Enteric trimethyl chitosan nanoparticles containing hepatitis B surface antigen for oral delivery

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Oral vaccination is the preferred route of immunization. However, the degradative condition of the gastrointestinal tract and the higher molecular size of peptides pose major challenges in developing an effective oral vaccination system. One of the most excellent methods used in the development of oral vaccine delivery system relies on the entrapment of the antigen in polymeric nanoparticles. In this work, trimethyl chitosan (TMC) nanoparticles were fabricated using ionic gelation teqnique by interaction hydroxypropyl methylcellulose phthalate (HPMCP), a pH-sensitive polymer, with TMC and the utility of the particles in the oral delivery of hepatitis B surface antigen (HBsAg) was evaluated employing solutions that simulated gastric and intestinal conditions. The particle size, morphology, zeta potential, loading capacity, loading efficiency, in vitro release behavior, structure, and morphology of nanoparticles were evaluated, and the activity of the loaded antigen was assessed. Size of the optimized TMC/HPMCP nanoparticles and that of the antigen-loaded nanoparticles were 85 nm and 158 nm, respectively. Optimum loading capacity (76.75%) and loading efficiency (86.29%) were achieved at 300 µg/mL concentration of the antigen. SEM images revealed a spherical shape as well as a smooth and near-homogenous surface of nanoparticles. Results of the in vitro release studies showed that formulation with HPMCP improved the acid stability of the TMC nanoparticles as well as their capability to preserve the loaded HBsAg from gastric destruction. The antigen showed good activity both before and after loading. The results suggest that TMC/HPMCP nanoparticles could be used in the oral delivery of HBsAg vaccine.

Introduction

Infectious disease of hepatitis B (HB) is a worldwide social health complication, particularly in developing countries where approximately 90% of the total number of HBV-infected patients reside.[1] Chronic HB disease induces persistent hepatitis, liver cirrhosis, and hepatic malignancy. Approximately 30% of the world's population has been infected with HBV and 350 million are persistent carriers.[2,3] At present, no effective therapy exists for HBV infection. Therefore, preventive vaccination continues to be the most cost-effective solution for HBV-related disease control.[4,5]

Currently, HBV vaccination requires 3 administrations (0, 1, and 6 months) for eliciting effective responses. However, completion rate of this standard immunization protocols among adolescents is as low as 11% due to the poor compliance of the multi-dose protocol.[5] This problem is more severe in developing countries, which generally lack permanent clinics and healthcare professionals to effectively implement the protocol. This, in turn, exacerbates the already poor vaccination coverage in such areas.[4] Development of single-dose vaccine formulations could alleviate this problem by eliminating the need for follow-up visits to clinics. However, the development of hepatitis B surface antigen (HBsAg) vaccine as a single-dose form with programmed-release profiles, which can stimulate the immune system equipotential to the 3 doses of a common adjuvant (aluminum hydroxide) vaccine, is a challenging task.[6]

Oral vaccine delivery is advised to be the most advantageous and comfortable way of vaccination, since it eliminates the local side effects created via the repetitive injection. Vaccines have inherently low absorption from the intestinal tract due to their hydrophilicity and large molecular size. Additionally, following oral administration, vaccines are extremely nonresistant to enzymatic deterioration in the stomach and intestinal environment.[7]

Once entrapped in suitable carrier nanoparticles, antigens will be protected versus the degradative condition of the GI environment prior to their absorption in their intact form. Furthermore, using mucoadhesive polymers for the preparation of the delivery vehicle lengthens the association of the particles to the surface of mucosa while achieving the greater regional concentration of antigen in the mucus bed and minimizing dilution and destruction of antigen by the intestinal lumen contents.[7,8]

Chitosan polymer is derived from chitin by deacetylation. It is widely investigated for applications in pharmaceutical domains. However, limited solubility has prevented its widespread use.[9,10] The mucoadhesive and absorption-promoting properties of chitosan have been extensively evaluated. The positive charge gained in acidic pH conditions, in which chitosan has high solubility, appears to be effective for enhanced absorption.[11] With due attention to very low solubility of chitosan above pH 6.5, its application is limited to absorption facilitator in the body. Therefore, chitosan derivatives with enhanced solubility at basic and neutral pH are essential.[12-15] It has been shown that TMC, a quaternary derivative of chitosan, enhances penetration and opens epithelial tight junctions in the environments with basic and neutral pH.[14]

