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ScienceDirect
European Journal of Pharmaceutics and Biopharmaceutics 70 (2008) 270–278
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Permeation enhancer effect of chitosan and chitosan derivatives: Comparison of formulations as soluble polymers and nanoparticulate systems on insulin absorption in Caco-2 cells

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Received 7 December 2007; accepted in revised form 5 March 2008
Available online 12 March 2008

Abstract

In this study four quaternized derivatives of chitosan: trimethyl chitosan (TMC), dimethylethyl chitosan (DMEC), diethylmethyl chitosan (DEMC) and triethyl chitosan (TEC) with degree of substitution of approximately 50 ± 5% were synthesized and their effect on the permeability of insulin across intestinal Caco-2 monolayers was studied and compared with chitosan both in free-soluble form and in nanoparticulate systems. Transepithelial electrical resistance (TEER) studies revealed that all four chitosan derivatives in free-soluble forms were able to decrease the TEER value in the following order TMC > DMEC > DEMC = TEC > chitosan, indicating their abilities to open the tight junctions. Recovery studies on the TEER showed that the effect of the polymers on Caco-2 cell monolayer is reversible and proves the viability of cells after incubation with all polymers. A similar rank order was also observed when measuring the zeta-potentials of the various polymers in solution form. Transport studies of insulin together with the soluble polymers across Caco-2 cell layers showed the following ranking: TMC > DMEC > DEMC > TEC > chitosan which is in agreement with the strength of the cationic charge of the polymer. In comparison to the free-soluble polymers, the nanoparticles prepared by ionic gelation of the chitosan and its quaternized derivatives had much lower effect on decreasing the TEER by opening of the tight junctions. This can be explained by the reduced available amount of positive charge at the surface of the nanoparticles. In accordance with these results, the insulin loaded nanoparticles showed much less permeation across the Caco-2 cell monolayer in comparison to the free-soluble polymers. Mass balance transport studies revealed that a substantial amount of the nanoparticles has been entrapped into the Caco-2 monolayer or attached to the cell surface. It can thus be stated that while free-soluble polymers can reversibly open the tight junctions and increase the permeation of insulin, the nanoparticles had basically only a low effect on the opening of the tight junction and the paracellular transport of insulin across the Caco-2 cell monolayer. These data convincingly show that nanoparticles consisting of chitosan and its quaternary ammonium derivatives loaded with insulin are less effective in facilitating paracellular transport across Caco-2 cell monolayers than the corresponding free polymers.

Keywords: Caco-2 cells; Insulin nanoparticles; Trimethyl chitosan; Dimethylethyl chitosan; Diethylmethyl chitosan; Triethyl chitosan; Uptake; Transcellular transport; Paracellular transport; Mass balance studies.

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doi:10.1016/j.ejpb.2008.03.004

1. Introduction

The low oral bioavailability of the peptide and protein drugs is mainly due to their poor absorption through the gastrointestinal epithelium as well as the rapid hydrolytic and enzymatic degradation in contact with the gastrointestinal fluids [1,2]. In order to overcome the above obstacles, different delivery platforms have been developed for hydrophilic drugs. An effective strategy to improve peptide permeation is the incorporation of suitable permeation enhancers in the delivery system. Modern permeation enhancers are multifunctional polymers with mucoadhesive properties, the ability to locally inhibit intestinal enzymes, reversibly open the tight junctions, show no toxicity and they are ideally not absorbed. Hence they do not show perturbances of the membranes of the epithelial cells and are therefore specific for inducing only the paracellular hydrophilic drug transport. The intestinal epithelium regulates the passage of natural compounds and acts as a barrier for paracellular passive transport of large hydrophilic molecules. This absorption barrier is composed of a single layer of columnar epithelial cells joined at the apical surface by a tight junctional complex. The junctional complex forms a continuous seal, which segregates the apical from and the basolateral compartment and conveys size and charge selectivity due to the presence of negative charge in its structure [3,4]. Nanoparticulate drug delivery systems have attracted an immense attention as novel carriers for the delivery of lipophilic and hydrophilic substances as well as vaccines [5].There is a strong belief that nanoparticles of appropriate size can pass the mucosal membranes intactly and deliver their drug load into the systemic circulation. In the case of hydrophilic drugs, nanoparticles should be able to protect such drugs from degradation in the intestinal fluids and improve their penetration and permeation across the intestinal mucosal epithelium [6–8]. Suitable nanoparticles have mucoadhesive properties which are due to their particle size and the particle's superficial charge [9]. It has been shown that nanoparticles may be internalized into the intestinal epithelial cells [10].

