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Original Article

Formation of particles prepared using chitosan and their trimethyl chitosan derivatives for oral vaccine delivery: Effect of molecular weight and degree of quaternization

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Abstract

The purpose of this study was to investigate the effect of molecular weight (MW) of chitosan and the degree of quaternization (DQ) of trimethyl chitosan (TMC) on the formation of particles. The amount of tripolyphosphate (TPP) was varied in order to optimize the preparation condition of particles. Particle size, zeta potential, loading efficiency (LE) of ovalbumin (OVA), a model antigen, and OVA release profiles were also investigated in order to select the best systems for oral vaccine delivery. The results showed that the amount of TPP added has an effect on the ability of chitosan and TMC polymer to form particles. The formation of chitosan particles required a higher amount of TPP than that of TMC particles. Using the same amount of TPP, the MW of chitosan did not have an effect on particle formation whereas the DQ of TMC had. Depending on the amount of TPP, the size of particles prepared from chitosan and their TMC were in the range of 0.5-2.3 μm and their positive charges were in the range of 3-48 mV. Adding the same amount of TPP, the size of the chitosan particles was larger than that of their respective TMC particles. The size of the TMC particles was decreased with increasing of DQ. The zeta potential of the TMC particles was higher than that of their starting chitosan particles and it increased with increasing DQ. The loading efficiency (LE) of OVA onto the TMC particles was charge-dependent. MW did not affect the LE of chitosan particles. The OVA release from the particles prepared depended on the solubility and loading capacity of the particles.

Keywords: chitosan, trimethyl chitosan, formation of particles, molecular weight, degree of quaternization

1. Introduction

The oral route is the most desirable one for vaccine delivery because it is easy to administer and carries no hurdles associated with needle-based injections, which may have a higher risk of infection with other diseases when non-sterile needles are used (Giudice *et al.*, 2006). However, immune responses following oral administration are usually poor due to the rapidly degradation of antigen when in contact with the gastrointestinal (GI) fluids and due to the low absorption of antigen in the GI tract (Norris *et al.*, 1998). Particulate delivery systems are known to enhance absorption and protect the associated antigen from degradation (O'Hagan *et al.*, 1998; Van der Lubben *et al.*, 2003; Delie *et al.*, 2005).

Chitosan, the second most abundant polysaccharide, was chosen to prepare particles in this study because it is available at low costs, it is biodegradable, biocompatible, and has a very low toxicity (Illum, 1998; David, 2006). Moreover, chitosan also possesses the ability for opening of the tight junctions and has mucoadhesive properties, which enhance the absorption across mucosa epithelium as well as prolong the residence time of delivery systems at the absorption sites (Kotzé *et al.*, 1997a; Van der Lubben *et al.*, 2004). Trimethyl chitosan (TMC), a partially quarternized chitosan derivative,

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was also used in this study. An increase in the positive charges on the polymer chain causes the molecular expansion in solution, resulting in a high water solubility compared to chitosan, especially at neutral pH, where chitosan is insoluble (Kotzé *et al.*, 1997b). It has been reported that the molecular weight (MW) of chitosan influences the potency and duration of immune responses. Vila *et al.* (2004) have demonstrated a stronger immune response from low MW chitosan (23 and 38 kDa) tetanus toxin loaded nanoparticles at earlier times than for loaded particles prepared with higher MW chitosans. The charge density of TMC, as determined by the degree of quaternization (DQ), plays an important role for the absorption enhancing properties of this polymer (Kotzé *et al.*, 1999; Hamman *et al.*, 2002). It has been reported that TMC with a higher DQ (ca. 60%) was more effective in enhancing the transport of a hydrophilic model compound mannitol, across Caco-2 cell monolayer, compared to TMC with lower DQ, about 40% (Thanou *et al.*, 2000). However, a study of Hamman *et al.* (2003), which investigated the enhancing effect of TMC polymers having DQ between 12% and 59% on the permeation of the hydrophilic mannitol or PEG-4000 across Caco-2, showed a maximum value for a DQ 48% and the effects did not increase further with higher DQ. As discussed above, MW and DQ seem to affect the efficiency of chitosan and TMC particles. However, a systematic study on the effect of MW and DQ on the formation of chitosan and their respective TMC derivatives particles has not been reported. Therefore, in this study, the formation of particles prepared by ionic gelation method using chitosan with low (Chi-L), medium (Chi-M), and high (Chi-H) MWs and their TMC derivatives with various DQs was investigated. The amount of TPP added was varied in order to optimize the preparation condition of particles. Characteristics of particles obtained, loading efficiency of ovalbumin (OVA), a model antigen, and release profiles were also investigated in order to select the best systems for oral vaccine delivery.

