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Preparation and Characterization of Trimethyl Chitosan Nanospheres Encapsulated with Tetanus Toxoid for Nasal Immunization Studies

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ABSTRACT

Background: Mucosal immunization requires potent delivery systems and/or adjuvants to result in protective immunity. Polymeric nanospheres could be used as a delivery system and adjuvant for antigens. These nanospheres could improve the interaction of antigens and antigen presenting cells (APCs) and increase their immune stimulation potentials. Here we report on the preparation of trimethylated chitosan (TMC) nanospheres loaded with tetanus toxoid (TT). At the present study TMC was synthesized and its nanospheres encapsulated with TT were prepared and characterized for nasal immunization. ***Methods*:** At the present study TMC was synthesized by a two-step method. TMC nanospheres encapsulated with TT were prepared by ionic gelation method. Nanospheres were studied by Dynamic Light Scattering (DLS) and SEM for size and morphology.andThe zeta potential were determined by Zetasizer.. Loading of TT was studied by BCA protein assay, release profile was studied for 4 h in 37 ^{o}C. Stability of loaded TT was evaluated by SDS-PAGE method. ***Results:*** Percent of quaternization for TMC was found to be 50.4 ± 10.4%. Mean diameter of blank and loaded nanospheres was 243.2 ± 35.1 nm and 295 ± 48.5 nm, respectively. Encapsulation efficiency of TT in nanospheres was 60.3 ± 12.7%. Percent of released TT after 0.5 h was 37.02 ± 27.63% and after 4 h reached to 86.19 ± 13.5%. Stability of encapsulated TT was confirmed by SDS-PAGE. ***Conclusion:*** The synthesized TMC polymer and its nanospheres loaded with TT showed desired characteristics as a mucosal antigen delivery system.

Introduction

Most of the present vaccines are administrated by injection because of their low stability in the gastrointestinal tract after oral administration and low absorption at mucosal sites.[1,2]

Obvious disadvantages of parenteral administration are the high production costs, low compliance of vaccine receivers, and need for trained personnel to administer the vaccines.[1,3] There is also the problem of inactivation of the proteins during storage and transport.[1,4]

Recently alternative methods of administration such as the mucosal routes, including nasal, oral and vaginal routes, have been tried to avoid such disadvantages.[4] Consequently, alternative routes of administration are being explored.[1,5,6] In particular, the nasal mucosa is an attractive site for the delivery of vaccines, because of its relatively large absorptive surface and low proteolytic activity.[1,7] Importantly, nasally administered vaccines can induce both local and systemic immune responses.[1,2,8] However, most proteins are not well absorbed from the nasal cavity if administered as simple solutions.[1]

Chitosan is the most abundant polysaccharide material for mucosal delivery system.[4] It has showed favorable biological properties, low toxicity and high susceptibility to biodegradation, mucoadhesive properties and an important capacity to enhance drug permeability and absorption at mucosal sites.[4,9] More importantly, chitosan nanoparticles can be spontaneously formed through ionic gelation using tripolyphosphate as the precipitating agent, so that the use of harmful organic solvent can be avoided during preparation and loading.[4]

Chitosan is able to open the tight junctions and in this way allows paracellular transport across the epithelium.[4,10,11] Both nasal and oral drug delivery researches have demonstrated that significantly higher amounts of macromolecular drugs can be transported after co-administration with chitosan.[11]

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In most of the studies, absorption enhancement was found only in acidic environment in which the pH was less or of the order of the pKa value of chitosan.[1,4,12] In contrast, N-trimethylchitosan chloride (TMC), a partially quaternized chitosan derivative, shows good water solubility over a wide pH range.[1,9] Hence, soluble TMC has mucoadhesive properties and excellent absorption enhancing effect even at neutral pH.[1,12] Moreover, TMC is an attractive alternative over chitosan for the design of protein loaded particles by ionic cross-linking.[1] Recently, nanoparticulate systems have been identified as suitable peptide/protein carriers.[13] In particular nanospheres are able to protect drugs from degradation, to improve permeation/penetration of the drugs across mucosal surface and also to control the release of the encapsulated or adsorbed drug.[13,14]

At the present study TMC polymer was synthesized and characterized. TMC nanospheres encapsulated with tetanus toxoid as an antigen delivery system and adjuvant were also prepared and their features were evaluated.

