OVA added} - \text{Amount of OVA remaining in the supernatant}}{\text{Total amount of OVA}} \times 100 \quad (2)$$

Determination of OVA Release from Particles

The in vitro OVA release profiles of chitosan and TMC particles were determined as follows. The OVA loaded chitosan or TMC particles were dispersed in 20 ml of PBS (pH 7.4). After that they were incubated at 37°C under magnetic stirring (300 rpm). At appropriate time intervals, samples were withdrawn and centrifuged at 4°C (10,000 rpm, 10 mins). The amount of OVA released from particles in the supernatant was analyzed by the Micro BCA protein assay. The supernatant that was obtained from non-loaded chitosan or TMC particles was used as a blank.

Statistical Analysis

All values were expressed as means ± SD. Statistical significance of data was evaluated by one-way ANOVA with post hoct Tamhane test

(SPSS 10.0, SPSS Inc., and Chicago, IL, USA). A p-value of 0.05 or less was considered significant. All experiments were repeated at least three times.

Results and Discussion

Synthesis and Characterization of TMC

In this study, TMC was synthesized by reacting chitosan with an excess of iodomethane in solution of NMP in the presence of sodium iodide and sodium hydroxide.[4, 11, 12] The quaternization of the nitrogen atom pertaining to amino groups originally presented in the chitosan chain results in permanently positive charged sites in the TMC chain (Figure 2). [9, 13, 14]

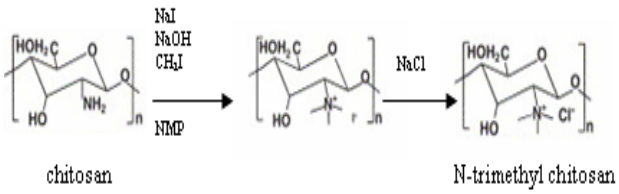


Figure 2. Synthesis reaction scheme of TMC [14]

Table 1 shows the characteristics of starting chitosans and their TMC derivatives. From NMR analysis a degree of quaternization of about 40% was obtained. GPC was used to determine the MW of chitosan and their TMC derivatives. It was presented that MW of TMC-L and TMC-H decreased compared with starting chitosan, Chi-L and Chi-H, respectively. A net decrease in the MW was also observed by Snyman and colleagues.[15] This is due to degradation of the polymer chain caused by the exposure time of the chemical reaction in the strong alkaline environment and temperature causing cleavage of the polymer backbone chain.

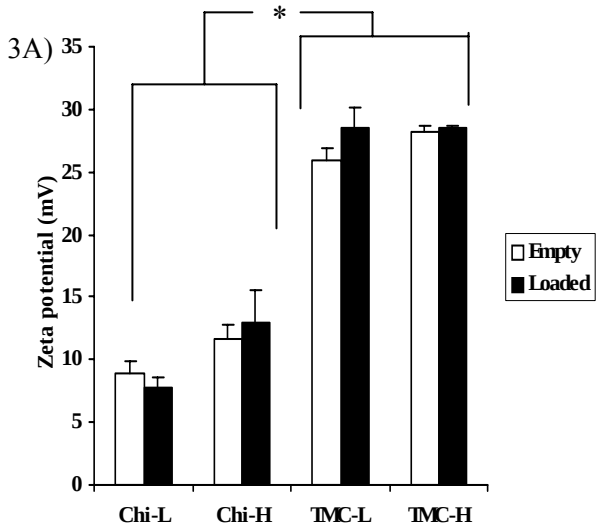
Table 1. The characteristics of chitosans and synthesized TMC

Type of chitosan	Code of product given	DQ (%)	MW (kDa)
Low MW chitosan	Chi-L	-	160
	TMC- L	37.8 ± 0.9	110
High MW chitosan	Chi-H	-	270
	TMC-H	40.0 ± 2.3	81

Preparation and Physicochemical Characterization of Chitosan and TMCP Particles

In this study, chitosan with difference MW and their TMC derivatives (DQ = 40%) particles were prepared by ionic gelation of cationic chitosan and TMC with TPP anions. This technique represents an easy method to prepare nasal delivery systems for antigen in mild condition by dropping TPP-OVA solution into chitosan or TMC solution at ambient temperature while stirring without using sonication, or organic solvents.[16, 17]

The zeta potential and particle size are shown in Figure 3. It was found that the MW in the studied range did not have an influence on the zeta potential of chitosan particles (Figure 3A). Comparing empty and OVA-loaded chitosan and TMC particles, a significant difference of zeta potential was not observed. However, all TMC particles presented higher zeta potential than chitosan particles ($p<0.001$). This is due to the permanently positive charged sites in the TMC chain as represented in Figure 2.[9] Similarly, the MW in the studied range has no effect on the size of the chitosan particles (Figure 3B). Also a significant difference of particle sizes between empty and OVA – loaded chitosan and TMC particles was not observed. Comparing chitosan with their TMC derivative particles, all TMC particles presented smaller sizes than chitosan particles. This result may be due to the strong interactions between TPP and TMC because TMC has a higher positive charge than chitosan.