The gastric enzymes and acidic conditions pose challenges to the oral administration of vaccines. In the present study, hydroxypropyl methyl cellulose phthalate (HPMCP) was used as an enteric coating to preserve the vaccines from being destroyed by gastric acids and to prevent gastrointestinal side effects.[16,17] HPMCP is used in the formulation of enteric coated pharmaceutical products to protect drug ingredients from destruction by stomach acidic contents. The HPMCP free carboxyl groups have the pKa of about 5.2.[7,8] The charge of free carboxylic acid groups of this polymer is converted into negative at pH values of higher than 5.2 and serves as the enforcement for the electrostatic bond with TMC.[8]

The objectives of the present work was to prepare TMC/HPMCP nanoparticles using the ionic gelation technique by cross-linking pH-sensitive HPMCP polymer with TMC and to evaluate the utility of the nanoparticles in the oral delivery of HBsAg. In order to reach optimized nanoparticles, nanoparticles with acceptable characteristics, size of the particles, morphology, zeta potential, loading capacity, and loading efficiency of the nanoparticles were analyzed and the in vitro release profile and biological activity of the released antigen were evaluated.

Results

NMR characterization of TMC and degree of quaternization
NMR spectroscopy was used for determining the degree of quaternization (percent of trimethylated amine groups) of the
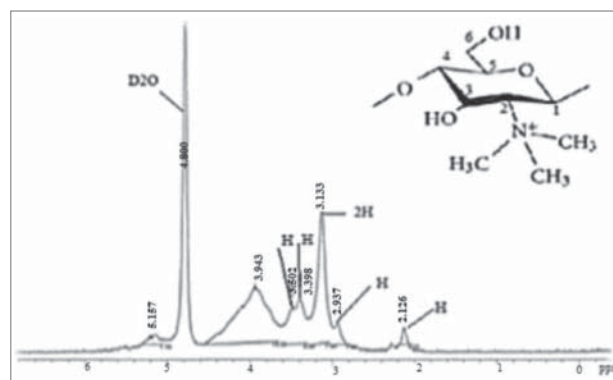

Figure 1. H- NMR spectrum of trimethyl chitosan.

synthesized TMC. This polymer's ^{1}H-NMR spectra is demonstrated in **Figure 1**. The peak at 3.3 ppm belonged to the $N(CH_3)_3$ group and a smaller signal at 5.2 ppm owning to the $N(CH_3)_2$ group. The degree of quaternization was estimated to be $50.95 \pm 9.85\%$ using the peak appointment and intensity.

Particle size and zeta potential
In the present study, to find optimum parameters, the effect of different TMC concentrations (1, 1.5, and 2 mg/mL) on nanoparticle formation was evaluated using the Zetasizer. As shown in **Table 1**, 1 mg/mL of TMC and HPMCP was observed as the suitable concentration for preparing optimized nanoparticles. On the other hand, effects of HBsAg's initial concentration (100, 300, 500, 700, and 1000 μg/mL) were evaluated on the characteristics of HBsAg-loaded nanoparticles at the optimized HPMCP and TMC concentration (1 mg/mL) (**Table 2**). The average particle size of the optimized empty and HBsAg-loaded TMC/HPMCP nanoparticles was approximately 85 nm and 158 nm, respectively. The PDI of TMC/HPMCP nanoparticles was 0.4 and that of the HBsAg-loaded nanoparticles was 0.3, which indicated narrow and favorable particle size distribution (PDI < 0.5). The present results demonstrated that, in optimum conditions, the zeta potentials of empty and HBsAg-loaded TMC/HPMCP nanoparticles were $+30.1 \pm 0.8$ mV and $+20 \pm 0.7$ mV, respectively. These results suggested that the loading of HBsAg led to a reduction in the zeta potential of the particles.

Effect of HBsAg concentration on loading capacity and loading efficiency
Effect of HBsAg's initial concentration (100, 300, 500, 700, and 1000 μg/mL) on loading efficiency and capacity was analyzed in optimized conditions (1 mg/mL concentration of TMC

Table 1. Formulations and properties of TMC/HPMCP nanoparticles, n = 3

Formulation	TMC mg/ml	HPMCP mg/ml	Mean particle Size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
F1	1	1	85 ± 10	0.40	+30.1 ± 0.8
F2	1.5	1	274 ± 19	0.50	+10 ± 0.5
F3	2	1	975 ± 29	0.80	+5.53 ± 0.8

Table 2. Formulations and characteristics of HBsAg-loaded TMC/HPMCP nanoparticles, n = 3

Formulation	TMC mg/ml	HPMCP mg/ml	HBsAg initial concentration (μg/ml)	Mean particle Size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
F4	1	1	100	132 ± 11	0.34	+25.3 ± 0.6
F5	1	1	300	158 ± 15	0.30	+20 ± 0.7
F6	1	1	500	180 ± 19	0.45	+19.33 ± 0.8
F7	1	1	700	242 ± 22	0.51	+18.55 ± 0.8
F8	1	1	1000	322 ± 31	0.62	+17.83 ± 0.9

and HPMCP) for preparing empty nanoparticles. As shown in **Figure 2**, optimum loading capacity (76.75%) and efficiency (86.29%) were obtained at 300 μg/mL initial concentration of the antigen.