Chitosan, a natural polyaminosaccharide, obtained by *N*-deacetylation of chitin is a non-toxic, biocompatible and biodegradable polymer that has good mucoadhesive properties in acidic environments [11–13]. Studies have shown that chitosan can promote the nasal absorption of insulin in rats and sheep and further enhance the paracellular transport of peptides in vitro and in vivo by opening the tight junctions [14–17]. However, chitosan is a polycation with an apparent pK_a of 5.5 and it loses its charge and precipitates in neutral and basic environments as prevailing in the intestine. Studies have shown that only protonated soluble chitosan in its uncoiled configuration can trigger the opening of the tight junctions and facilitate the transport of hydrophilic compounds [18,19]. Hence, chitosan can be used as an enhancer only in the proximal part of the intestine where the pH is close to its pK_a value. Subsequently, quaternized derivatives of chitosan, synthesized by introducing various alkyl groups to the NH_2-group of the chitosan molecule structure, were studied extensively. These derivatives were characterized to be drastically more soluble in neutral and alkaline environments of the intestine and hence more useful for drug delivery and absorption across the intestinal epithelium of the jejunum and ileum than the mother compound chitosan [19,20]. The permeation enhancing properties of these chitosan derivatives have been attributed to their ion pair interactions with the tight junctions and cellular membrane components to increase the paracellular permeation of hydrophilic compounds [20]. Trimethyl chitosan (TMC), Dimethylethyl chitosan (DMEC), Diethylmethyl chitosan (DEMC) and Triethyl chitosan (TEC) were synthesized by partial quaternization of chitosan as described by Sieval et al., Bayat et al. and Avadi et al., respectively [21–24]. The polymer charge density, determined by the substitution degree is a key factor in obtaining both the mucoadhesion and penetration enhancement towards the intestinal epithelium [25,26]. Chitosan derivatives were synthesized from low molecular weight chitosan and all had the same degree of quaternization of approximately $50 \pm 5\%$ which has been shown for TMC to have the highest penetration rate across the intestinal epithelium in vitro [25,27]. The molecular weight of the synthesized derivatives did not change significantly.

Accordingly, the aims of this study were to develop and compare the nanoparticulate systems based on chitosan, TMC, and the newly described DMEC, DEMC and TEC loaded with insulin generated by the polyelectrolyte complexation (PEC) method described previously [28] with a weight ratio of polymer to insulin of 50% (w/w) which has been described by the same authors as optimal and to measure their intestinal transmucosal insulin transport across Caco-2 cell layers. The flux data were compared afterwards with the insulin fluxes across Caco-2 cell layers obtained after application of the various aqueous insulin/polymer solutions. The obtained results convincingly show that nanoparticles consisting of chitosan and its quaternary ammonium derivatives loaded with insulin are much less effective in facilitating paracellular transport across Caco-2 cell monolayers than the corresponding free polymers.

2. Materials and methods

2.1. Materials

ChitoClear® chitosan (viscosity 1% w/v solution, 22 mPa s, 98% deacetylated) was purchased from Primex, Iceland. Human insulin was a generous gift from Exir Pharmaceutical Company (Lorestan, Iran). The TMC, DMEC, DEMC and TEC were synthesized in our laboratory as described previously [21–24].

2.2. Determination of the molecular weight of chitosan and its derivatives

The biopolymer analysis was performed with a triple detection size-exclusion-chromatography (SEC^3) on a

TDA max SEC system (Viscotek, USA). The detection was operated by a differential refractometer at $\lambda = 660$ nm and a right angle light scattering detector (RALS) with a 3-mW He/Ne laser at $\lambda = 670$ nm. A dn/dc value of 0.163 was used. Intrinsic viscosities were measured on a four-capillary, differential Wheatstone bridge viscometer. A degassed 0.3 M AcOH/0.3 M AcONa buffer (pH 4.5) with 1% ethylene glycol was used as eluent. To ensure a low light scattering noise level the eluent was filtrated through a 0.2 μm filter. Chitosan samples dissolved in the eluent at a concentration of 0.3–1 mg/ml were filtrated through a 0.45-μm filter prior to analysis to remove aggregates. Injection volume varied from 10 to 100 μl and the flow rate was maintained at 0.7 ml/min.

