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Chitosan and Trimethyl Chitosan Particles as Oral Vaccine Delivery Systems: Comparison of the Potential to Initiate Immune Responses

Supavadee BOONTHA, Hans E. JUNGINGER, Neti WARANUCH, Assadang POLNOK and Tasana PITAKSUTEEPONG*

Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok 65000, Thailand

Abstract

In this study, particles having low MW (Chi-L), high MW (Chi-H) and their respective trimethyl chitosan (TMC) with a degree of quaternization (DQ) of about 40% (TMC-L-40 and TMC-H-40), and prepared by using chitosan, were used to compare their potentials for provoking immune responses to ovalbumin (OVA) following oral administration. The results showed that the Chi-L particles gave higher serum immunoglobulin G (IgG) responses to OVA than those induced with Chi-H, TMC-L-40 and TMC-H-40 particles. However, Chi-L and Chi-H particles gave similar secretory immunoglobulin A (sIgA) to OVA, and sIgA titers obtained from mice immunized with OVA-loaded TMC were negligible. This study demonstrated that chitosan particles seem to be better delivery systems for oral vaccination, and the MW of chitosan appeared to have an effect on the ability to elicit immune responses to OVA in BALB/c mice following oral immunization**.**

Key words: Chitosan, Trimethyl chitosan, Oral vaccine, Immune responses

Introduction

Vaccination presents the most efficient and cost-effective disease control tool. In this regard, oral vaccinations are safe and convenient methods. However, immune responses following oral administration are usually poor due to the rapid degradation of antigen when getting into contact with the gastrointestinal (GI) fluids, and low absorption of antigen in the GI tract [(1)]. Particulate delivery systems are known to protect the associated antigen from degradation and to enhance absorption [(2-4)]. In order to design these systems, chitosan was chosen for the preparation of particles because it is biodegradable, biocompatible and safe for use in humans [(5, 6)]. Chitosan is the value added product from frozen food biowaste, e.g. shrimp shell, crab shell and squid pen. Annually, Thailand exports large amounts of frozen food, such as shrimp and squid, totaling approximately 311,000 and 66,000 tons, respectively [(7)]. The trend of frozen food export is increasing and subsequently generates large amounts of biowaste. Thus, the application of the biowaste is very interesting and useful. The United States Food and Drug Administration (USFDA) approved chitosan use in 1983 as a feed additive and has recently recognized it as a GRAS (Generally Recognised As Safe) component [(8)]. In the pharmaceutical field, chitosan is used as excipients and drug carriers. Moreover, chitosan also possesses opening tight junctions and mucoadhesive properties which enhance the absorption across mucosa epithelium as well as prolong the residence time of delivery systems at the absorption sites [(9, 10)]. However, chitosan is only soluble in acidic medium. Thus, trimethyl chitosan (TMC) has received attention for usage as vaccine delivery system since TMC, a partially quaternized derivative of chitosan, has high water solubility even in neutral pH when compared with chitosan [(11)]. Moreover, TMC has proved an effective penetration enhancer of hydrophilic and/or high molecular weight molecules across the intestinal epithelium by means of the same mechanism of chitosan [(12)]. Following investigation of the effect of TMC with DQ of 20, 40 and 60% on immune responses to OVA via nasal immunization by our group, the results showed that TMC with DQ of 40% (TMC-40) could elicit the highest immune titers [(13)]. Also, it has been reported that molecular weight (MW) of chitosan influenced the potency and duration of immune responses [(14)]. Therefore, the aim of this study was to compare the potential of particles prepared using chitosan having different MW and their respective TMC-40

* Corresponding author: Tel: +66 5596 1877; Fax: +66 5596 3731; E-mail: Htasanap@nu.ac.th

derivative for inducing immune responses to ovalbumin (OVA), a model antigen, via the oral route.

Materials and Experimental Procedures

Chi-L (MW = 160 kDa, degree of deacetylation (DD) = 96%) (Aqua Premier, Chonburi, Thailand), Chi-H (MW = 270 kDa, DD = 93%) (Primex, Haugesund, Norway) and TMC with DQ of 40% synthesized by reductive methylation of Chi-L named (TMC-L-40, MW = 110 kDa) and Chi-H named (TMC-H-40, MW = 81 kDa) [13] were used in this study. OVA–loaded particles were prepared by ionic gelation with tripolyphosphate (TPP) (Sigma, MO, USA). Particle sizes and zeta potential were determined using a photon correlation spectroscopy (PCS) (ZetaPlus, Brookhaven instrument, UK). Loading contents and release profiles of OVA were determined by the Micro Bicinchoninic Acid (BCA) assay (Pirece, Rockford, IL, USA).

For evaluation of immune responses to OVA, seven female BALB/C mice were immunized orally with 500 µg of OVA in phosphate buffer solution (PBS) at pH 7.4 and in particles prepared from Chi-L, Chi-H, TMC-L-40 and TMC-H-40 or immunized subcutaneously (s.c.) with of 100 µg of OVA in alum on weeks 0 and 2. Blood and fecal samples were collected on weeks 0, 2, 3 and 4. The immune to OVA were analyzed by ELISA in order to determine the levels of OVA-specific IgG and sIgA antibodies [15].