2. Materials and Methods

2.1 Materials

In this study, three types of chitosan having low (Chi-L), medium (Chi-M) and high (Chi-H) MW were used. Chi-L (MW=160 kDa, degree of deacetylation (DD) = 96%) and Chi-H (MW=500 kDa, DD=96%) were obtained from Aqua Premier (Chonburi, Thailand). Chi-M (MW=270 kDa, DD= 93%) was received from Primex (Haugesund, Norway). A range of TMC with DQ approximately 20% (TMC-20), 40% (TMC-40), and 60% (TMC-60) was synthesized from three types of chitosan in our laboratory as previous described (Boonyo *et al.*, 2007). The resulting TMC polymers were named "TMC-MW-XX" according to the MW of starting chitosans and the DQ obtained. For example, TMC-L-20 is TMC synthesized from low MW chitosan (i.e. Chi-L) at DQ 20%. The DQ, average molecular weight, and intrinsic viscosities of the synthesized polymers were determined by ^{1}H-Nuclear magnetic resonance spectroscopy (^{1}H-NMR), gel permeation chromatography (GPC), and intrinsic viscometry. These characteristics are presented in Table 1. For the preparation of particles, glacial acetic acid was purchased from J.T. Baker (NJ, USA). Sodium tripolyphosphate pentabasic (TPP) and ovalbumin (grade V) were purchased from Sigma (MO, USA). Tween 80 was obtained from Srichand United Dispensary (Bangkok, Thailand).

2.2 Formation of particles

The particles of chitosan with different MW and their respective TMCs having various DQs were prepared by ionotropic gelation with TPP using the conditions optimized in a previous study (Boontha *et al.*, 2006). The effect of the amount of TPP on the formation of particles was investigated in this study. Briefly, chitosan particles were prepared by dissolving chitosan in 150 ml of 2% (v/v) acetic acid solution at 1% w/v. After stirring overnight, 2% w/v of Tween 80 was added to the chitosan solution. Then, 25 to 150 mg of TPP in 50 ml distilled water was added drop wise into the chitosan solution under stirring using the Mixer Heidolph® RZR 2101 (Diethelm, NY, USA.). Particle formation was identified visually and classified into three systems including solutions, suspensions, and gel precipitation. The formation of solutions and gel precipitation indicated that no particles were formed. The formation of suspensions indicated that the particles were formed, which is the desired system. Turbidity of the suspensions, indicating the number or size of particles formed was also visually observed and was given a score rating from '+' to '++++' indicating the amount of particles formed from less to more. TMC particles were prepared in the same manner except that TMC was dissolved in distilled water instead of acetic acid.

2.3 Characterization of suspension samples

The suspension samples only were subjected to characterization and the particle characteristics obtained from these samples are morphology, size, and zeta potential.

2.3.1 Particle size and zeta potential

The particle suspension obtained was centrifuged at 4°C by a high speed centrifuge (Beckman, CA, USA) at a speed of 18,000 rpm for 30 min. The particles were washed three times with distilled water and then the particle size and zeta potential was determined by using a photon correlation spectroscopy (PCS) Zetasizer (Model Zs90 nano, Malvern Instrument, UK) following by dispersing the sediment with distilled water.

2.3.2 Particle morphology

Following washing, particles were dispersed in distilled water. A drop of the particles suspension was placed

on a cover glass and allowed the water to evaporate completely. The samples were then coated with gold for viewing with a scanning electron microscope (SEM, LEO 1455VP, LEO Electron Microscopy Ltd, UK).

2.4 OVA loading efficiency

Loading of OVA onto the chitosan and TMC particles was performed by dispersing the washed empty particles in phosphate buffer solution (PBS; pH 7.4) containing OVA at a concentration of 0.3 mg/ml. The particle suspension was incubated at room temperature under magnetic stirring (300 rpm). Following stirring for 12 hrs, the particles were separated from the medium using centrifugation at speed 18,000 rpm for 30 min at 4°C and free OVA in the supernatant were quantified by the Micro Bicinchoninic Acid (BCA) assay (Pirece®, Rockford, IL, USA) using a microplate reader (Spectra count, Perkin Elmer, USA) at 540 nm. The amount of OVA adsorbed on the particles was expressed as loading efficacy (LE) and loading capacity (LC). The calculation was as follows:

$$\%LE = \frac{Total\ amount\ of\ OVA\ added - Amount\ of\ OVA\ remained\ \ in\ \ supernatant}{Total\ amount\ of\ OVA\ added} \bullet 100 \quad (1)$$

$$\%LC = \frac{Total\ amount\ of\ OVA\ \ added - Amount\ of\ OVA\ remained\ \ in\ \ supernatant}{Weight\ of\ \ particels} \bullet 100 \quad (2)$$

2.5 OVA release profiles

OVA-loaded chitosan and TMC particles were dispersed in 20 ml PBS at pH 6.8 and transferred to a double-wall beaker with controlled temperatures at 37°C, under stirring at 200 rpm. At time 0, 0.08, 0.25, 0.5, 1, 3, and 6 hrs, 400 µl samples of the dispersion were withdrawn and centrifuged at 10,000 rpm for 10 min at 25 °C. The OVA released in the supernatant was determined by the Micro BCA protein assay using a microplate reader with a 540 nm filter.

