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Preparation, characterization and antibacterial activities of chitosan, *N*-trimethyl chitosan (TMC) and *N*-diethylmethyl chitosan (DEMC) nanoparticles loaded with insulin using both the ionotropic gelation and polyelectrolyte complexation methods

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Abstract

TMC and DEMC, quaternized derivatives of chitosan, have been shown to have penetration enhancement properties and able to open the tight junctions of the intestinal epithelia at neutral and alkaline pH environments. The use of the nanoparticulate systems has the advantage of protecting the peptidic drugs from the harsh environment of the gastrointestinal tract. Hence, the aim of this study was to synthesize and characterize TMC and DEMC, both with quaternization degrees of $50 \pm 5\%$, which were then used to prepare insulin nanoparticles with two different methods: ionotropic gelation and the polyelectrolyte complexation (PEC) techniques. The obtained nanoparticles were then characterized for size, zeta potential, insulin loading and release as well as antibacterial activities. The results showed that nanoparticles prepared by the PEC method had higher insulin loading efficiency and zeta potential than those made by the ionotropic gelation method and may subsequently be used for further in vitro, ex vivo and in vivo studies. Moreover, the antibacterial studies suggest that the polymers in free form have higher antibacterial activity against Gram-positive bacteria than in the nanoparticulate form.

Keywords: Trimethyl chitosan (TMC); Diethylmethyl chitosan (DEMC); Insulin nanoparticles; Polyelectrolyte complexation; Ionotropic gelation; Antibacterial activity

1. Introduction

Oral drug delivery is the most favorite route for drug administration. In the past decade, biodegradable polymers such as chitosan and its quaternized derivatives have been studied extensively for their role as multifunctional permeation enhancers to enhance the permeation of hydrophilic macromolecules in peroral drug delivery. Chitosan, a natural polyaminosaccharide obtained from *N*-deacetylation of chitin, is a non-toxic, biocompatible and biodegradable polymer that has been used in biomedical fields in the form of sutures, wound covering, and as artificial skin (Dodane and Vilivalam, 1998; Domb and Bentolila, 1998; Varum et al., 1991; Rinaudo and Domard, 1989). Chitosan has mucoadhesive properties which are mediated by the spreading ability of chitosan over the mucus layer and additionally through its positive ionic interactions with the negative charges of the mucus or of the cell surface membranes (Lehr et al., 1992, 1993). Furthermore, it has been shown that chitosan and its derivatives can act as antibacterial agents against both Gram-negative and Gram-positive bacteria (Henriksen et al., 1996; Jung et al., 1999). Chitosan is a polycation with an apparent pk_a 5.5; hence, in neutral and basic

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environments prevailing in the intestine, the chitosan molecules lose their charge and precipitation will occur. However, studies have convincingly shown that quaternized derivatives of chitosan, synthesized by introducing alkyl groups to the NH_2-group of the chitosan structure, are drastically more soluble in the neutral and alkaline environments of the intestine and hence more useful for drug delivery of peptides and proteins (Avadi et al., 2004; Thanou et al., 1999). Moreover, the soluble chitosan salts may act as permeation enhancers to increase the transmucosal absorption of peptide drugs that normally do not pass the tight junctional barrier. The enhancing properties of chitosan and its derivatives have been attributed to their interactions with the tight junctions and cellular membrane components to reversibly open the tight junctions and hence to increase the paracellular permeation of hyrodrophilic compounds (Park et al., 2002).

Today, biotechnological processes enable us to produce in large amounts endogenous peptides and proteins that are required for the treatment of chronic diseases. Although the peroral route for drug delivery is the most convenient and desirable one for patients, the harsh environment of the gastrointestinal tract, the high molecular weight of the peptides as well as their hydrophilicity which prevents them to cross the lipophilic barrier of the mucosal walls, are major problems in developing an effective delivery system.