FTIR spectroscopy

The FTIR spectra of TMC, TMC/HPMCP nanoparticles, and HBsAg-loaded TMC/HPMCP nanoparticles are shown in **Figure 3A,B,C**, respectively. The FTIR spectrum of TMC showed evidence of methylation, particularly in the 1,700–1,200 cm^{-1} region. A new peak appeared at the high wave number, 1631cm^{-1}, which was attributed to the quaternary ammonium salt. The strong, sharp peaks in 3450 cm^{-1} region and a peak at 2923 cm^{-1} were also observed.

The FT-IR spectra of TMC/HPMCP nanoparticles (**Fig. 3B**) was unlike those of the TMC polymer (**Fig. 3A**). In the spectrum of TMC/HPMCP nanoparticles, the tip of the peak at 3450 cm^{-1} was wider, which indicated enhanced hydrogen bonding. A new peak represented at 1735 cm^{-1} was attributed to C=O stretching. The FT-IR spectra were consistent with the modification of TMC film by HPMCP.

Figure 3C shows the FTIR spectrum of HBsAg-loaded TMC/HPMCP nanoparticles. The spectrum showed the characteristic peaks of the substituted secondary amide in the 3300–3400 cm^{-1} region. The peaks at 1145 cm^{-1} and 600 cm^{-1} were attributed to the C-S stretching vibrations of sulfides and disulfides.
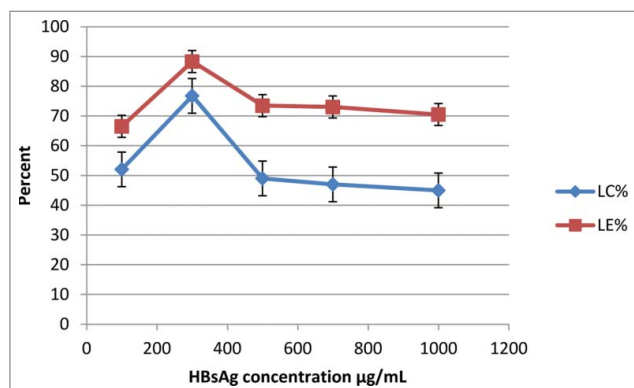
Morphology

Figure 4A,B demonstrate representative scanning electron micrographs of empty and HBsAg-loaded TMC/HPMCP nanoparticles, respectively. These particles were spherical and had a smooth surface.

In vitro antigen release

The profiles of antigen release, under simulated intestinal (pH 6.8) and stomach (pH 2) environments, from HBsAg-loaded TMC/HPMCP nanoparticles are shown in **Figure 5**. The amount of the antigen released from the nanoparticles after 6 h of incubation at pH 2 did not exceed 25% of the loaded protein. The particles showed good physical stability in gastric pH conditions and their size did not noticeably change. The present results showed that, under simulated intestinal conditions, 70% of the loaded antigen was released after 10 h.

Reactivity of HBsAg before and after loading

The antigen reactivity before and after loading was evaluated by Ouchterlony double immunodiffusion test (**Fig. 6A,B**). Sample of HBsAg before entrapment was showed sharp antigen-antibody precipitin line, but HBsAg released in gastric simulated condition and PBS did not demonstrate antigen antibody reaction (**Fig. 6A**). The ability of the TMC/HPMCP nanoparticles in terms of preserving the loaded HBsAg from the degradative gastric conditions is shown in **Figure 6B**. The results showed good precipitin lines and antigen-antibody reactivity for the samples before loading, antigen released in intestinal simulated condition, and antigen released in intestinal simulated condition after treatment in gastric simulated condition (**Fig. 6B**).