Materials and Methods

Materials

Chitosan (Fluka) (degree of deacetylation (DD) 95%, molecular weight 200 kDa) was used for synthesis of TMC. The tripolyphosphate (TPP), n-methyl-2-pyrrolidone (NMP), methyl iodide (MeI), sodium iodide (NaI), tween 80, sodium carbonate were purchased from Merck (Germany). Tetanus toxoid was provided by Razi Institute (Hesarak, Karaj). All other chemicals were of reagent grade and were used as received.

Synthesis and Characterization of TMC

As described previously, TMC was synthesized according to the method by Sieval et al[2,15] with slight modifications.[16] Briefly, 2 g of chitosan was dissolved in 80 ml of NMP and stirred in a 2-necked flask in a constant temperature water bath at 60 °C. The flask was connected to a condensation column. 11 ml of 15% NaOH solution was added to the flask and it was followed by addition of 11.5 ml of methyl iodide (as the methylation agent) and 4.8 g of NaI. The mixture was stirred for 75 min and precipitated with addition of 200 ml of ethanol, centrifuged, washed with acetone on a sintered glass filter and dried. In the second stage, 80 ml of NMP was added to the precipitate (mainly dimethyl chitosan iodide) and the mixture was stirred at 60 °C. Then 11 ml of 15% NaOH, 7 ml of methyl iodide and 4.8 g NaI were added successively and the mixture was stirred for 30 min. An additional 2 ml of methyl iodide and 0.6 g pellets of NaOH were added and stirring was continued for 1 h. This mixture was precipitated with 200 ml of ethanol, centrifuged and the solid substance was filtered on a sintered glass filter and washed with acetone to obtain a powdery substance, which is trimethyl chitosan iodide. To exchange iodide with chloride, trimethyl chitosan iodide was dissolved in 40 ml solution of 10% NaCl. The solution was precipitated with 200 ml of ethanol. Excess ethanol should be avoided because it will also precipitate NaCl. The mixture was centrifuged and the supernatant containing the excess NaCl was completely removed. The precipitate, which was TMC, was filtered and washed with acetone, dried and milled to obtain an off-white water-soluble powder. To determine the degree of quaternization (% DQ), 1H NMR spectrum of TMC was measured in D2O, using a 600 MHz spectrometer (Bruker-Biospin, Rheinstetten, Germany) at 80 °C. The %DQ was calculated from Eq. (1):

$$DQ= [[(CH3)3 / [H] \times 1/9] \times 100 \quad (1)$$

DQ is the degree of quaternization, in mole percentage of free amine, [(CH3)3] is the integral of chemical shift of the hydrogens of trimethyl amino groups at 3.3 ppm,; [H] is the integral of H-1 peaks between 4.7 and 5.7 ppm, related to hydrogen atoms bound to carbon 1 of the chitosan molecule, which is taken as the reference signal.[2]

Preparation of TMC Nanospheres

According to previous studies, formation of nanospheres in concentrations more than 3 mg/ml of TMC has been difficult, and resulted in unstable suspension.[17] In this study, TMC with 2 mg/ml concentration and TPP with 1 mg/ml concentration were used. During preparation, Tween 80 (1% w/v) was added to TMC solution. Tween 80 as a suspending agent and surfactant could increase the stability of nanospheres suspension and prevent the aggregation of nanospheres during gelation step.[18,19]

The TMC nanospheres were prepared by ionic gelation of TMC with tripolyphosphate (TPP) polyanion. Briefly, 10 mg of TMC was dissolved in 5 ml of distilled water. Subsequently, 2 ml of TPP solution (1 mg/ml) was added dropwise to the above solution under magnetic stirring at room temperature and in the presence of Tween 80% (1% V/V) to prevent particle aggregation.[4,13,19] For loading of tetanus toxoid (TT) in nanospheres, TT was added to TPP solution. After 5 min stirring, nanospheres were separated by centrifugation at 16000g for 40 min.