Special strategies are hence required to overcome the above obstacles. It is the current opinion that nanosized polymeric particles, designed as delivery platform for those hydrophilic peptides, have the highest chance to succeed in overcoming these hurdles. Moreover, nanoparticles are capable of protecting drugs from degradation, improving permeation and penetration of the drugs across the mucosal surface as well as controlling the release of the encapsulated or adsorbed drugs (Schipper et al., 1997; Florence et al., 1995; Takeuki et al., 2001). The uptake of chitosan nanoparticles seems to be related to their size and the superficial charge; hence, the higher the superficial charge, the stronger the affinity of the nanoparticles for the negatively charged cell membrane (Janes et al., 2001; Sakuma et al., 1997). Various techniques are available for producing nanoparticles including solvent evaporation, interfacial polymerization and emulsion polymerization (Sakuma et al., 1999; Julienne et al., 1992; Ibrahim et al., 1992); however, most of these techniques involve the use of organic solvents, heat and vigorous agitation that may be harmful to peptides (Leroux et al., 1995). In 1997, the first chitosan nanoparticles were prepared by Alonso et al. using ionotropic gelation of chitosan (polycation) with tripolyphosphate TPP (polyanion). Ever since, almost all chitosan nanoparticles were prepared accordingly. However, recently polyelectrolyte complexation (PEC) resulting from self-assembly of proteins with natural and synthetic polymers has drawn increasing attention (Calvo et al., 1997). PEC is formed when oppositely charged polyelectrolytes are mixed and interact via electrostatic interactions. Both processes are easily applied; they have the advantage of not using sonication or organic solvents, both harmful for the proteins and peptides. The PEC formation results in an optically homogeneous and stable nano-dispersion (Mao et al., 2001). At a pH below 6.5, chitosan becomes positively charged due to protonation of the amino groups; on the other hand, most proteins become negatively charged at pH above 6.5. Hence, the electrostatic interactions between both entities at a suitable pH value can be used as driving force for PEC formation.

The aim of the present work was to develop nanoparticulate systems based on chitosan, diethylmethyl chitosan (DEMC) and trimethyl chitosan (TMC) using both the polyelectrolyte complexation and the ion gelation methods and to load the nanoparticles with insulin. The nanoparticles were characterized in terms of particle size, zeta potential, insulin loading and release as well as stability for further in vitro, ex vivo and in vivo investigations. Moreover, the antibacterial effects of these polymers were compared both in free polymer solution and in the nanoparticulate form.

2. Materials and methods

2.1. Materials

ChitoClear® chitosan (viscosity 1% (w/v) solution, 22 mPa s) was purchased from Primex, Iceland. Human insulin was a generous gift from Exir Pharmaceutical Company (Lorestan, Iran). Ethyliodide, methyl iodide, and sodium borohydride were obtained from Sigma (Vienna, Austria). Sodium hydroxide, *N*-methyl pyrrolidone (NMP) and sodium iodide were purchased from Merck (Darmstadt, Germany). *Staphylococcus aureus* ATCC 29737 was obtained from Persian Type Culture Collection (PTCC, Iran). The antibiotic broth # 5273 was purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade and were used as received.

2.2. Synthesis and characterization of N-trimethyl chitosan

TMC with degree of substitution of $55 \pm 5\%$ was synthesized as previously described using minor adjustments (Sieval et al., 1998). Briefly, low molecular weight chitosan was dispersed in a basic solution of methylpyrrolidone and sodium hydroxide, methyl iodide and sodium iodide were, subsequently, added at 60 °C for 6 h. The polymer was then precipitated with acetone and separated by centrifugation. For exchanging I^- with Cl^-, the polymer was dissolved in aqueous NaCl (5.0%, w/w) solution and subsequently, precipitated with acetone and dried to obtain a water-soluble white powder with quantitative yield of 98% and substitution degree of $50 \pm 5\%$. The ^1H NMR spectrum was obtained in D_2O using a 500 MHz spectrum (Varian unity plus) and the degree of quaternization was calculated.

2.3. Preparation and characterization of N-diethylmethyl chitosan

DEMC was prepared by a two-step method reported previously by Avadi et al. In summary, low molecular weight chitosan was dissolved in 1% acetic acid solution and formaldehyde solution. After stirring for 120 min, the pH of solution was adjusted to 4.5 by adding 1 M NaOH solution. Methyl chitosan precipitate

was obtained by adding 1 M NaOH solution and adjusting the pH of solution to 7.0. The precipitate was washed with distilled water and Soxhlet-extracted with ethyl alcohol and diethyl ether (1:1, v/v) for 72 h. The ^{1}H NMR spectrum of methyl chitosan was obtained in CF_3COOD and D_2O as solvent. In the second step, methyl chitosan was dispersed in *N*-methyl pyrrolidone and subsequently, sodium hydroxide, ethyl iodide and sodium iodide were added. The reaction was carried out under gentle stirring for 5 h at 60 °C. Finally acetone was added and the precipitate of the chitosan derivative was collected. For exchanging I^- with Cl^-, the polymer was dissolved in NaCl solution (5%, w/w) and subsequently precipitated with acetone, centrifuged and dried to obtain a white, water-soluble powder. The ^{1}H NMR spectrum was obtained in D_2O using a 500 MHz spectrometer (Varian unity plus) and the degree of quaternization was calculated to be 55 ± 5%.