Discussion

TMC-based nano- and micro-particles have been broadly studied for their use in mucosal vaccination.[8] TMC particles prepared via chemical cross-linking can be potentially toxic and the nanoparticles could also chemically modify the entrapped proteins.[15] Therefore, physical techniques, similar to spray drying and ionic cross-linking, are considered relatively more suitable and conservative for protein. TMC nano- and micro-particles have been fabricated by ionic cross-linking with different polyanions, such as polyglutamic acid, TPP, sodium sulfate, dextran sulfate, and anionic cyclodextrins.[18] The acidic environment of the stomach and enzymes pose significant challenges to the oral administration of vaccines.
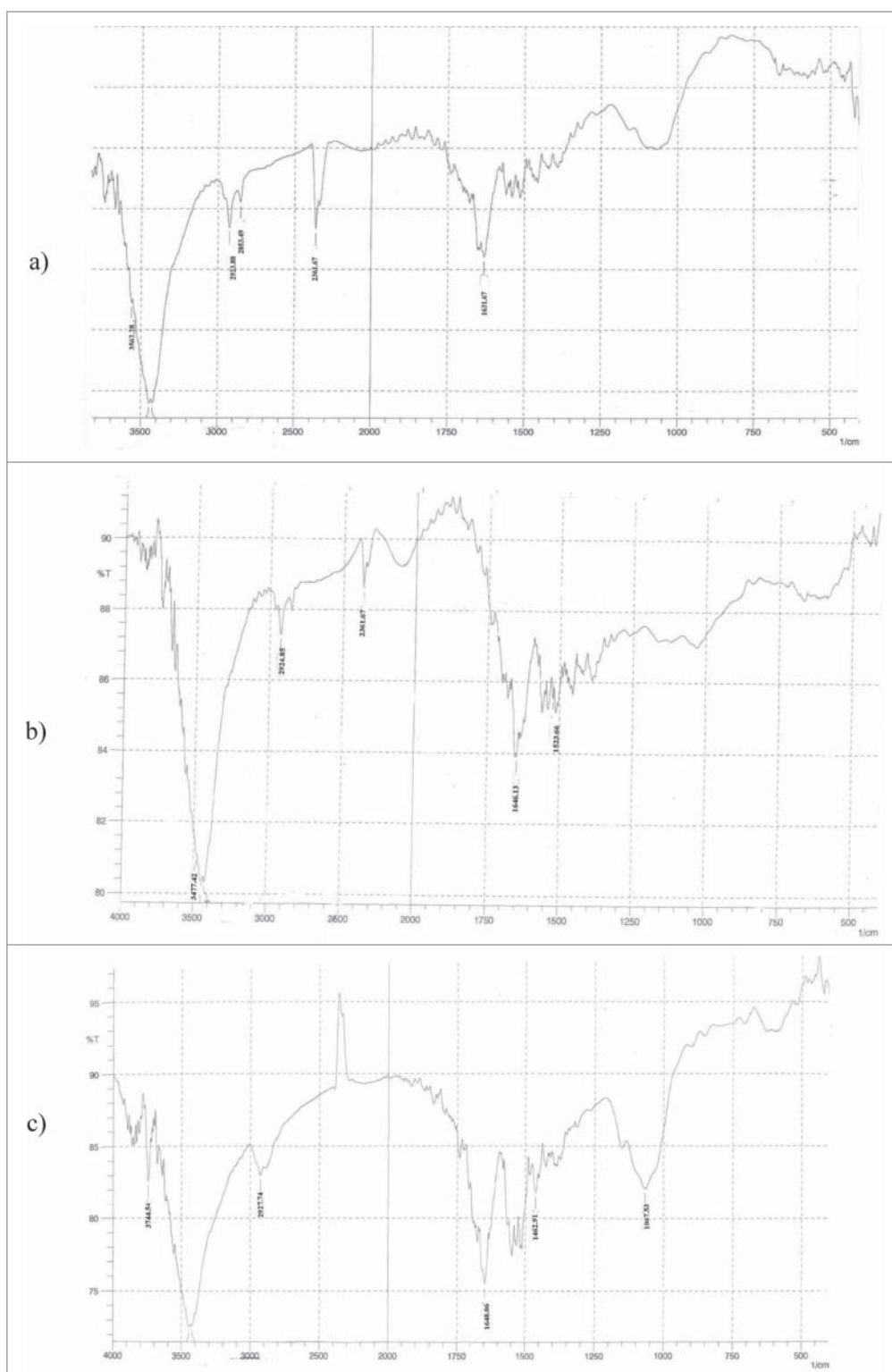

Figure 2. Effects of HBs Ag concentration (100, 300, 500, 700 and 1000 μg/mL) on the antigen loading efficiency (LE) and loading capacity (LC), (TMC 1mg/ml, HPMCP 1mg/ml), mean ± SD, n = 3.

Figure 3. FTIR spectrum of (**A**) trimethyl chitosan, (**B**) TMC/ HPMCP) nanoparticle and (**C**) TMC/ HPMCP nanoparticle containing HBs Ag (TMC 1 mg/ml, HPMCP 1 mg/ml, HBs Ag 300 μg/ml).