2.3. Preparation and characterization of insulin loaded nanoparticles

The insulin loaded nanoparticles were prepared from chitosan, TMC, DMEC, DEMC and TEC using polyelectrolyte complexation as described earlier [28]. The complex formation was a result of the positively charged polymer and the negatively charged insulin. To obtain such a complex, a solution of chitosan and each quaternized derivatives was prepared in 0.25% acetic acid and water, respectively, and the pH was adjusted to 5.0 so that more than 90% of the amine groups were protonated. The insulin powder was dissolved in 0.01 N HCl to obtain a solution and the pH was adjusted to 8.0 using 1 N NaOH. The insulin solution was subsequently added to the polymer solution at a 1:1 ratio (w/w) in a beaker under gentle magnetic stirring at room temperature. The concentration of the polymer and insulin was adjusted to obtain a final insulin concentration of 2.0 mg/mL. An opalescent, colloidal suspension was immediately formed to indicate the formation of nanoparticles. The morphology of the nanoparticles was observed by TEM-100CXII (JEOL, Tokyo, Japan). Insulin loading was calculated 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, Milford, Massachusetts, 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 [29]. The column used was C8 (Capital HPLC, Edinburg, Scotland) 250 × 4.6 mm equipped with a C8 (Capital HPLC, Edinburg, Scotland) 10 × 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 the insulin association efficiency was calculated as follows:

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

The characteristics of the obtained nanoparticles are presented in Table 1.

2.4. Zeta potential measurement

Particle size and the zeta potential of the nanoparticles were measured using a Malvern Zeta sizer (3000 HS, Malvern, Worsc, UK) with a folded capillary cell (DTS, 1060). The zeta potential of the free-soluble polymers was measured by dissolving chitosan in 0.25% acetic acid, while the derivatives were dissolved in deionized water. The obtained solutions (2.0 mg/mL) were then diluted at a 1:10 ratio using deionized water that was passed through a 0.22-μm filter and ultrasound for 6–10 min. The measurements were done on the polymer solutions after 20 min to account for possible polymer aggregation. It should be noted that zeta potential measurements are not dependent on the size of the particles but the surface charge of the particles. The nanoparticle suspensions were diluted in pre-filtered deionized water as described above to obtain a dilution of 0.01 mg/ml as recommended by the instrument's instruction to avoid multiple scattering.

2.5. Caco-2 cell cultures

Caco-2 cells, with a passage number 36–42, were obtained from American Type Culture Collection (Rockville, MD, USA). The cells were cultured on polycarbonate membrane filters (pore size 0.4 μm, area 4.7 cm^2) in Corning Transwell 6-well plates (Corning Incorporated, Europe) at a seeding density of 10^4 cells/cm^2. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, pH 7.4), supplemented with 1% v/v non-essential amino acids, 10% v/v heat-denatured fetal calf serum

Table 1
The mean molecular weight (M_w) and intrinsic viscosities ([η]) of chitosan and its alkylated derivatives

Polymer	M_w (kg/mol)	M_n (kg/mol)	M_w/M_n	η (dl/g)	Rh (nm)	M–Ha	Zeta-potential
TMC	105	40	2.76	1.15	11.3	0.73	43.2 ± 1.1
DEMC	123	47	2.61	1.58	13.4	0.72	40.0 ± 1.2
TEC	135	59	2.28	2.37	15.9	0.65	39.9 ± 2.1
DMEC	121	49	2.83	3.03	23.9	0.69	41.1 ± 1.1
Chitosan	126	36	3.47	2.82	16.0	0.72	24.2 ± 0.9

M_w, molecular weight, [η], intrinsic viscosity, number average M_w, Rh, hydrodynamic radius (nm), MH-a, Mark–Houwink constant a.