3. Results and Discussion

According to the characteristics of the polymers obtained in Table 1, using the same type of chitosan, it was found that the MW of the synthesized TMCs was lower than that of their starting chitosan and it decreased with increasing DQ. The results obtained may be explained by the degradation of the polymer backbone in the synthesis step. During the methylation process, the MW of the polymer chain increased due to the addition of methyl groups to the amino group of repeating monomers. However, the decrease in the MW of the TMC synthesized following the methylation process is due to the degradation of the polymer backbone caused by the exposure to the strong basic environment and elevated temperature during the synthesis of the TMC (Hamman and Kotze, 2001; Snyman *et al.*, 2002). Hence, the increase of MW by the methylation process is overcompensated by the degradation of the polymer backbone.

Table 1. Summary of the characteristics of the synthesized TMC derivatives.

Product Code	DQ (%)	MW (kDa)
Chi-L	-	160
TMC-L-20	18.34 ± 2.60	130
TMC-L-40	37.84 ± 0.91	110
TMC-L-60	62.99 ± 5.70	85
Chi-M	-	270
TMC-M-20	21.30 ± 0.59	110
TMC-M-40	40.05 ± 2.31	81
TMC-M-60	62.80 ± 3.68	79
Chi-H	-	500
TMC-H-20	16.33 ± 1.65	260
TMC-H-40	37.75 ± 4.27	200
TMC-H-60	62.26 ± 3.09	180

3.1 Formation of particles

As shown in Table 2, particles prepared using Chi-L, Chi-M, Chi-H, and their respective TMC-20 and TMC-40 synthesized analogues were formed at a wide range of TPP amount added. On the contrary, no particles were observed when using TMC-60. Using TMC-20 and TMC-40, particles could be formed with lower amount of TPP added compared with the particles prepared from their starting chitosan. For example, the lowest amount of TPP needed for the particle formation when using Chi-L, TMC-L-20, and TMC-L-40 were 50, 30 and 30 mg, respectively. This may be explained by the different conformation of these polymers in solution. Chitosan was found to be in an extended conformation whereas TMC was found to be coiling up into compact globules (data not shown). This is due to the fact that in an acetic acid solution the amino groups of chitosan are protonated resulting in a polyelectrolyte characteristic. The electrostatic repulsion between the positive charges along the chitosan chain may affect the flexibility of the backbone. Thus, the chain will tend to adopt an extended conformation. Following addition of TPP into the chitosan solution, TPP interacts with the chitosan chain by ionic interaction between the negatively charged ($-P_3O_{10}^{5-}$) of TPP and positively charged ammonium groups ($-N^+H_3$) of chitosan. As the charge reduces, the chitosan chains start to fold, resulting in the formation of particles. Thus at an amount of TPP less than 50 mg where chitosan particles were not formed, we assume that the amount of TPP added is not sufficient to suppress the electrostatic repulsion along the polymer chains. On the other hand, the morphology of TMC in solution was observed to be a condensed conformation. The spherical structure of TMC in an aqueous medium is confirmed by the value of Mark-Houwink ex-

Table 2. Effect of the amount of TPP added on particles formation.

Product code	Amount of TPP added (mg) (weight ratio of polymer to TPP)					
	150(1:1)	75(2:1)	50(3:1)	37.5(4:1)	30(5:1)	25(6:1)
Chi-L	++++	++	+	-	-	-
TMC-L-20	+++	+++	+++	+++	++	-
TMC-L-40	+++	+++	+++	+++	+	-
TMC-L-60	Gel	Gel	Gel	Gel	Gel	-
Chi-M	++++	++	+	-	-	-
TMC-M-20	+++	+++	+++	+++	++	+
TMC-M-40	Gel	Gel	Gel	++	-	-
TMC-M-60	Gel	Gel	Gel	+	-	-
Chi-H	++++	+++	+++	-	-	-
TMC-H-20	+++	+++	+++	++	+	-
TMC-H-40	+++	++	++	++	-	-
TMC-H-60	Gel	Gel	Gel	Gel	-	-