As shown in **Figure 1**, using the peak appointment and intensity, degree of quaternization was estimated to be be about 51%. The present results indicated that TMC was successfully synthesized and can be used for the preparation of nanoparticles.

In this work, the effect of different TMC concentrations (1, 1.5, and 2 mg/mL) on nanoparticle formation was assessed using the Zetasizer. The obtained results showed that, at constant HPMCP concentration (1 mg/mL), size of the nanoparticles increased with TMC concentration (**Table 1**). It appears that enhancing the TMC concentration increased the viscosity of the solution, which led to increased resistance against dispersion in the liquid phase and formation of larger particles. In the epithelial cells, smaller particles could be absorbed faster than the larger particles.

Results of the study on the influence of various initial concentrations of HBsAg on the characteristics of HBsAg-loaded nanoparticles are shown in **Table 2**. Accordingly, at constant HPMCP and TMC concentration (1 mg/mL), enhanced antigen concentration led to increased size and PDI of the HBsAg-entrapped nanoparticles. In this study, the optimal size of the empty nanoparticles (85 nm) and HBsAg-loaded nanoparticles (158 nm) was obtained at 1 mg/mL concentrations of both TMC and HPMCP.

The PDI of optimized TMC/ HPMCP nanoparticles was 0.4 and that of the HBsAg-loaded nanoparticles was 0.3, which indicated a narrow and favorable particle size distribution (PDI < 0.5). The present results demonstrated that, in optimum condi-

Therefore, in this study, HPMCP was used as the enteric coating agent for protecting HBs antigen from degradation by stomach acids.

tions, the zeta potentials of empty and HBsAg-loaded TMC/ HPMCP nanoparticles were +30.1 ± 0.8 mV and +20 ± 0.7 mV, respectively.

The data obtained using the Zetasizer revealed that the HBsAg-loaded TMC/HPMCP nanoparticles were larger than their empty counterparts, probably as a result of the high molecular weight and large size of the HBsAg molecules, HBsAg surface adsorption during the incubation, and negligible increase in the viscosity by HBsAg during the loading process. The zeta potential of HBsAg-loaded TMC/HPMCP nanoparticles can extremely affect their stability in suspension by electrostatic repulsion among the particles.[19,8] Present results demonstrated that the zeta potentials of empty and HBsAg-loaded TMC/HPMCP nanoparticles were $+30.1 \pm 0.8$ mV and $+20 \pm 0.7$ mV, respectively. These results suggested that the loading of HBsAg led to a reduction in the zeta potential of the particle. Because HBsAg isoelectric point is approximately 4.5, in the solutions with pH of higher than 4.5, antigen had negative charge. Therefore, encapsulation of HBsAg led to decreased zeta potential of the nanoparticles.

Figure 4. (**A**) SEM image of TMC/HPMCP nanoparticles and (**B**) HBs Ag-loaded TMC/HPMCP nanoparticles (TMC 1mg/ml, HPMCP 1mg/ml, HBs Ag 300 μg/ml).

Effect of various HBsAg initial concentrations on loading efficiency and capacity was analyzed. The optimum loading capacity (76.75%) and efficiency (86.29%) were obtained at 300 μg/mL initial concentration of the antigen (**Fig. 2**). The high antigen loading of TMC/HPMCP nanoparticles was probably resulted from the electrostatic interactions between the antigen and TMC. Additionally, HPMCP containing chains of high molecular weight (45 kDa) could contribute to effective interactions with TMC. Such three-dimensional structures could facilitate antigen loading in nanoparticles.[8] The higher loading capacity is important advantage for antigen delivery systems, for the reason that by use of small quantity of antigen entrapped nanoparticle with high loading capacity great amount of antigen can be deliver to body.