(FCS), 160 U/mL benzyl-penicillin and 100 U/ml streptomycin sulfate (Sigma Chemical, St. Louis, MO, USA). The culture medium was added to both the apical and the basolateral compartments and was changed every second day for 21 days. The cells were maintained at 37 °C, in an atmosphere of 95% air and 5% CO_2 at 90% relative humidity. One hour before the experiments the medium was changed to the transport medium: Hank's balanced salt solution (HBSS) buffered with 30 mM *n*-(2-hydroxyethyl) piperazine-*n*-(2-ethanosulfonic acid) (HEPES) at pH 5.5 and the cells were allowed to equilibrate for 1 h.

2.6. Measurement of the insulin release from the nanoparticles

The in vitro release profiles of insulin from all nanoparticles have been described in previous publications [30] and will be only shortly mentioned. The release curves of insulin obtained with all five polymers are the same and can be superimposed. In 0.01 N HCl as dissolution medium, approximately 90% of the insulin has been released within 30 min. At pH 6.8 (PBS buffer), about 50% of insulin is released within 30 min.

2.7. Measurements of the transepithelial electrical resistance (TEER)

The transepithelial electrical resistance (TEER) was measured using a Milicell® ERS meter (Millipore Corp. Bedford, MA, USA) connected to a pair of chopstick electrodes to ensure the integrity of the monolayers formed on the filters. TEER measurements were also performed during the experiments to check the effect of soluble polymers and the nanoparticles on opening the tight junctions at predetermined time intervals of 0,1, 2, 4, 8 and 24 h. The experiments were done in triplicate.

2.8. Permeability studies

For the transport studies insulin was used in free form, in the presence of free-soluble polymer and in the form of nanoparticle suspensions prepared from chitosan, Trimethyl chitosan (TMC), Dimethylethyl chitosan (DMEC), Diethylmethyl chitosan (DEMC) and Triethyl chitosan (TEC). Consequently, 2.0 mL of 0.25 mg/mL final concentration of insulin and 0.25 mg/mL polymer were added to the apical side of the cell culture dish. Samples of 200 μl were withdrawn from the basolateral part at predetermined time of 15, 30, 45, 60, 120 and 240 min and replaced with equal volumes of fresh HBSS–HEPES. The samples were analyzed for the insulin content using the HPLC method as described previously [29]. After completion of the transport studies, the samples were carefully removed from the apical part and the cell monolayers were rinsed with HBSS–HEPES and this medium was then replaced with culture medium. The monolayers were allowed to incubate for 24 h at 37 °C in regular cell culture conditions. The TEER was monitored during this period up to 24 h. Results were corrected for dilution and expressed as cumulative transport with time. All the experiments were done in triplicate.

Apparent permeability coefficients (P_{app}) were calculated according to the following equation:

$$P_{app} = (dQ/dt) \cdot (1/A \cdot C_0 \cdot 60)$$

where P_{app} is the apparent permeability in cm/s, dQ/dt is the permeability rate, A is the diffusion area of the monolayer (cm^2), and C_0 is the initial concentration of the insulin. Statistical differences were calculated using one way ANOVA at a significant level of $P < 0.05$.

2.9. Mass balance studies

After the completion of the transport study, samples were removed from both the basolateral and the apical compartments. The samples were analyzed for insulin contents using HPLC as described previously [29]. The measured insulin on the apical part was compared to the initial insulin on the apical part as well as the amount permeated from the basolateral compartment to decide upon the fate of the insulin.

2.10. Trypan blue exclusion

After the completion of the insulin transport experiments, both the apical and the basolateral compartments of the monolayer were rinsed gently with sterile 0.01 M phosphate-buffered saline (PBS), pH 7.4. A solution of 0.1% Trypan blue in PBS buffer was added to the apical part and the monolayers were incubated for 30 min at 37 °C. Subsequently, the solutions were removed from both sides and the monolayers were rinsed gently with PBS to remove excess dye. The filters were cut carefully from the inserts and transferred on a coverslip. The monolayers were then checked by a light microscopy for the exclusion of Trypan blue. If the intact monolayers showed no inclusion of dye they were considered to be viable, if the cells, however, showed dye inclusion they were considered to be damaged.