2.4. Preparation and characterization of nanoparticles with chitosan, TMC, and DEMC using polyelectrolyte complexation and ionotropic gelation method

The polyelectrolyte complexation method was used to make nanoparticles from chitosan and its two derivatives. To obtain such a complex, the pH of chitosan and its derivatives were adjusted to 5.0 so that over 90% of the amine groups were protonated and the insulin pH was adjusted to 8.0. Chitosan was dissolved in 0.25% acetic acid and the derivatives were dissolved in water. The pH was, subsequently, adjusted to 5.0 using 1N NaOH. The insulin powder was dissolved in 0.01N HCl to obtain a solution of 1.0 mg/mL and the pH was adjusted to 8.0 using 1N NaOH. The insulin was added to the polymer solution at a 1:1 ratio in a beaker under gentle magnetic stirring at room temperature. Once an opalescent suspension was formed, the samples were centrifuged at 14,000 rpm for 30 min at 4 °C and the pellet was freeze-dried overnight to obtain a white powder and further examined as nanoparticles (Shirui et al., 2005).

Chitosan-insulin nanoparticles were also prepared by an ionotropic gelation method (Julienne et al., 1992). Briefly, insulin (1.0 mg/mL) was pre-mixed with TPP (1.0 mg/mL) at a 1:4 ratio. A solution of chitosan (1.0 mg/mL) was prepared in 0.25% acetic acid. The pre-mixed solution of TPP-Insulin was added to chitosan solution drop-wise at a ratio of 1:3 and under gentle magnetic stirring at room temperature.

Chitosan derivatives, TMC and DEMC, were dissolved in water to obtain a solution of 1.0 mg/mL and the pH was adjusted to 5.2 using 0.01N NaOH. Insulin (1.0 mg/mL) was pre-mixed with TPP (1.0 mg/mL) at a 1:4 ratio. The pre-mixed solution of TPP-Insulin was added to derivative solution drop-wise at a ratio of 1:4 and under gentle magnetic stirring at room temperature.

Once a stable colloidal suspension was obtained, the samples were centrifuged at 14,000 rpm for 30 min at 4 °C and the pellet was freeze-dried overnight to obtain a white powder. Particle size and the zeta potential of the nanoparticles were measured using a Malvern Zetasizer (3000HS, UK) of the freshly prepared suspension of polymer-insulin nanoparticles. The morphology of the nanoparticles was observed by TEM-100CXII.

2.5. Insulin loading of the nanoparticles

The freeze-dried samples were used to measure the loading efficiency of insulin in the nanoparticles. The amount of insulin associated with the PEC was calculated by the difference between the amount of insulin added to the suspension and the amount found in the freeze-dried samples in the case of the direct method. For the indirect method, the calculation was done by measuring the difference between the total amount of insulin added to the solution and the amount of insulin in the free form in the supernatant. The quantity of insulin was measured using a HPLC-UV apparatus (Waters 600 Controller, USA) at 210 nm. Isocratic elution was performed using 28% acetonitrile and 72% buffer containing 0.1 M KH_2PO_4 and 1% triethylamine adjusted to pH 3.0 with phosphoric acid (Dorkoosh et al., 2002). The column used was C8 (Capital HPLC, UK) 250 mm × 4.6 mm equipped with a C8 (Capital HPLC) 10 mm × 4.6 mm precolumn. Flow rate and injection volume were 1.5 mL/min and 50 μL, respectively. The detection limit was 0.10 μg/mL. The amount of insulin was quantified by peak integration and insulin association efficiency was calculated as follows:

a) Direct method

$$\text{Loading efficiency} = \frac{\text{Amount of insulin detected}}{\text{Total amount of insulin}} \times 100$$

b) Indirect method:

$$\text{Association efficiency} = \frac{\text{Total amount of insulin} - \text{Free insulin in supernatant}}{\text{Total amount of insulin}} \times 100$$

All experiments were done in triplicate.

2.6. Insulin release studies

A comparative insulin release study was done in three different pH values of 1.5, 3.0 and 6.8 with 0.01N HCl, PBS (pH 3.0 and 6.8), respectively. A known amount of freeze-dried nanoparticles was transferred to a 25 mL tube that was designed to fit into the Erweka apparatus (DT6, Heusentamm, Germany) and 10 mL of the respective dissolution buffer was added to the tube. The temperature and rotation were adjusted to 37 °C and 50 rpm, respectively. At predetermined time of 5, 15, 30, 45, 60, 90, 120, and 240 min 0.5 mL of sample was removed and replaced with fresh pre-warmed buffer. The samples were further analyzed using HPLC.