The FTIR spectra of TMC was the evidence of methylation, particularly in the 1,700–1,200 cm^{-1} region. A new peak appeared at a high wave number, 1631cm^{-1}, which was attributed to the quaternary ammonium salt. The strong, sharp peaks in 3450 cm^{-1} region were attributed to the axial stretching vibrations of O–H and N–H bonds and the peak at 2923 cm^{-1} was attributed to the axial stretching of C–H bonds.[20] The FT-IR spectrum of TMC/HPMCP nanoparticles (**Fig. 3B**) was different from that of the TMC matrix (**Fig. 3A**). In the spectrum of TMC/HPMCP nanoparticles, the tip of the peak at 3450 cm^{-1} was wider, which indicated enhanced hydrogen bonding. A new peak that became apparent at 1735 cm^{-1} was attributed to C=O stretching. The FT-IR spectra were consistent with the modification of TMC film by HPMCP. The modification could be related to the formation of linkage among negatively charged carboxylic groups and positively charged ammonium ions.[21] The FTIR spectrum of HBsAg-loaded TMC/HPMCP nanoparticles
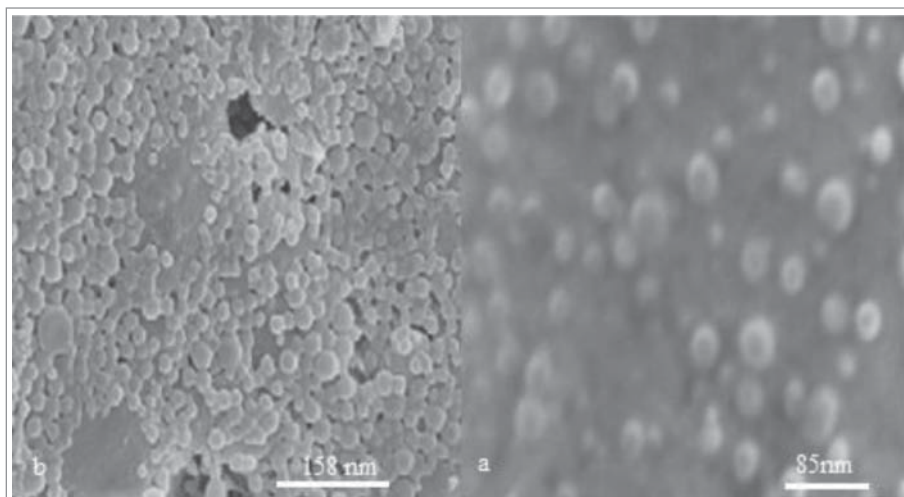
(**Fig. 3C**) showed characteristic peaks of the substituted secondary amide in the 3300–3400 cm^{-1} region. The peaks at 1145 cm^{-1} and 600 cm^{-1} were attributed to C-S stretching vibrations of sulfides and disulfides. The FTIR results confirmed the encapsulation of HBsAg in nanoparticles.

The profiles of antigen release, under simulated intestinal (pH 6.8) and stomach (pH 2) environments, from HBsAg-loaded TMC/HPMCP nanoparticles (**Fig. 5**) showed that the amount of the antigen released from the nanoparticles after 6 h of incubation at pH 2 did not exceed 25% of the loaded protein. The particles demonstrated good physical stability in gastric pH conditions and their size did not noticeably change. Considering HPMCP insolubility in acidic environments, physical association with insoluble HPMCP polymeric construction could probably reduce the solubility of TMC at lower pH values. Therefore, antigen release from TMC/HPMCP nanoparticles at pH 2 likely could involve the mechanism of diffusion, rather than corrosion
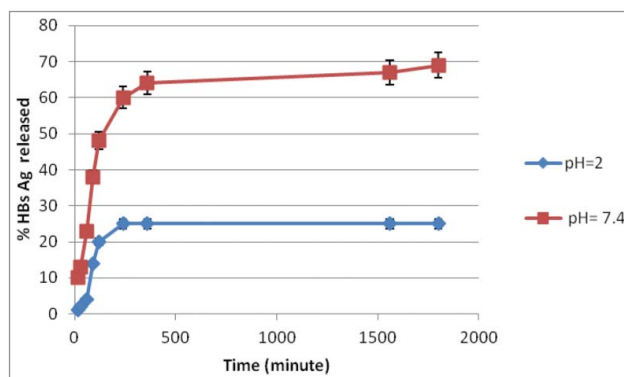