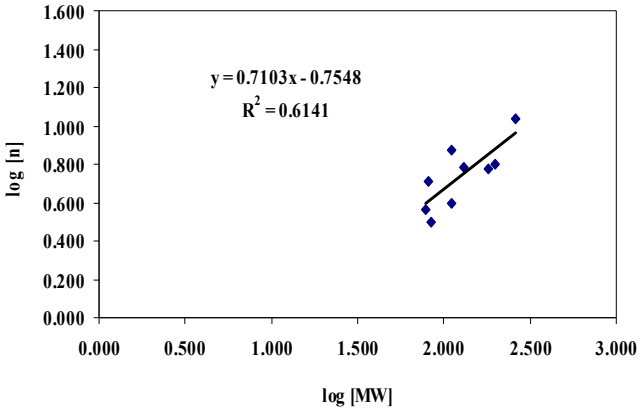
Note: Particles formed = '+' to '++++' means turbidity low to high; solution = (-); Gel = Gel precipitation.

ponent (a) which was calculated using Mark-Houwink Equation as followed:

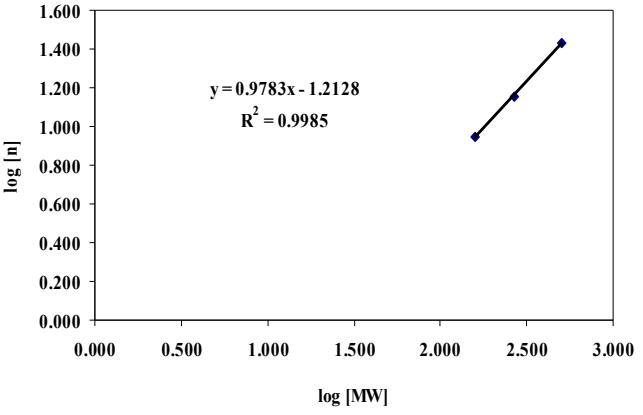
$$[\eta] = KM_V^a \quad (3)$$

where $[\eta]$ is the intrinsic viscosity, K and a are constants and Mark-Houwink parameters depending on the nature of the polymer and solvent as well as on temperature, and M_V is the so-called viscosity-average molecular weight, which can be substituted with the weight-average molecular weight. According to the equation, the exponent a is used as a parameter to determine the conformation of a polymer and polymers with exponent a values of 0, 0.5-0.8 and 1.8 have the shape of a sphere, random coil and rod, respectively (Tsaih *et al.*, 1997; Chen *et al.*, 1998). In this study, the value of a is obtained from the slope of the curve plotted against log $[\eta]$ and log [MW]. TMC has an a value of 0.71 (Figure 1a) whereas chitosan has a value of 0.98 (Figure 1b) indicating that the conformation of TMC particles is more compact. This may be due to the steric effect of pendent methyl groups which minimized electrostatic repulsion forces of the protonated amino groups of TMC (Jintapattanakit *et al.*, 2008). Moreover, the hydrophobic interaction of the methyl groups may cause cohesive force, which forces the polymer chains to coil up into compact globules (Song *et al.*, 2002). Therefore, TMC is able to form particle easier than chitosan and thus the amount of TPP required for the preparation of TMC particles is lower than that required for the preparation of chitosan particles.

Using TMC-60, only gel precipitation was observed instead of the formation of a particles suspension. This may be explained by the higher water solubility of TMC-60 (Jintapattanakit *et al.*, 2008). Following the addition of TPP,



(a)



(b)

Figure 1. Relation of log[n] and log [MW] for TMC (a) and chitosan (b) samples.

ionic interaction between TMC-60 and TPP occurred rapidly and hence some amount of water was trapped into the intra- and/or intermolecular of TMC-60 resulting in gel formation. Using the same type of polymer, the turbidity of the suspension decreased with decreasing the amount of TPP added (Table 2). This may be due to the decrease of ionic crosslinking that can interact with lower numbers of polymer chain, resulting in lower number or smaller size of particles.

3.2. Characterization of suspension samples

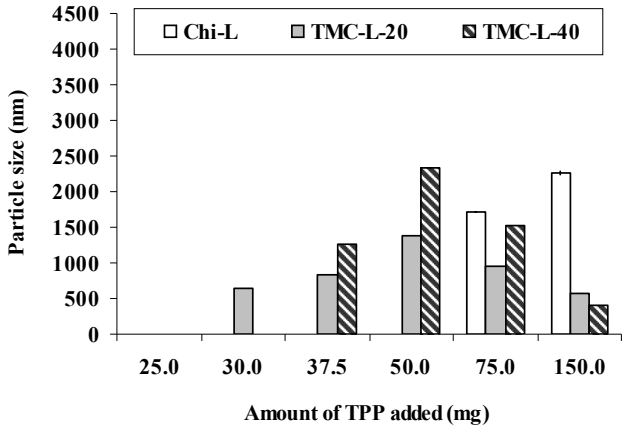
3.2.1 Particle size and zeta potential

The size of the particles prepared using chitosan and their TMC-20 and TMC-40 derivatives increased with increasing amount of TPP added as shown in Figure 2a-c. From these results, it can be estimated that a higher amount of TPP may interact with a higher number of polymer chains, resulting in larger particle size. Using the same amount of TPP, the particles size using TMC was smaller than that prepared using their starting chitosan. Using the same type of starting chitosan, the size of TMC particles decreased with increasing DQ. MW of chitosan seems to have no effect on the particle size.