3. Results and discussion

3.1. The polymer characterization

Table 1 presents the result of zeta potential, the molecular weight and intrinsic viscosities of the polymers. Accordingly, there was a significant difference between the zeta potential of chitosan and the derivatives ($p < 0.05$). The zeta potential of the chitosan derivatives, however, was very close to one another. This may be explained as chitosan by nature contains a less positive surface charge while the derivatives contain a higher positive surface charge. The higher the positivity of the surface charge in a polymer may result in a higher ability to inter-

act with the tight junctions and cellular membrane components to increase the paracellular permeation of hydrophilic compounds. Although the size and the configuration of the polymers are also important factors in their interaction with the cellular components, methyl groups are usually very compact and show less steric hindrance in comparison with the ethyl substituted groups.

Moreover, the M_w and the intrinsic viscosities of chitosan and chitosan derivatives are presented in Table 1. The results suggested that the molecular weight of the synthesized derivatives did not change significantly. The derivatives showed a lower intrinsic viscosity in comparison with the initial chitosan. This can be due to the fact that the derivatives have a more dense structure and become heavier through the synthetic reactions.

3.2. Morphology of the nanoparticles

According to the TEM picture shown in Fig. 1, morphologically the nanoparticles look round to oval in shape and have a relatively smooth surface.

3.3. Effect of polymers in free form and in nanoparticle suspension on TEER of Caco-2 cell monolayer

The effects of soluble polymers and nanoparticles in opening the intestinal tight junctions were studied by measuring the TEER values across the Caco-2 cell. The results are presented as the percentage of the initial values at $t = 0$ min and are shown in Fig. 2. According to Fig. 2, the effect of the free-soluble polymers in opening the tight junction was immediate. After the apical addition of the free-soluble polymers to the monolayers together with the insulin solution, a significant TEER decrease was observed within the first hour which remained stable during the course of the experiment. Moreover, among the polymers used chitosan had the least effect on opening the tight junctions (about 75% of the initial value) and TMC had the strongest effect (about 45% of the initial value). The effect of the other three polymers namely, DMEC, DEMC and TEC was comparable and was about 60% of the initial value. These results were significant in comparison to the free insulin used as the negative control ($p < 0.05$) that had no effect on the reduction of the TEER. Hence, the chitosan derivatives with a substitution degree of about $50 \pm 5\%$ were able to significantly decrease the TEER at pH values present in the small intestine. This ability of chitosan derivatives to decrease the TEER can be explained by the possible interactions of their positive surface charge with the anionic components of the glycoproteins on the surface of the epithelial cells or the fixed negative charges of the interior of the tight junctions [31]. It is known that the surface charge of the molecules is independent of their size. Hence it is possible to measure the surface charge of the polymer solutions which are not really solutions but macromolecular dispersions. The surface charge density of the polymers has a direct effect on their ability to interact with the components of the tight junction, i.e. to open the paracellular pathway and increase the permeability of hydrophilic peptide drugs. The results obtained from the zeta potential of the polymers, Table 1, correlate very well with the ability of chitosan and each derivative to reduce the TEER and open the tight junction. The TEER measurement after the apical application of the nanoparticle suspensions on top of the monolayers indicates that the TEER was reduced much less than in the presence of free-soluble polymer (Fig. 3). Accordingly, the polymers in the nanoparticulate forms had much less effect on opening of the tight junction and hence a much lower reduction was observed in the TEER measurement during the course of the experiment. This is also explained by the surface charge density of the nanoparticles. According to Table

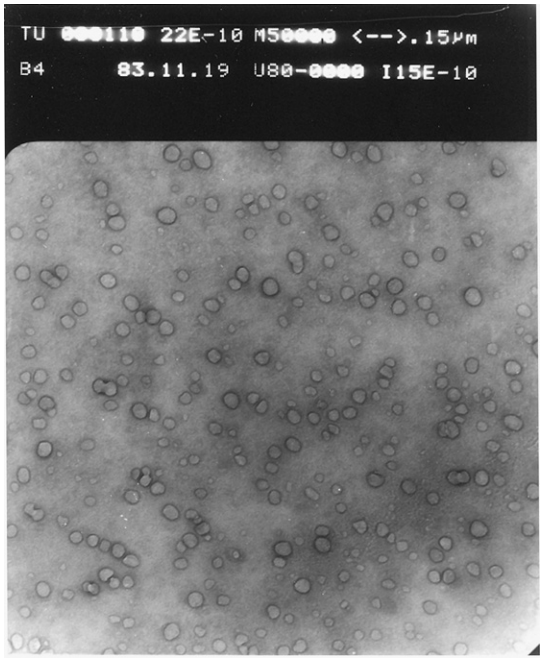

Fig. 1. The TEM picture of the insulin-loaded nanoparticles made by the PEC method.