2.7. Stability test

In order to determine the stability of the nanoparticles made by the PEC and ionotropic gelation methods, the freeze-dried samples, kept at −4.0 °C, were analyzed monthly for 6 months and the amount of insulin was measured using HPLC. Briefly, 1.0 mg of freeze-dried samples was analyzed for the insulin

content and the results were compared with the initial loading obtained on the first day of the experiment.

2.8. The antibacterial effect of chitosan and its derivatives as free polymer and in nanoparticulate form

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were determined as described previously (Avadi et al., 2004). Chitosan was dissolved in 0.25% acetic acid and the chitosan derivatives were dissolved in water. The concentrations of the polymers used in this study were 1000–62.5 μg/mL. The micro-organism was *S. aureus* ATCC 29737. The experiments were performed in triplicates. To compare the inhibitory properties of the polymers with their nanoparticle forms, the PEC method was used and the nanoparticles were applied as suspensions form. As antibacterial standard tetracycline hydrochloride was used. Four concentrations (1000, 500, 250 and 125 μg/mL) of TMC and the standard tetracycline were prepared in water. Since TMC had shown the highest antibacterial effect among the polymers, it was used for the comparison studies with the tetracycline. The Kirby-Bauer diffusion method was used to study the inhibitory zones at different antimicrobial concentrations. Accordingly, a standard concentration of staphylococcus bacteria (1.0 μg/mL) was uniformly spread on the surface of an agar plate (antibiotic broth #1). Consequently, 4 discs each impregnated with a predetermined concentration of the polymers were placed on the surface of the medium. During incubation, polymers diffuse outward from the disc at a rate inversely proportional to their size, forming a concentration gradient around each disc. A clear zone of inhibition around an antimicrobial disc reflects the degree of susceptibility of the micro-organism to the polymer.

3. Results and discussion

3.1. Characterization of trimethyl chitosan chloride and diethylmethyl chitosan chloride

The ^{1}H NMR spectra of chitosan, TMC and DEMC are presented in Fig. 1a, b and c, respectively. In Fig. 1b, the signal at 1.9 ppm is attributed to the acetyl group of the chitin; the peak at 3.6 represents the $N(CH_3)_3$ group together with a smaller peak at 3.4 ppm assigned to the $N(CH_3)_2$ group. According to the peak assignment and intensity the degree of quaternization was calcu-

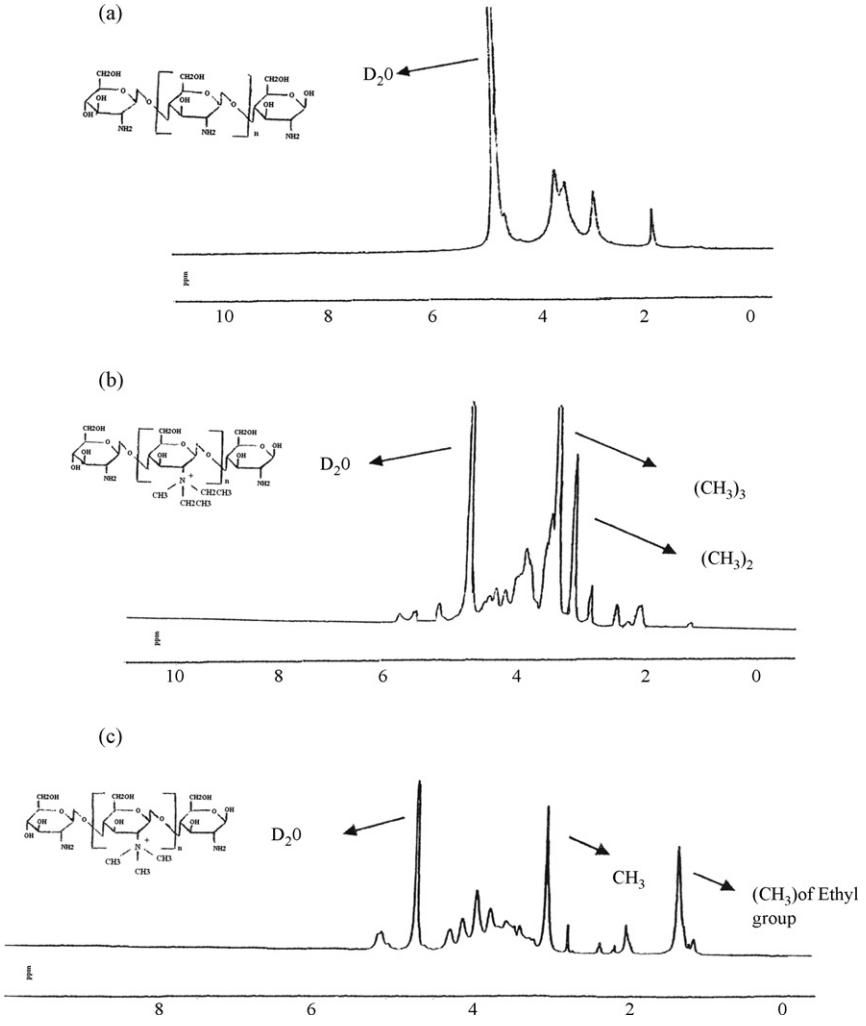

Fig. 1. The H NMR spectrum of chitosan (a), TMC (b) and DEMC (c).