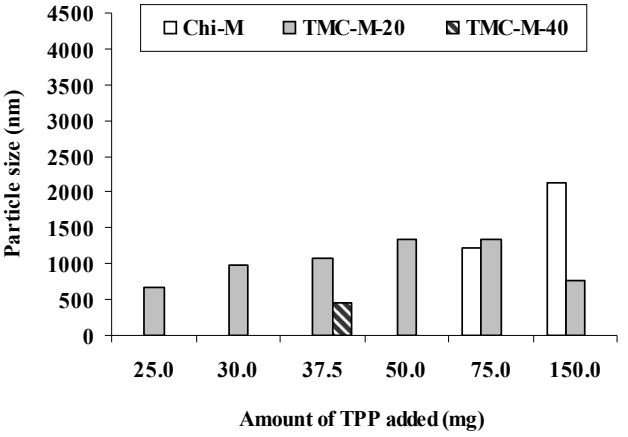
For determination of zeta potential, particles prepared using three types of chitosan have a similar positively charge when using the same amount of TPP. Thus, one can state that the zeta potential of the chitosan particles is not affected by the MW in the studied range. The surface charge of TMC-20 and TMC-40 particles is higher than that of its starting chitosan particles as shown in Figure 3a-c. These results are in agreement the measurement of charge of these polymers dissolved in solution at various pH values (Figure 4a-c). At a pH of about 6 where the measurement of zeta potential of particles was performed in this study, it was found that the zeta potential of chitosan is lower than that of TMC-20 and TMC-40, respectively. The addition of negatively charged TPP in the preparation of particles resulted in the reduction of the zeta potential of chitosan and their respective TMC particles compared to their solution form. Using Chi-L, Chi-M, and Chi-H, the zeta potential values of particles prepared obviously decreased with increasing amount of TPP added. However, the zeta potential of particles prepared using TMC-20 and TMC-40 was not changed as shown in Figure 3a-c. As discussed above, this may be due to the more compact conformation of TMC in an aqueous medium and thus TMC particles can be formed with lower amounts of TPP compared to chitosan particles, resulting in a slight reduction of the positively surface charge of the TMC particles.

3.2.2 Particle morphology

The optimum amount of TPP at 150 mg polymer was chosen to prepare chitosan and TMC particles for morphology determination. Particles from all formulations have a similar morphology and were polyhedrons in shape with a



(a)



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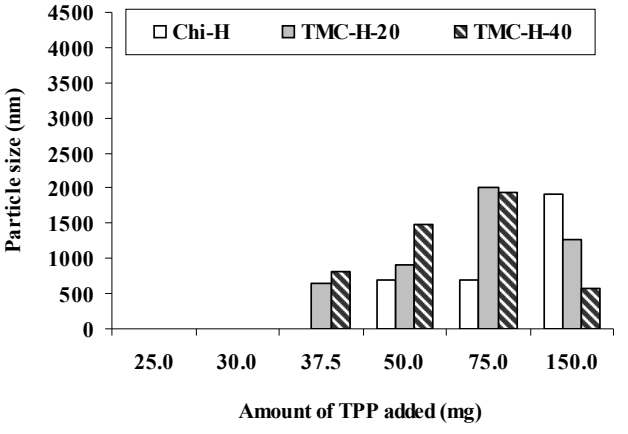
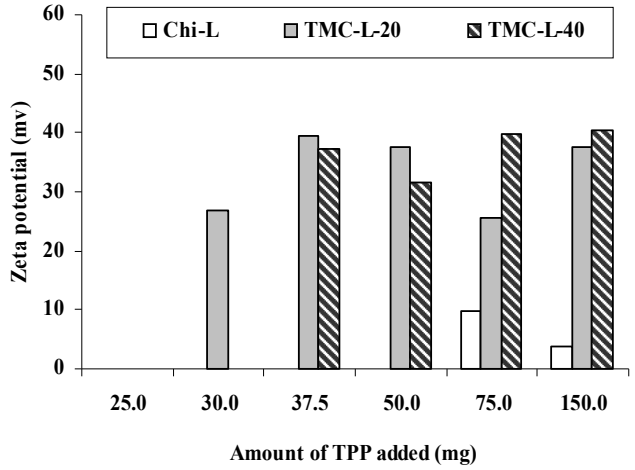
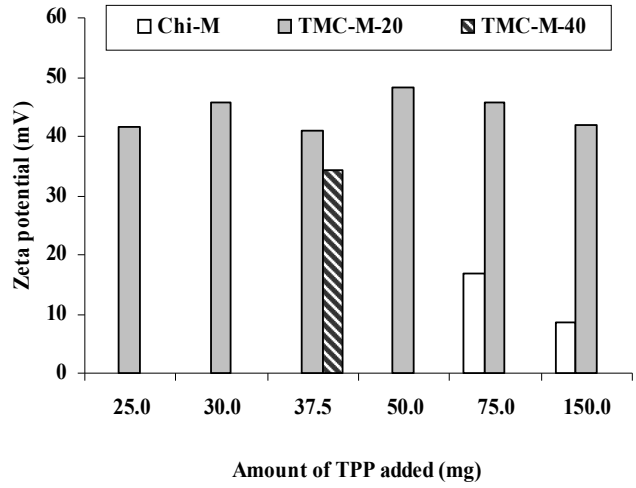