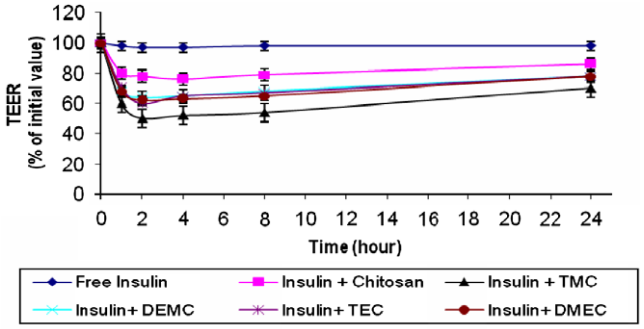

Fig. 2. Effects of free-soluble polymers on TEER of Caco-2 cell monolayer. After 240 min, the monolayers were rinsed with HBSS–HEPES and culture medium was applied on the monolayers. Data are expressed as mean ± SD of 3 experiments the difference ($p < 0.05$) is considered significant.

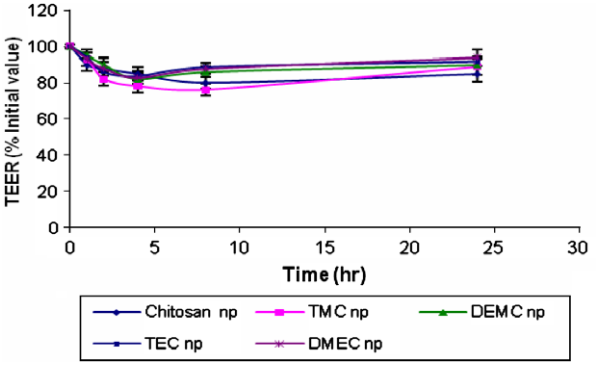

Fig. 3. Effects of nanoparticles on TEER of Caco-2 cell monolayer. After 240 minutes, the monolayers were rinsed with HBSS–HEPES and culture medium was applied on the monolayers. Data are expressed as means ± SD of three experiments the difference ($p < 0.05$) is considered significant.

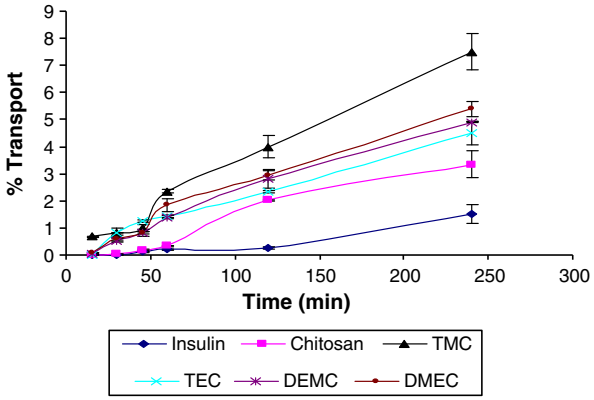

Fig. 4. Cumulative transport of insulin across Caco-2 cell monolayers using free-soluble polymers in HBSS–HEPES medium. Data are expressed as means ± SD of three experiments the difference $p < 0.05$ is considered significant.

Table 2
The results of insulin loading, size of nanoparticles (np), polydispersity and zeta potential of the nanoparticles prepared by PEC method using chitosan, TMC, DMEC, DEMC and TEC ($n = 3$)

Nanoparticles	% Insulin loading	Size (nm)	Poly dispersity	Zeta potential (mV)
Chitosan (np)	90 ± 5	220 ± 20	0.25	+15.9 ± 0.5
TMC (np)	70 ± 5	195 ± 20	0.32	+22.0 ± 1.2
DMEC (np)	75 ± 5	205 ± 15	0.40	+21.2 ± 1.2
DEMC (np)	75 ± 5	220 ± 20	0.45	+19.0 ± 0.9
TEC (np)	69 ± 5	210 ± 10	0.20	+21.0 ± 1.0

nm, nanometer, mV, millivolt.