Table 1
The zeta potential of chitosan, TMC and DEMC in free form and the nanoparticle forms

Polymer	Zeta potential (mV)
Chitosan	+24.2
Chitosan np	+15.9
TMC	+43.2
TMC np	+22.0
DEMC	+40.0
DEMC np	+19.0

lated to be 50 ± 5%. In Fig. 1c, the signal at 1.3 ppm is attributed to the CH_3 group of the diethyl substituted N-atom, while H2–H6 protons of the polysaccharide backbone superimpose the CH_2 groups. The intense band at 4.8 ppm is related to the solvent HDO. The integral of the CH_3 of the ethyl groups versus the other protons was used to calculate the degree of quaternization to be 50 ± 5%. The zeta potential of the polymers was measured using a Malvern Zeta Sizer and the results are presented in Table 1. As shown in this table, chitosan has the least positive zeta potential and TMC has the highest positive zeta potential among the derivatives. DEMC has a zeta potential in between. The positivity of the chitosan in zeta potential relates to the portion of the chitosan that has been protonated and dissolved in water. In the alkylated salts of chitosan, TMC and DEMC, the NH_2-group was converted to a quaternary ammonium group and that describes the higher positivity of the derivatives in comparison to the chitosan itself. The higher positivity of TMC compared to DEMC can be explained by the fact that methyl groups have a smaller volume than the ethyl groups which makes the positive charge of the TMC more accessible.

3.2. Nanoparticle characterization

3.2.1. Insulin loading

Table 2 represents the diameters (nm), zeta potential and the insulin loading of the nanoparticles prepared by both the ionotropic gelation and the PEC methods. The size of the nanoparticles made by either method is comparable and is around 200 ± 20 nm. Also the size of the insulin nanoparticles prepared with chitosan was not different with the ones made by TMC or DEMC. Thus, one can state that there was no distinct effect on the size of the nanoparticles dependent on the preparation method.

The insulin loading of the nanoparticles made by PEC was shown to be higher than the nanoparticles prepared by the ionotropic gelation method. In the PEC method, the electrostatic interactions between the negatively charged insulin and the positively charged polymer are used as the driving force for the formation of the nanoparticles. Consequently, in this method, the insulin is directly interacting with the positive polymer and this can assign to the higher insulin loading in the obtained nanoparticles. In the ionotropic gelation method, the main interaction is between the negative phosphate groups of the tripolyphosphate (TPP) and the positive amino groups of the polymers. As the TPP (negative charge) interacts with chitosan (positive charge), a matrix system is obtained. The hydrophilic insulin is entrapped in this network and tends to diffuse to the external medium. This can explain the lower insulin loading efficiency of the nanoparticles using the ionotropic gelation method. Furthermore, using the PEC method, the insulin loading efficiency of the nanoparticles prepared with the chitosan was higher than in the ones prepared by the chitosan derivatives, TMC and DEMC. One can argue that in the PEC method when the pH of the polymers is adjusted to 5.2 the NH_2-groups of the chitosan are mostly protonated and are better accessible to interact with the insulin. With the quaternized derivatives, the N^+ atoms are surrounded by methyl and ethyl groups and are hence less available to interact with insulin. This may account for the higher insulin loading of chitosan in comparison to the derivatives.

According to Table 2, the zeta potential of the insulin nanoparticles made by the PEC method is higher in comparison to the nanoparticles prepared by the ionotropic gelation method. The zeta potential is a good indicator of the available surface charges on the nanoparticle. The positivity of the nanoparticles is important for their interaction with the cellular membrane components and the tight junctions in triggering the paracellular permeation of the hydrophilic compounds. In the nanoparticles made by the PEC method, the ratio of negative charge (insulin) and the positive charge of the chitosan and its derivatives remains in the favor of the positive charge of the polymers because only a fraction is neutralized by binding insulin. However, in the ionotropic gelation method, the ratio of TPP, both the negatively charged TPP and the insulin bind with the positive charged polymers resulting in a lower positivity of the nanoparticles.

3.2.2. Morphology of the nanoparticles

According to the TEM picture shown in Fig. 2a and b, the morphology and the size of the nanoparticles are comparable in both ionotropic gelation and PEC methods. Morphologically, the nanoparticles look round to oval in shape and have a relatively smooth surface.