(c)

Figure 2. Effect of the amount of TPP added on the size of particles prepared using chitosan having low (a), medium (b) and high (c) MW and their respective synthesized TMC-20 and TMC-40 derivatives (n=2).



(a)



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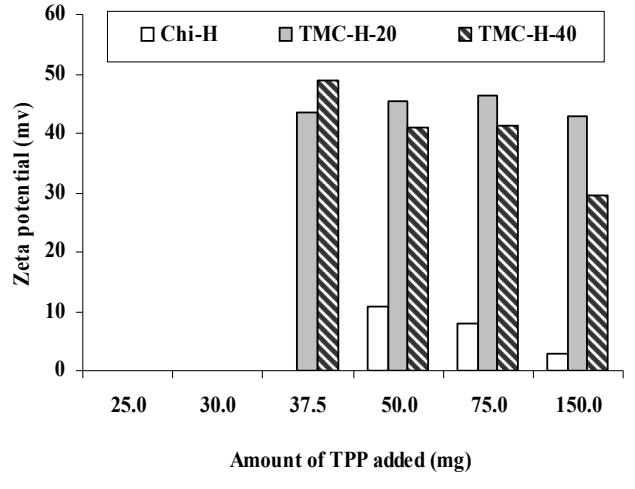
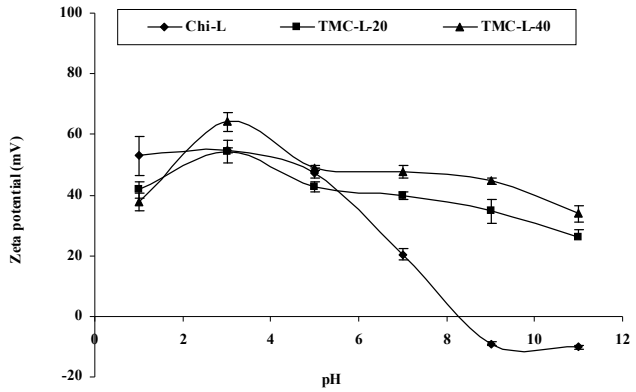
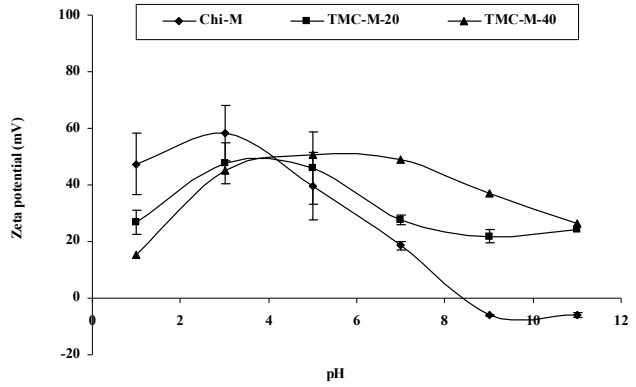


(c)

Figure 3. Effect of the amount of TPP added on the surface charge of particles prepared using chitosan having low (a), medium (b), and high (c) MW and their respective synthesized TMC-20 and TMC-40 derivatives (n=2).



(a)



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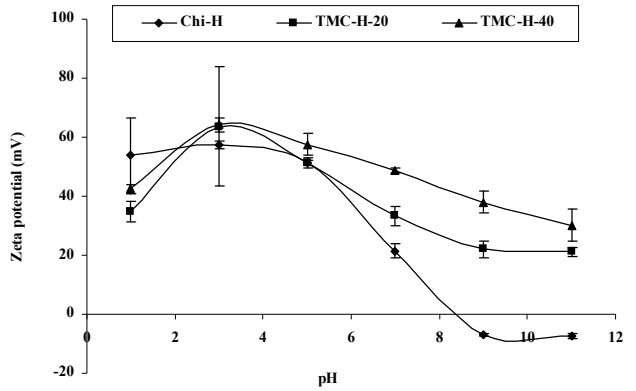


(c)

Figure 4. Effect of pH on the zeta potential of chitosan having low (a), medium (b), and high (c) MW and their respective synthesized TMC-20 and TMC-40. The zeta potential is expressed as mean ± S.D. (n=3).