2, the zeta potential of chitosan and its quaternized derivatives in the nanoparticle form is less than in their free-soluble form. As the nanoparticles were prepared by the polyelectrolyte complexation, i.e. the positive charge of the polymers interacted with the negatively charged insulin to result in the nanoparticle suspension. Subsequently, the amount of available positive charge on the surface of the nanoparticles was less in comparison to that of the free-soluble polymers. Moreover, it can be stated that nanoparticles – if at all – are mainly transported via an intracellular pathway, i.e. endocytosis or transcytosis across the Caco-2 cells and not along the paracellular pathway. This is in accordance with the study of Ma and Lim [10]. These authors also have shown that chitosan nanoparticles can reduce the TEER, however to a stronger extent than what was found in this study. However, this can be due to the difference in size and zeta potential of the obtained nanoparticles in their study. Their conclusion stating that basically no insulin was transported across the cellular monolayer using nanoparticles correlates quite well with our findings.

3.4. Insulin transport studies

Human insulin was used as a relevant model drug to determine the increase of paracellular permeability within Caco-2 cell monolayers using the polymers in free-soluble and in nanoparticulate forms. As shown in Fig. 4, the chitosan and to a greater extent its derivatives caused a significant increase in the transport of insulin across the monolayer. According to Fig. 4, TMC showed the highest increase on the permeability of insulin and chitosan had the least influence on the transport of insulin across the Caco-2 cell monolayer. The influence of DMEC, DEMC and TEC in inducing permeation of insulin across the monolayer was within a narrow range. The following ranking: TMC > DMEC > DEMC = TEC > chitosan could be obtained. These results correlate well with the results obtained from the TEER measurements. TMC with the highest reduction of the TEER values was also most effective in inducing the permeation of insulin across the monolayer. Conversely, chitosan with the weakest reduction on the TEER across the monolayer caused the least permeation of insulin across Caco-2 cells. Moreover, the other three derivatives with the same effect on the TEER had very similar influence on the permeation of insulin.

In Fig. 5, the effect of nanoparticles on the induction of paracellular transport of insulin across the Caco-2 cell

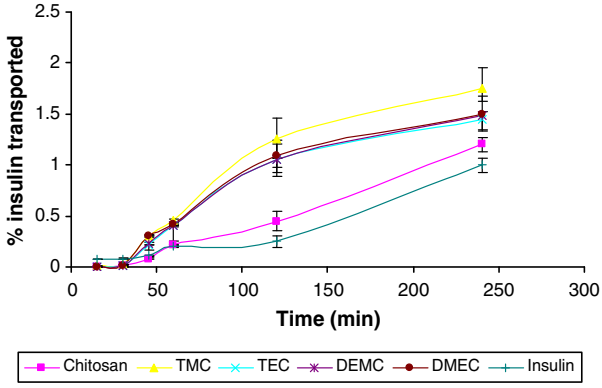

Fig. 5. Cumulative transport of insulin across Caco-2 cell monolayers using nanoparticles in HBSS–HEPES medium. Data are expressed as means ± SD of three experiments the difference $p < 0.05$ is considered significant.

Table 3
Apparent permeability (P_{app}) for Insulin transport across Caco-2 cell monolayers using free-soluble polymer and nanoparticulate suspensions (n = three experiments) the difference $p < 0.05$ is considered significant

Polymer	Average $P_{app} \times 10^{-8}$ cm/s
Insulin	0.3 ± 0.05
Chitosan (np)	0.2 ± 0.19
TMC (np)	0.45 ± 0 .12
DMEC (np)	0.39 ± 1.1
DEMC (np)	0.35 ± 0.21
TEC (np)	0.40 ± 0.25
Chitosan	0.8 ± 0.4
TMC	1.84 ± 1.1
DMEC	1.33 ± 0.9
DEMC	1.11 ± 0.82
TEC	1.2 ± 0.55