Table 2
The results of insulin loading, size of nanoparticles, polydispersity and zeta potential of the nanoparticles prepared by both ionotropic gelation and PEC methods using chitosan, TMC and DEMC

Polymer	Insulin loading (%)	Size (nm)	Poly dispersity	Zeta potential (mV)
Chitosan (ionotropic)	50 ± 5	250 ± 20	0.46	+15.9
Chitosan (PEC)	90 ± 5	220 ± 20	0.25	+23.1
TMC (ionotropic)	55 ± 5	215 ± 20	0.1	+22.0
TMC (PEC)	70 ± 5	195 ± 20	0.32	+29.0
DEMC (ionotropic)	50 ± 5	203 ± 20	0.23	+19.0
DEMC (PEC)	75 ± 5	220 ± 20	0.45	+26.0

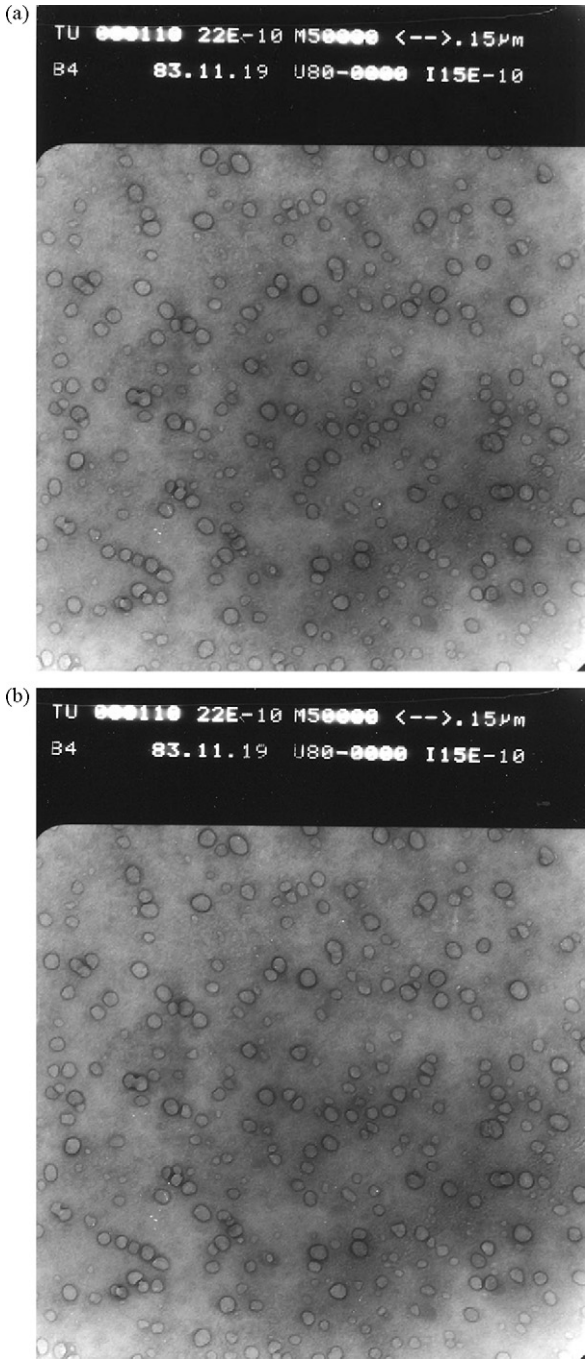

Fig. 2. The TEM picture of the insulin loaded nanoparticles made by the ionotropic gelation (a) and the PEC method (b).

3.2.3. The stability of nanoparticles

The stability of nanoparticles prepared by both ionotropic gelation and the PEC methods was followed up to 6 months and the results indicate that the insulin was stable in both methods for this period of time and a $10 \pm 4\%$ decrease in loading was observed (data not presented).

3.3. Insulin release studies

Fig. 3 shows a comparison between the insulin released from the nanoparticles made by the PEC method at three different

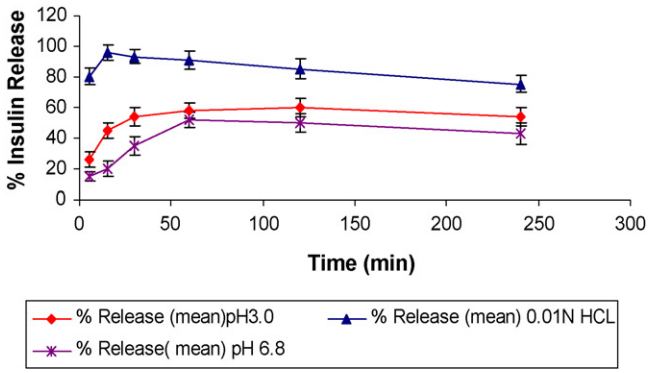

Fig. 3. The release profile from insulin nanoparticles using PEC method at 3 different pH mediums (0.01N HCl, PBS pH 3.0 and PBS pH 6.8).