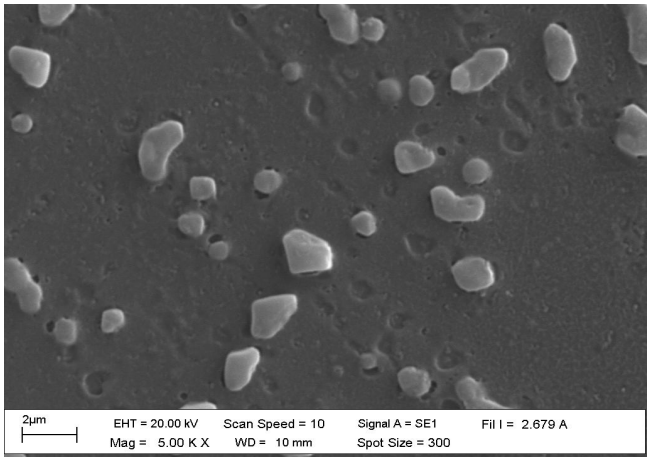


Figure 5. Scanning electron microscope image of empty chitosan particles prepared using Chi-L.

smooth surface as an example shown in Figure 5.

3.3 OVA loading efficiency and loading capacity

Based on the formation of particles and high particle yield, the amount of TPP added at 150 mg polymer was chosen to prepare empty chitosan and TMC particles for loading with OVA. As shown in Figure 6a, particles prepared using the TMC derivatives showed to have higher LE than the particles prepared using its respective starting chitosan. LE of TMC-40 particles was higher than that of TMC–20 particles. Similar results were obtained for the determination of LC (Figure 6b). These results can be explained by the fact that chitosan particles were incubated with OVA in PBS at pH 7.4 and at this pH the amino group on the surface of chitosan particles was deprotonated whereas TMC particles have a permanent positively charge. Therefore, chitosan particles have a lower available positive charge than theirs respective TMC particles, resulting in lower ionic interaction with OVA. Particles prepared using three types of chitosan have a similar loading content of OVA. This can be explained that OVA was loaded by the adsorption method and therefore the LE of particles prepared depended on the interaction between the negative charge of OVA and the positive charge of chitosan particles. The empty particles prepared using different types of chitosan have the same particle surface charge as described above. Thus, MW of chitosan seems to have no effect on LE and LC of the particle prepared.

3.4 OVA release profiles

For the release profiles as shown in Figure 7a-c, an initial burst released of OVA from all particles prepared using chitosan and their synthesized TMC-20 and TMC-40 derivatives was found. Using the same type of starting chitosan, particles prepared using TMC-40 released OVA quicker than those prepared using TMC-20 and the respective starting

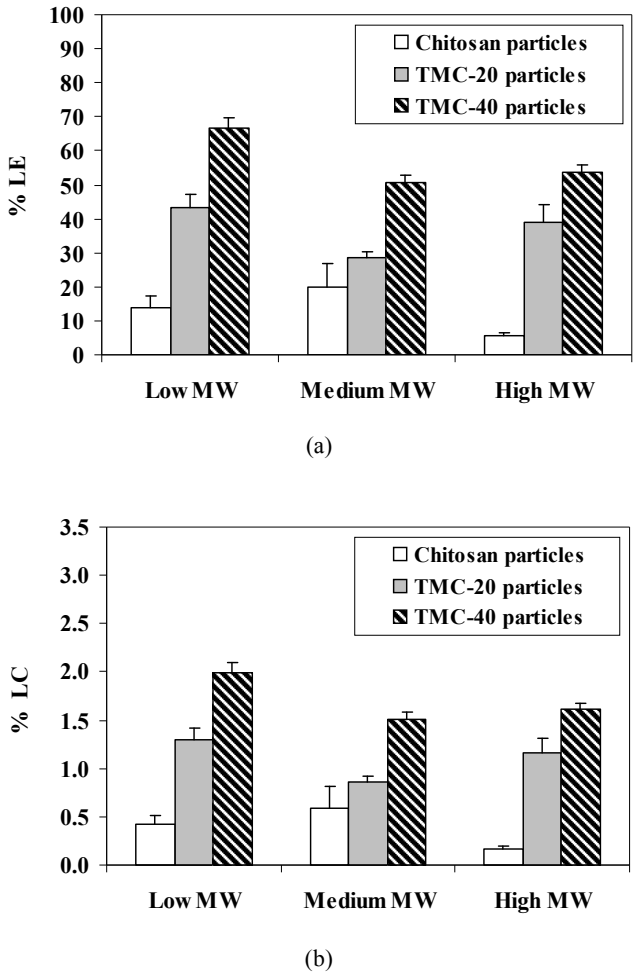
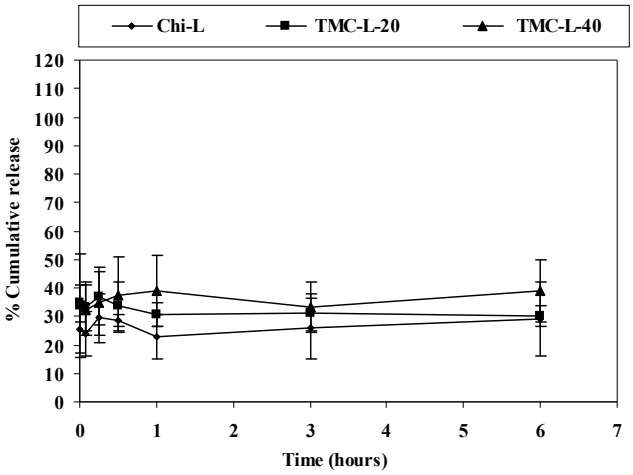