Physicochemical Characterization of TMC Nanospheres

Particle Size, Zeta Potential

The size and zeta potential of the TMC nanospheres were measured by differential laser scanning (DLS) method (Malvern zetasizer Nano ZS, UK).

Morphology

Morphological examination of the nanoparticles was performed by scanning electron microscopy (SEM) (Hitachi H-600, Japan).

Encapsulation Efficiency and Loading of Tetanus Toxoid in Nanospheres

The Encapsulation efficiency and loading of the TT loaded in TMC nanospheres were determined by separating the nanospheres from the preparation media, by centrifugation at 16000g for 40 min.[20, 21] The amount of free protein in the supernatant was measured by micro-BCA protein assay method.[22] The supernatant of blank TMC nanospheres was used as blank. The encapsulation efficiency (EE) was calculated from standard curve and percent of EE was calculated from Eq. (2):

$$EE\% = (TT_{entrapment} / TT_{total}) \times 100 \quad (2)$$

Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The fragmentation and aggregation of loaded TT was evaluated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method. Tetanus toxoid-loaded TMC nanospheres were destabilized by adding 1 ml of 10% (w/v) NaCl to 6.8 ml of nanoparticle suspension, resulting in a solution with a protein concentration of about 0.3 mg/ml. The dissolved antigen was run on a 7.5% SDS-polyacrylamide gel. The protein bands were visualized by silver nitrate staining.

In Vitro Release Profile of Antigen from TMC Nanospheres

30 mg of TT-loaded nanospheres were suspended in 1 ml of PBS pH 7.4 and incubated in 37 °C under continuous shaking. Each 30 min until 4 h, the suspensions were centrifuged in 13000 rpm for 15 min and 900 µl of supernatants were drawn and replaced with fresh PBS buffer. The amount of released TT in supernatant was determined by micro-BCA protein assay method. The experiments were performed in triplicate.[23] After the entire drug payload was released at pH 7.4, the percent of the TT was reported.

Statistical Analysis

The Student t test was used for comparisons. P values smaller than 0.05 were considered as significant.

Results

NMR Characterization of Synthesized TMC and Degree of Quaternization

TMC was synthesized based on reaction of chitosan with CH_3I.[2] NMR spectroscopy was used to determine the degree of quaternization (percent of trimethylated amine groups) of synthesized TMC.[1,2,23] Figure 1 shows the H-NMR spectra of TMC. The signal of 3.3 ppm is attributed to the (N(CH3)3) group together with a smaller peak at 5.2 ppm assigned to the (N(CH3)2) group.[23] According to the peak assignment and intensity, the degree of quaternization was calculated to be 50.45 ± 10.45%.

Characterization of TMC Nanospheres

Figure 2 shows the TMC nanospheres encapsulated with TT.

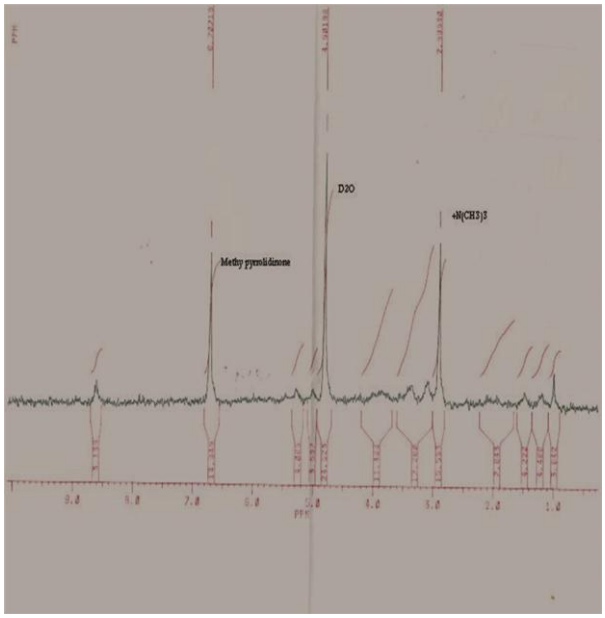

Figure 1. H NMR spectrum of N-trimethylchitosan chloride after a two step synthesis