pH media: 0.01N HCl, phosphate buffer pH 3.0 and phosphate buffer pH 6.8. Each study was done three times. In 0.01N HCl, a burst release of insulin in the first minutes of the study (about 70%) was observed and after 30 min almost 100% of the insulin was released from the nanoparticles. When phosphate buffer pH 3.0 was used, the burst release was less, approximately 20% and a maximum of 50% insulin release was observed after 45 min. Finally with phosphate buffer 6.8, a burst release of about 15% was observed and the maximum insulin release was 40% after 45 min. This observation can be best described by the fact that both insulin and the polymer are soluble in the acidic pH (0.01N HCl) and hence a better insulin release was observed. Using phosphate buffer pH 3.0, although both insulin and polymer are soluble, their solubility is lower than in the HCl medium and consequently a lower insulin release is observed from the nanoparticles. Finally, the lowest insulin release in phosphate buffer pH 6.8 can be described by the poor solubility of both insulin and the polymer at this pH. The release profiles of insulin from nanoparticles prepared by the ionotropic gelation method at pH media 3.0 and 6.8 were similar to those of the PEC method (data not presented).

Figs. 4 and 5 show the insulin release from nanoparticles made from chitosan, TMC and DEMC by both the ionotropic gelation method and the PEC method, respectively. According to these figures the insulin release profiles from the chitosan, TMC

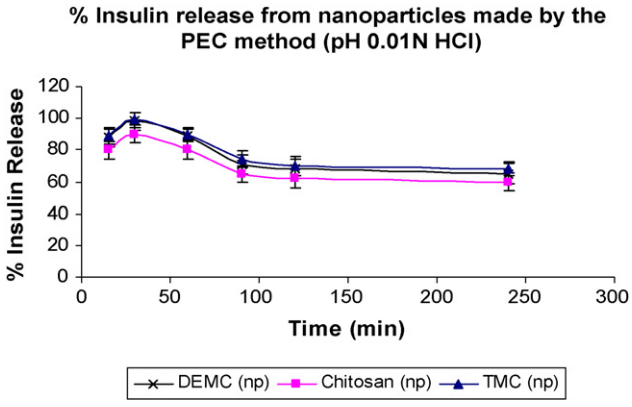

Fig. 4. Comparison of insulin release from chitosan, TMC and DEMC nanoparticles prepared by the ionotropic gelation method in 0.01N HCl.

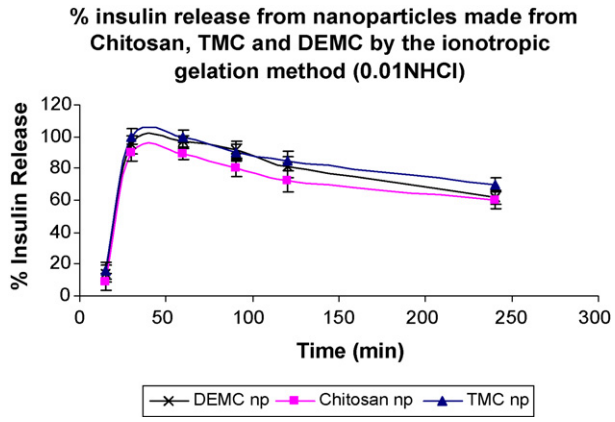

Fig. 5. Comparison of insulin release from chitosan, TMC and DEMC nanoparticles prepared by the PEC method in 0.01N HCl.

and DEMC nanoparticles prepared by the ionotropic gelation and the PEC methods were very similar in 0.01N HCl. Fig. 6 compares the insulin release from the nanoparticles made by both ionotropic gelation and the PEC methods in 0.01N HCl medium. According to Fig. 6, a burst release is observed early during the release study from the nanoparticles made according to the PEC method. The release of insulin was slower from nanoparticles made according to the ionotropic gelation method. In both methods, the maximum insulin release was 100% and comparable. The release studies were done with nanoparticles made from chitosan, TMC and DEMC and the results were comparable in all three polymers. The difference in the insulin release profiles between the PEC and the ionotropic gelation method is that the nanoparticles prepared by the ionic gelation method due to the additional cross linking of the polymer with TPP possess a denser matrix structure and hence a lesser availability of the incorporated insulin at the beginning of the release experiments.