Statistical Analysis

All values were expressed as means ± SD. Statistical significance of data was evaluated by one-way ANOVA with post hoc LSD test (SPSS 11.0, 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

Characteristic of OVA - Loaded Particle

Size and charge of chitosan and TMC prepared particles are shown in Table 1. It was found that the MW of chitosans in the studied range did not have an influence on the size of chitosan particles. Surprisingly, comparing chitosans with TMCs, particles prepared from TMCs were smaller than those prepared from chitosans. This may be explained by the different conformation of these polymers in solution. Our previous study illustrated that when these polymers dissolved in solution chitosans tended to be in an extended conformation whereas TMCs were found to be coiling up into compact globules [16]. Thus, TMC particles could be formed with a lower amount of TPP added compared with the particles prepared from their starting chitosan. From these results, it can be estimated that a lower amount of TPP may interact with a lower number of polymer chains, resulting in smaller size of TMC particles. Similarly, the MW of chitosans in the studied range has no effect on the charge of the chitosan particles. However, comparing chitosan with their TMC derivative particles, all TMC particles had higher surface charge than chitosan particles. This may be due to the fact that TMC has a permanent positive charge which stems from the substitution of the methyl group on the C-2 atom on the amino group of chitosan. These results are in agreement with the charge measurement of these polymers dissolved in solution at various pH values as described in our previous study[16]. At a pH of about 6, at which the measurement of zeta potential of particles was performed in this study, it was found that zeta potential of TMC-40 was higher than that of chitosan. The addition of negatively charged TPP in the preparation of particles resulting in the reduction of zeta potential of chitosan and their respective TMC particles corresponded with their solution form. The amount of OVA loaded on the particles was calculated and expressed as loading efficacy (LE) and loading capacity (LC) (Table 1). It was observed that MW and types of polymer have an influence on LE and LC. Particles prepared using TMC-H-40 possessed the highest LE and LC. This may be explained by the fact that the preparation of TMC-H-40 particles made use of a lower amount of TPP [16]. As a result, an intermolecular and/or intramolecular linkage between TPP and TMC was less, and thus the binding site on TMC for reacting with OVA was higher than other TMC [17]. Particles prepared using TMC released a higher amount of OVA than those prepared using the starting chitosan (Figure 1). This may be due to the fact that TMC particles have smaller size and thus higher surface area than chitosan particles, resulting in a higher amount of OVA release. In addition, higher release of OVA from TMC particles may also be due to the higher solubility of TMC than chitosan.

Table 1. The characteristics of OVA - loaded particles

Product Code	Size (nm)	Charge (mV)	LE (%)	LC (%)
Chi-L	2431 ± 124	+ 8.2 ± 0.5	81 ± 17	10 ± 2
Chi-H	2345 ± 100	+ 9.9 ± 0.7	29 ± 10	4 ± 1
TMC-L-40	861 ± 87	+ 31.8 ± 0.8	64 ± 10	12 ± 2
TMC-H-40	888 ± 47	+ 25.3 ± 2.1	85 ± 10	56 ± 5

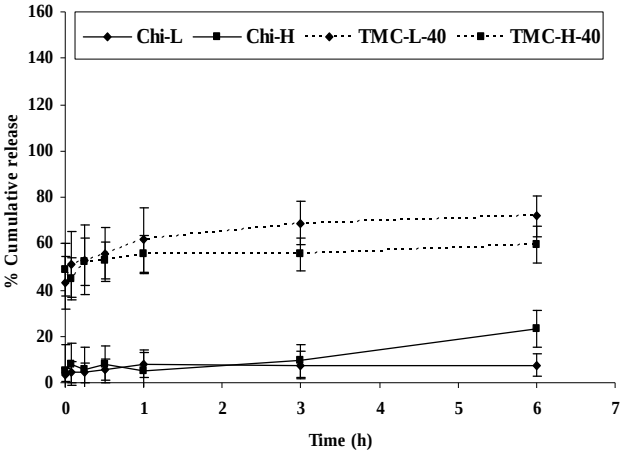


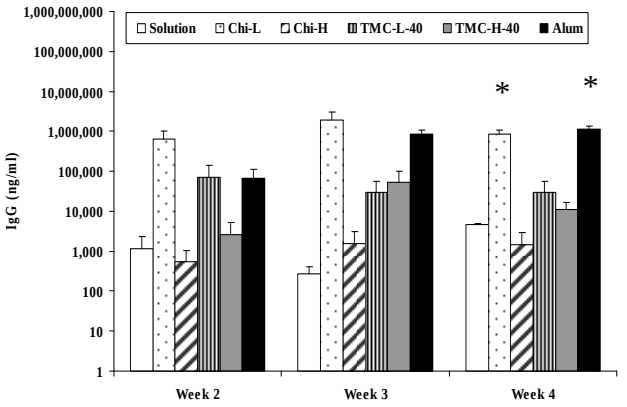
Figure 1. Release profiles of OVA from particles prepared using chitosan having low and high MW and their respective synthesized TMC-40 derivatives. The amounts of OVA released were expressed as mean ± S.D. (n=3).