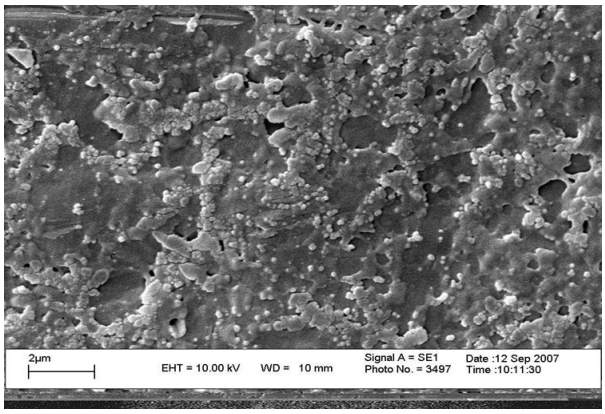

Figure 2. SEM image of TT-loaded TMC nanospheres.

The size and zeta potential of TMC nanospheres were determined using a Malvern zetasizer (Table 1). Both blank and TT-loaded nanospheres had a similar diameters of 250-300 nm ($P>0.05$). After centrifugation, the mean diameters of both kinds of nanospheres were significantly increased to about 700 nm ($P<0.01$).

Table 1. Mean diameter of TMC nanospheres before and after centrifugation

Mean diameter (nm)	Before centrifugation	After centrifugation
Blank TMC nanospheres	243±35	676±43
TT- loaded TMC nanospheres	295±48	793±53

Encapsulation efficiency of TT was calculated as 60 ± 12%. This high encapsulation efficiency could be attributed to positive charge of TMC and negative charge of TT. It seems that electrostatic interactions have a positive effect on TT loading.

The stability problem has been reported for tetanus toxoid in acidic conditions during preparation of chitosan nanoparticles.[24] At the present study SDS-PAGE results indicated that the integrity of TT has been maintained during preparation (Figure 3).

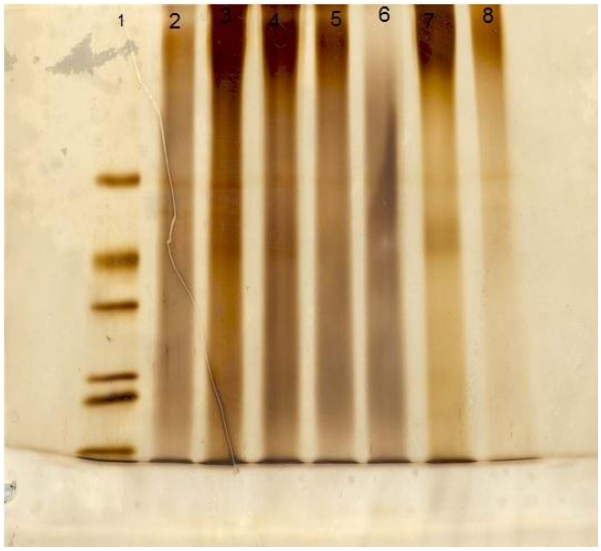

Figure 3. SDS-PAGE gel. 1. Standard protein, 2. Supernatant of TT loaded nanospheres, 3-5. TT loaded nanospheres, 6. Blank nanospheres, 7-8. Original TT

In Vitro Release of TT from TMC Nanospheres

As it is seen in figure 4, the amount of released TT in the first 0.5 h of study was about 25%. Nearly all of encapsulated TT has been released within 2 hours. After 2 hours, the graph reached to plateau.

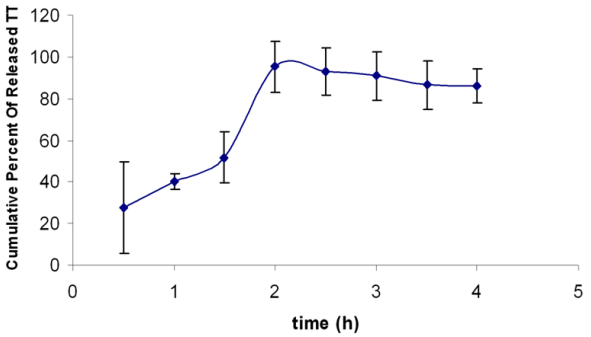

Figure 4. Release profile of TT from TMC nanospheres. The TT-loaded nanospheres were suspended in 1 ml of distilled water and incubated in 37 ºC under continuous shaking. The amount of released TT in supernatant was determined by micro-BCA protein assay method each 30 min until 4 h (error bars represent the SD, n=3).