3.4. Comparison of the antibacterial effect of chitosan, TMC and DEMC in free form and as nanoparticles

Table 3 presents the antibacterial effect of chitosan and the two chitosan derivatives, TMC and DEMC, in free form and as nanoparticle form prepared by the ionotropic gelation method. According to the results, TMC shows the highest inhibition and chitosan the least inhibition against *S. aureus*. Since the chitosan derivatives as quaternary ammonium compounds, have a stronger positive charge than chitosan, their increased antibacterial effects can be attributed to the formation of polyelectrolyte complexes between the polymers and the negative peptidoglycans of the bacterial cell wall. This interaction may in turn disrupt the cell wall and result in the inhibition of the bacterial growth. As for the derivatives, with N-substitution degrees of approximately 50%, the charge densities are very close to each other; thus, their different inhibitory effects may be explained by the size of the substituted group. In another word, as the trimethyl group is smaller than the ethyl group, it may react better with the bacterial cell wall in comparison to the more voluminous diethylmethyl (DEM) group. Also, less steric hindrance may be expected when TMC with three methyl groups interacts with the bacterial cell wall in comparison to the (DEM) group containing 2 ethyl groups and a methyl group. The antibacterial effect of the polymers was compared to their nanoparticle forms as well and the results are presented in Table 3. The results show that the nanoparticles have less inhibitory effect than the polymers in free form. One can assume that since the nanoparticles are prepared by electrostatic interactions, the amount of positive charge available for interacting with the bacterial cell wall is reduced. This can be explained by a lower zeta potential of the nanoparticles in comparison to the free polymer form. In all three cases, the antibacterial effect of the nanoparticles was about one dilution lower than their respective free polymer forms.

Table 3
The antibacterial effect of chitosan, TMC and DEMC in free form and as nanoparticles forms against Gram-positive *Staphylococcus aureus*

Polymer	MIC (μg/mL)	MBC (μg/mL)
Chitosan	1000	1000
Chitosan np	2000	2000
TMC	250	250
TMC np	500	500
DEMC	500	500
DEMC np	1000	1000

Fig. 7 presents the difference between TMC (1000 μg/mL) and tetracycline hydrochloride (1000 μg/mL) in the inhibitory effect against *S. aureus*. Evidently, the inhibition zones produced by tetracycline are much bigger than the ones from TMC which indicates higher inhibition potency by tetracycline. Using the diffusion disc method, no zones were seen at lower concentrations of TMC. This can be best described by the interaction and probably entanglement of the polymer chains with the agar which further reduce the ability of the polymer to diffuse through

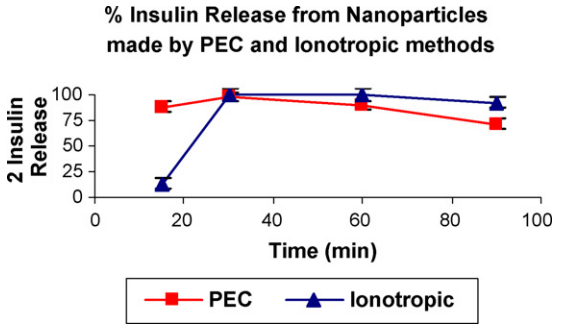

Fig. 6. Comparison of insulin release from chitosan nanoparticles prepared by both the ionotropic gelation and the PEC methods in 0.01N HCl.

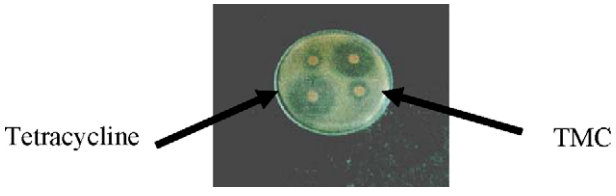

Fig. 7. The comparison of TMC and tetracycline antibacterial effect against *Staphylococcus aureus* using the disc method.

the agar. As expected chitosan and DEMC have lower inhibitory effects than TMC compared to tetracycline.

4. Conclusions

In summary, the results suggest that while both the ionotropic gelation and the PEC methods were easy to be used for the production of insulin loaded nanoparticles, the PEC method seems to be more suitable for future studies. The obtained nanoparticles by the PEC method had higher insulin loading efficiency and zeta potential both required for an effective permeation enhancement across the intestinal epithelium. Moreover, the antibacterial studies suggest that the nanoparticles have less inhibition effect on Gram-positive bacteria than the polymers in free soluble form since nanoparticles have less positive charge available to bind to the negative bacterial cell wall. However, among the polymers used, TMC, with the highest zeta potential and smallest alkyl groups showed the highest antibacterial inhibition against *S. aureus*.

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