3B)

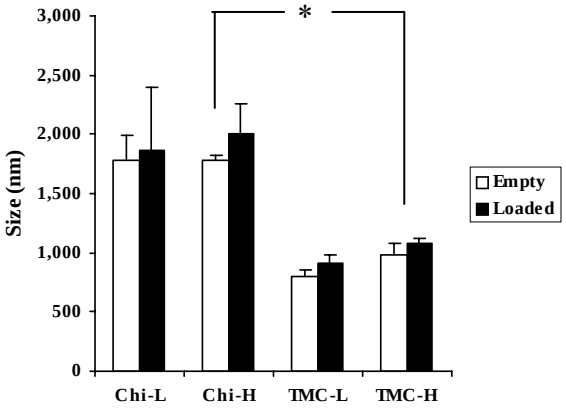


Figure 3. Zeta potential (3A) and particle size (3B) of chitosans and theirs TMC particles. Significant difference was expressed as *p<0.05.

Loading Efficiency of Ovalbumin from Chitosan and TMC Particles

Loading efficiency (LE) of the particles prepared using chitosan and their TMC derivatives are presented in Figure 4. The MW of polymers did not show an influence on LE of OVA in chitosan particles. Similarly, a significant difference of LE between TMC-L and TMC-H was not observed. Comparing chitosan and their TMC derivative, all TMC derivatives particles presented higher LE than chitosan particles. However, a significant difference of LE could not be observed.

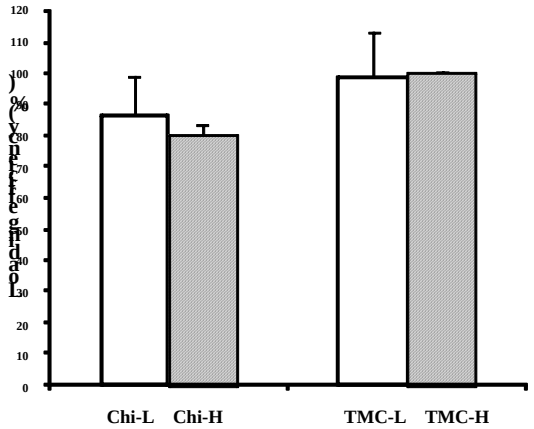


Figure 4. Loading efficiency of OVA in chitosans and theirs TMC particles .

OVA Release Profiles from Chitosan and TMC Particles

The release profiles of OVA from chitosan and TMC particles in PBS, pH = 7.4, are presented in Figure 5. It could be seen that OVA released from Chi-L and Chi-H particles in PBS buffer, pH 7.4, was negligible. In contrast, an initial burst of OVA - approximately 40% to 50% from TMC-L and TMC-H particles - was observed. This result may be explained by the fact that the chitosan matrix is not soluble at pH 7.4[18] while TMC immediately dissolves under this condition.[19] It indicated that ionic exchanges between TMC and release medium may lead to solubilization of the reversible physical cross-link of TMC and TPP. Therefore, TMC-OVA binding at particle surface was loose and burst release of OVA occurred. As TMC particles possess higher LE than chitosan particles, another factor assessed should be the OVA concentration gradient between particles and the release medium that caused a higher diffusion rate of TMC than that of chitosan particles. Comparing TMC-H and TMC-L, OVA released from TMC-H was higher than that released from TMC-L. However, a statistic significance was not observed.

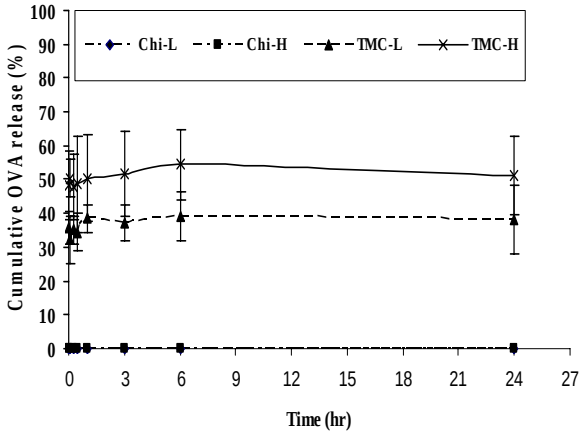


Figure 5. Ovalbumin (OVA) released from particles prepared using chitosans and theirs TMC particles

Conclusions

In this study, the particles of chitosan with difference MW and their TMC derivatives (DQ = 40%) were prepared using ionic gelation technique which is of mild condition. Their features were characterized in terms of zeta potential, particle size, loading efficiency and release profile. MW of

chitosan in the studied range did not present a significant influence on particle characteristics. All TMC particles showed higher zeta potential, smaller size, higher loading efficiency and higher release profile than chitosan particles. Nasal adjuvants will further be studied in animal models.

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