Discussion

At the present study the trimethylated derivative of chitosan (TMC) was synthesized to increase its water solubility. TT loaded TMC nanospheres were prepared and characterized as a mucosal vaccine delivery system.

The semi-synthetic N- trimethyl chitosan chloride (TMC) polymer has several advantages over chitosan polymer of them could be referred to desirable solubility and easy preparation.[23] There are three methods to synthesize TMC: one step method, two step method, and three step method. In two step method, the minimum degree of quaternization is 40% and water solubility of polymer is optimal. In one step method, both quaternization and water solubility are low. In three step method the resultant polymer has a higher quaternization degree (more than 80) due to complete O-methylation; but its water solubility decreases due to decrease of hydrogen bonds with water.[23] Therefore, in this study the two step method was used for preparation of TMC. The synthesized polymer had a quaternization degree of 50.45 ± 10.39%.

In the first steps, the synthesized TMC by two step method did not have desirable water solubility. This problem was due to residue of methyl pyrolidone in polymer chain. The problem was resolved by repeated washing of synthesized polymer with acetone.

According to previous studies, higher rate of encapsulation is seen in TMC with lower degree of quaternization. Higher quaternization degree could lead to decreased particle size and zeta potential.[4]

The ionic gelation method was used for preparation of TMC nanospheres. Nanospheres were fabricated through ionic interactions between TPP (polyanion) and quaternary amine groups of TMC (cation). This method has several advantages including delete of organic solvents, high efficacy and high encapsulation rate.[21]

For particulate antigen delivery systems, the particle size is an important factor in their interaction with antigen presenting cells and also their uptake from epithelial surfaces. According to data obtained from particle size analyzer (PSA), the mean diameter of blank nanospheres and TT loaded nanospheres was 243.1 ± 35.1 and 295 ± 48.5 nm, respectively. This small size of nanospheres is desirable. Formation of small nanospheres can be due to using of ionic gelation method. This method produces smaller nanospheres than other methods.[23] In this study encapsulation efficiency of TT was calculated as 60.3 ± 12.7%, which is an acceptable loading. In previous studies, proteins such as BSA and TT have been encapsulated better, when dissolved in TPP solution. Usually based on net charge of encapsulate, it will be added to chitosan solution (for positively charged encapsulates) or to TPP solution (for negatively charged encapsulates). In physiologic pH, TT has a net negative charge and therefore was added to TPP solution.[18]

One of the important features of particulate delivery systems like nanospheres and microspheres is the rate and profile of the release. At the present study, to give particulate nature to TT solution, it was encapsulated

with TMC nanospheres. It has been shown in several studies that particulate antigens could better interact with APCs and also could be better uptaken by microfold cell of mucosa associated lymphoid tissues (MALT),[25,26] therefore it is preferred that most of antigen keep its particulate nature and the least release rate is the most optimum condition. In the release profile of TT from of TMC nanospheres (Figure 4), nearly all of encapsulated TT has been released within 2 hours. However, as a nasal antigen delivery system, based on the mucociliary clearance in nasal cavity, the normal residence time is very short.[27] Therefore, the nanospheres have a short stay in nasal cavity and during that time most of antigen will interact with M cells in particulate form.

Conclusion

TMC was successfully synthesized from chitosan by the two-step method. NMR spectrum of TMC showed 51% degree of quaternization. The synthesized TMC was used in the preparation of nanospheres loaded TT. TMC nanospheres loaded TT was prepared under mild conditions using TPP as a polyanion cross-linker. Stable TMC nanospheres with a small size and a narrow size distribution were obtained. The TMC nanospheres have an acceptable loading capacity for proteins, and a positive surface charge, suitable to attach to nasal mucosa. The integrity of the loaded antigen was preserved. In vitro studies confirmed that this system is capable of delivering the antigen with TMC to nasal mucosa. Further studies are underway to assess the in vivo feasibility of TMC nanospheres.

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