Figure 5. HBs Ag release profile from TMC/HPMCP nanoparticles at pH =2 and 7.4 (TMC 1mg/ml, HPMCP 1mg/ml, HBs Ag 300 μg/ml), mean $\pm$ SD, n = 3.
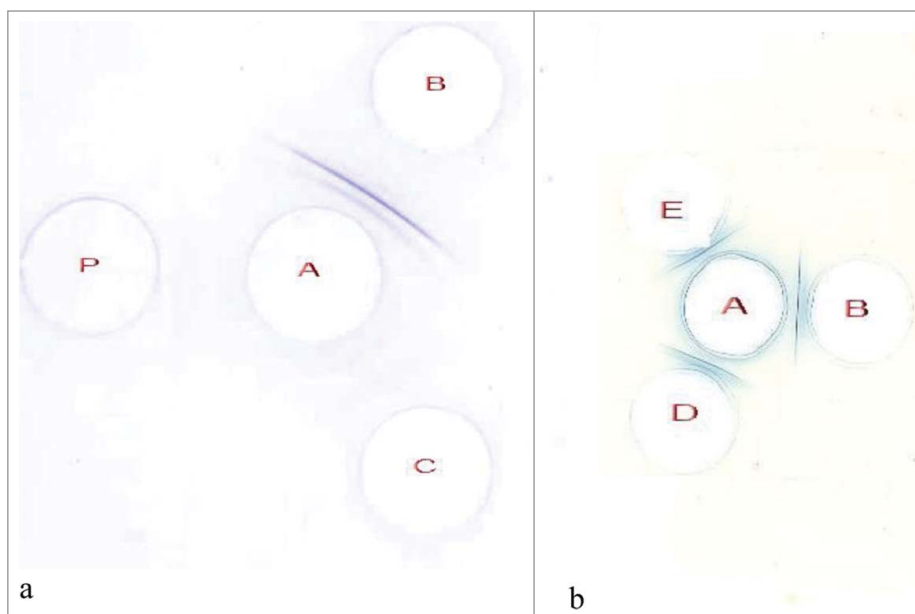
Figure 6. Double immunodiffusion test images of antigen reactivity: (**A**): before loading (B), after release in simulated gastric condition (C) and PBS (P), (**B**): before loading (B), after release in simulated intestinal (D), release in intestinal simulated condition after treatment in gastric simulated condition (E), [Anti-HBs Ag (A), HBs Ag (B)].

or destruction of the polymeric matrix. Similar results have been obtained for chitosan/alginates[22] and chitosan/HPMCP.[8] The results showed that, under simulated intestinal conditions, 70% of the loaded antigen was released after 10 h. The TMC/HPMCP nanoparticles were stable at acidic pH and rapidly dissolved in simulated intestinal environment. Such fast solubility of TMC matrix could justify the facile release of the loaded antigen at pH 6.8.

The results showed good and sharp precipitin lines and antigen-antibody reactivity for the samples before loading, antigen released in intestinal simulated condition, and antigen released in intestinal simulated condition after treatment in gastric simulated condition. Also, HBsAg released in gastric simulated condition did not show any antigen antibody reaction (**Figs. 6A,B**). It could be suggested that TMC/HPMCP nanoparticles had protective ability for entrapped HBsAg against degradation by gastric simulated condition. These results were consistent with that reported for papain,[21] insulin,[8,23] cyclosporine A, calcitonin, and somatostatin.[24]

In this study, a novel TMC/HPMCP nanoparticle system was successfully developed for oral administration of HBsAg. Oral administration is a favorite method of drug delivery. Advantages of oral delivery of vaccines include better management of vaccination, greater compliance, elimination of complications associated with local injection, cost-effective manufacturing, and affordability. However, the challenges faced while developing suitable oral vaccine delivery systems are significant. The present results showed that formulation with HPMCP significantly improved the stability of TMC nanoparticles in the acidic condition and their potentiality in terms of preserving the loaded

antigen from gastric destruction. The present results clearly showed that pH-sensitive TMC nanoparticles are favorite delivery vehicles that could load and preserve HBsAg from degradation. Therefore, HBsAg-loaded TMC/HPMCP nanoparticles could be used as an alternative vaccine delivery and adjuvant system to the conventional parental dosage form. Further in vivo studies are underway for advancing the development of this vaccine.

Materials and Methods

Synthesis of TMC
Concisely, polymer of chitosan was dispersed in NMP. Then, sodium hydroxide solution, sodium iodide, and methyl iodide were added to the dispersion. The mixture was stirred at 60°C for 6 h. The polymer thus obtained was precipitated with acetone and dried at room temperature to yield a water-soluble white powder. The dried products were then solubilized in 20 mL of 5% (w/v) sodium chloride solution and stirred for 1 h to replace the iodide with chloride ion. Then, the polymer was precipitated, washed twice with acetone, and dried at room temperature.[15,16]

Characterizing TMC by ^{1}H-NMR spectroscopy
The TMC synthesized in this study was characterized by ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectroscopy. The NMR spectra of the solution of 5 mg of TMC in 700 µL of D$_2$O (dissolved by heating at 80°C) were acquired. The peak corresponding to water was suppressed. The degree of quaternization (DQ) was estimated by Equation (1).[17]