Immune Responses to OVA

Comparing between chitosans and TMCs, it was shown that particles prepared using Chi-L could induce higher IgG responses to OVA than its TMC derivatives (i.e. TMC-L-40) (Figure 2(a)). This may be explained by the fact that delivery systems administered via oral route are prone to acid and enzymatic degradation. As shown in Figure 1, the particles prepared using TMC-L-40 dissolved quicker and released higher amount of OVA than those prepared using Chi-L. Thus, following delivery in gastrointestinal (GI) tract, some TMC-L-40 particles may be dissolved, and free OVA behaves like OVA solution co-administered with TMC solutions, resulting in lower IgG elicited. However, particles prepared using Chi-H could induce lower IgG responses to OVA than its TMC derivatives (i.e. TMC-H-40). This result suggests that very low amount of OVA is released from Chi-H particles along GI tract. According to the different MW of chitosan, Chi-L particles could elicit significant higher IgG responses to OVA than Chi-H particles. The result is in agreement with the finding of Coombes et al. [18] who observed that the lower MW poly (*D*, *L*-lactide co-glycolide) (PLG) particles degraded faster than high MW PLG particles, resulting in more OVA released from the particles and subsequently higher serum IgG responses to OVA elicited [18]. Therefore, in this study, it can be explained that the higher IgG responses to OVA elicited by Chi-L are affected by a degradation rate of the particles higher than for Chi-H.

The sIgA titers elicited by OVA-loaded particles prepared using two types of chitosan showed no significant difference (Figure 2(b)). Interestingly, sIgA titers obtained from mice immunized with OVA-loaded TMC particles were negligible. In general, following antigen delivered through the intestinal lumen, there are two distinctive pathways to allow the transport of antigen into the lymphoid tissue depending on the nature of antigen. Soluble antigen may be able to penetrate the intestinal epithelium into the lamina propria (LP), and may interact with the antigen presenting cells (APCs) such as macrophages and dendritic cells. The APCs migrate to the lymph node where the antigen is presented to the T cells as a start of the activation of the IgG immune response cascade. In contrast, antigen in particulate form is largely taken up by M-cells for transportation to gut-associated lymphoid tissue (GALT), and is subsequently transferred to underlying APCs for the initiation of antigen-specific mucosal sIgA and IgG responses. This may imply that most chitosan particles may mainly be taken up via M-cells, and interact and process by underlying APCs to provoke IgG and sIgA immune responses [14, 19]. However, TMC particles may dissolve along GI tract and behave like OVA solutions co-administered with TMC. The absorption of OVA may occur along the intestinal epithelium. Thus, IgG responses from OVA-loaded TMC particles were lower than Chi-L, but higher than OVA solutions. Few or none of the TMC particles may reach M-cells and thus sIgA responses are negligible. The levels of IgG titers obtained from TMC-L-40 and TMC-H-40 particles did not differ significantly. This result was expected and is due to their similar characteristics. No IgA response was observed when mice were immunized with OVA in alum. Once more, this is an expected result because immunization via s.c. route cannot initiate mucosal immune responses.

(a)



(b)

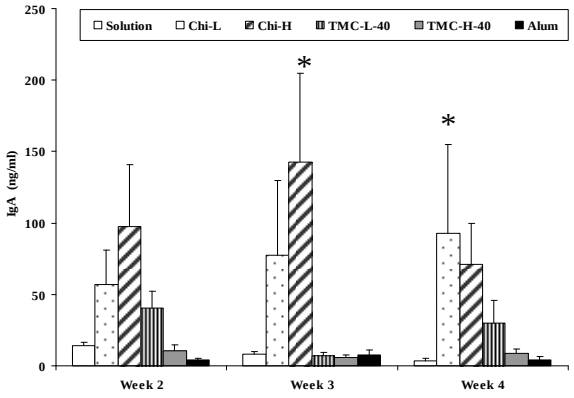


Figure 2. Serum IgG (a) and secretion IgA (sIgA) (b) antibodies obtained from mice following oral immunization with OVA in different formulations. IgG at week 2 and 3 were obtained from pooled serum, while IgG at week 4 were obtained from individual sera. IgG at week 0 were not detectable. The IgG at week 2 and 3 were expressed as mean ± S.E.M (n=3) while the IgG at week 4 were expressed as mean ± S.E.M (n=7). sIgA were obtained from individual faecal extracts. sIgA at week 0 were not detectable. The sIgA were expressed as mean ± S.E.M (n=7). Significant differences with solution group (control) are designated as * ($p < 0.05$).

Conclusions

Chitosan and TMC have different potentials in the initiation of immune responses via oral immunization. Chitosan particles seem to be better delivery systems for oral vaccination. The MW of chitosan appeared to have an effect on the ability to elicit immune responses to OVA in BALB/c mice following oral immunization**.**

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