Figure 6. Loading efficiency (a) and loading capacity (b) of OVA on particles prepared using chitosan having low, medium, and high MW and their respective synthesized TMC-20 and TMC-40 derivatives. The LE and LC are expressed as mean ± S.D. (n=3).

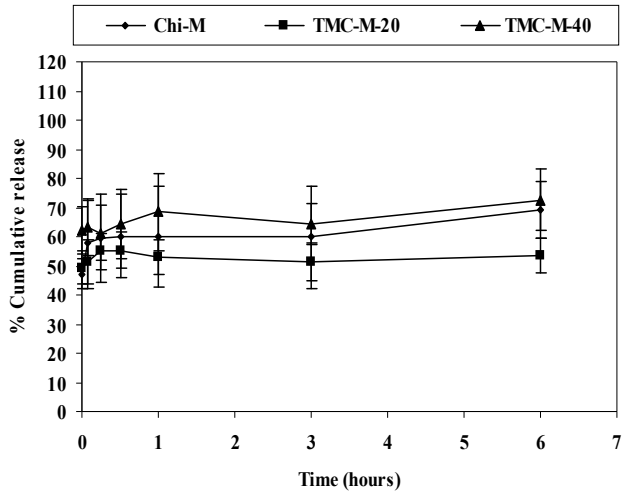
chitosan. This may be due to the fact that TMC-40 and TMC-20 have a higher solubility than their starting chitosan. Moreover, the LC of the particles prepared with TMC-40 was higher than that of particles prepared using TMC-20 and their respective starting chitosans. The high LC may cause a high concentration gradient between the surface of the particles and the release medium resulting in a high release rate. Using three types of chitosans, the particles prepared released OVA with the same rate. This may be due to the fact that the particles prepared using three types of chitosans have a similar loading content of OVA. Thus, there was no distinct difference on the release rate of OVA from the particles.

4. Conclusions

The amount of TPP added has an effect on the ability of chitosan and TMC polymer to form particles. Particles



(a)



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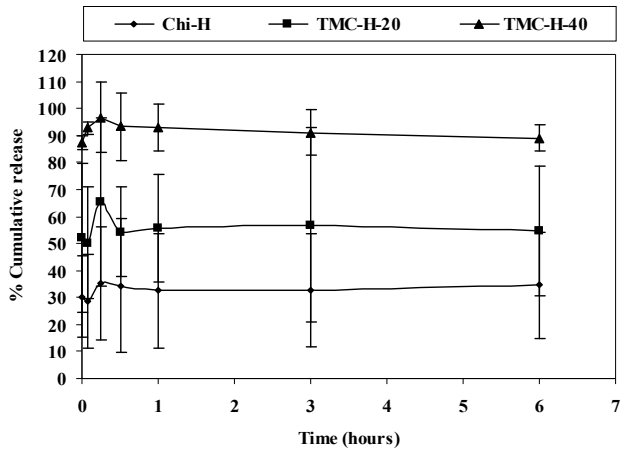

(c)

Figure 7. Release profiles of OVA from particles prepared using chitosan having low (a), medium (b), and high (c) MW and their respective synthesized TMC-20 and TMC-40 derivatives. The amount of OVA released is expressed as mean ± S.D. (n=3).

were not formed when an insufficient amount of TPP was added. Using the same amount of TPP added the MW of chitosan did not have an effect on the particles formation whereas the DQ of TMC did. Comparing between the two types of polymers, TMC could form particles with a lower amount of TPP than chitosan due to the more compact conformation of TMC in solution. Size, surface charge, and LE of the particles prepared were affected by the type of polymer (i.e. chitosan or TMC), amount of TPP added, and DQ of TMC. The differences in the release profiles of OVA were the consequences of the solubility and loading capacity of the particles prepared. For future investigations, these particles will be chosen to study the ability for immune induction *in vivo*.

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