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

where DQ (%) is the degree of quaternization expressed as percent, [(CH$_3$)$_3$] is the integral of the chemical shift of the trimethylamino group at 3.3 ppm, which is attributed to the 9 hydrogen atoms of the methyl groups of the trimethylamino group, and [H] is the integral of the ^{1}H peaks between 4.7 and 5.7 ppm (reference signals), which is due to the protons attached to the carbon atom of the glucosamine unit of the glucopyranose ring.[17,25,26]

Preparing TMC/HPMCP nanoparticles and HBsAg-loaded nanoparticles
The nanoparticles were fabricated by the ionic gelation of the positively charged TMC with the negatively charged pH-sensitive polymer HPMCP. TMC solutions of various concentrations (1, 1.5, and 2 mg/mL) were prepared. For preparing the nanoparticles, an HPMCP aqueous solution (1 mg/mL) was combined

with TMC (1 mg/mL) aqueous solution slowly under constant shaking (1000 rpm, 1 h) at room temperature. Once an opalescent suspension was created, the samples were centrifuged at 15000 rpm for 30 min at 4°Ċ and the precipitate was lyophilized and weighed.[8] In the first step, the effects of the initial concentrations of TMC (1, 1.5, and 2 mg/ml) on the characteristics of nanoparticles were studied in terms of access to the optimum concentration of TMC for synthesising TMC/HPMCP nanoparticles.

In the second step, HBsAg-entrapped nanoparticles were fabricated by adding the HPMCP solution to the TMC solution containing different concentrations of HBsAg in optimized conditions in order to synthesize empty TMC/HPMCP. The effects of the initial concentrations of HBsAg (100, 300,500, 700, and 1000 μg/mL) on the characteristics of HBsAg-entrapped TMC/HPMCP nanoparticles were also examined. In all the steps of this work, while studying the effect of one parameter, the other parameters were kept constant.[27]

Physicochemical properties of empty and HBsAg-loaded TMC/HPMCP nanoparticles

Structure of nanoparticles

The IR spectrum of the samples was prepared on a Fourier transform infrared spectrophotometer (FTIR- 410, Jasco Colchester, United Kingdom) with attenuated total reflection (ATR) using KBr pellets.

Particle size and zeta potential

The mean size of the particles, size distribution [polydispersity index (PDI)], and the zeta potential of the particles were determined by Zetasizer (Malvern Instruments, UK).

Morphology

The morphologies of the nanoparticles were analyzed using a scanning electron microscope (SEM) (Hitachi S- 4160, Japan) after coating with a thin layer of platinum.

HBsAg loading efficiency and loading capacity of nanoparticles

HBsAg-entrapped nanoparticles were isolated from aqueous suspension using centrifuge at 65,000g and 4°C for 30 min. The protein content (free HBsAg) supernatant was analyzed by the Bradford protein assay.[28,29] The HBsAg loading efficiency (*LE*) and loading capacity (*LC*) of the nanoparticles were estimated as follows:

$$\%LE = [(A - B)/A] \times 100$$
$$\%LC = [(A - B)/C] \times 100$$

where A is the total amount of HBsAg, B is the free amount of HBsAg, and C is the weight of nanoparticles.[30]

In vitro antigen release

For the in vitro antigen release assessment, a known quantity of HBsAg-entrapped TMC/HPMCP nanoparticles was suspended in separate tubes containing equal volumes of 0.2 mol/L PBS (pH 7.4, simulating intestinal conditions) and HCl solution (pH 2, simulating gastric conditions). Then, the tubes were incubated at 37°C under constant shaking. One tube was removed at specific intervals (15, 30, 60, 90, 120, 240, 360, 1440, and 1800 min) and centrifuged at 65,000g and 4°C for 30 min. The amount of HBsAg released into the supernatant was then measured.[30]

Reactivity of the HBsAg before and after loading

The antigen reactivity (before and after loading) was evaluated by the Ouchterlony double immunodiffusion test. For this purpose, after preparing agar gel plate, the reactivity of the antigen before loading and that of the antigen released into the solutions that simulated gastric, intestinal after gastric incubation stage and intestinal conditions was determined. The precipitin lines were visualized after staining with Coomassie brilliant blue solution.[31,32]

Stability of the entrapped HBsAg in TMC/HPMCP nanoparticles against simulated gastric condition was investigated to evaluate the protective ability of the prepared nanoparticles against gastric degradation. The precipitate of the sample of in vitro release study in gastric simulated condition (after 360 min) was suspended in the solution of intestinal simulated condition. Then, the suspension was incubated at 37°C under constant shaking for 240 min, centrifuged (65,000g, 4°C for 30 min), and the amount of HBsAg released into the supernatant was measured. Sample of the supernatant was used for antigen reactivity experiment.

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