monolayer is presented. According to Fig. 5, the effect of nanoparticles in facilitating insulin transport across the monolayer was much lower than that of the soluble polymers. The TMC nanoparticle has a slightly higher ability to transport insulin than chitosan and the other derivatives. This, however, is not significant and may be explained by the increase in the cationic charge of TMC-nanoparticles' zeta potential. The decrease in permeation with nanoparticles in comparison to the free-soluble polymers can be explained by the much lower TEER reduction caused by the nanoparticles. Additionally, the enhancing ability of polymers in the nanoparticulate and soluble forms can be explained by the strong difference in the surface charge density of free polymers compared to the nanoparticles. Release studies of insulin from the nanoparticles in 0.01 N HCL showed a quick insulin release up to 90% of the incorporated dose within 30 min and to about 50% in PBS buffer pH 6.8 (data not shown). As a result of these release profiles the insulin transport across the Caco-2 cell layers is controlled by the transport across the opened tight junctions and not by the insulin release from the nanoparticles.

The calculated apparent permeability values of the free-soluble polymers and nanoparticles prepared with different polymers are presented in Table 3. According to Table 3, insulin and TMC showed the lowest and highest apparent permeability values, respectively. The values for DMEC, DEMC and TEC, although significantly increased in comparison to chitosan, are lower than that of TMC. Chitosan also showed an increase in permeation enhancement of insulin, however, its apparent permeability value is the lowest of all polymers investigated. Moreover, the apparent permeability coefficients of the nanoparticles prepared with different polymers indicate that, even though the nanoparticles are effective in permeating insulin across the Caco-2 cell monolayer, their enhancing effect is less pronounced than the free-soluble polymer.

3.5. Mass balance studies

Mass balance transport studies measuring the apical and basolateral insulin concentrations after application of the insulin nanoparticles revealed that a substantial amount of the nanoparticles must have been entrapped into the Caco-2 cell monolayers and/or they are attached to the surface of the cells and the insulin has not been transported across the cell monolayer. In the case of the free-soluble polymer, the amount of insulin present on the apical part was higher than the amount found with the nanoparticles; as a result of this, the insulin transported across the Caco-2 cell monolayer was also found to be higher than in the nanoparticulate form. This can indicate that the transport of insulin using free-soluble polymer is mainly via opening the tight junctions and via the paracellular pathway, while the insulin nanoparticles are mainly transported by the intracellular pathway. Previous studies have shown that once the nanoparticles are inside the cells they may get entrapped within the cellular components such as the Golgi apparatus and endoplasmic reticulum [31]. Also our in-vitro release studies indicate that only 50% of the insulin is released from the nanoparticles at pH 5.0 within the first hour [data not presented]. This may also account for the lower permeation of insulin nanoparticles across the Caco-2 cell monolayer in comparison to the free-soluble polymer. The apical and the basolateral concentrations and the differences between both values regarding the total amount of added insulin are summarized in Table 4. This difference is less pronounced in the amount of insulin on

Table 4
Insulin concentration in the apical and basolateral compartments (incubation time 240 min, $n = 3$)

Polymer	μg Insulin apical part (t_0)	μg Insulin basolateral part	μg Insulin apical part ($t = 240$ min)	μg Insulin apical + basolateral	% Insulin missing
Insulin	2000	30 ± 0.3	1960 ± 20	1990 ± 20.3	0.5
Chitosan	2000	67 ± 0.5	1874 ± 53	1941 ± 53.5	2.9
TMC	2000	150 ± 0.7	1690 ± 62	1840 ± 62.7	8.0
DMEC	2000	108 ± 0.4	1788 ± 75	1896 ± 75.4	5.2
DEMC	2000	90 ± 0.3	1657 ± 76	1747 ± 76.3	12
TEC	2000	98 ± 0.4	1758 ± 74	1856 ± 74.4	7.2
Chitosan (np)	2000	24 ± 0.1	1591 ± 66	1615 ± 66.1	19
TMC (np)	2000	35 ± 0.1	1380 ± 46	1415 ± 46.1	29
DMEC (np)	2000	30 ± 0.2	1456 ± 55	1476 ± 55.2	26
DEMC (np)	2000	29 ± 0.1	1490 ± 65	1519 ± 65.1	24
TEC (np)	2000	29 ± 0.2	1440 ± 74	1469 ± 57.2	27

the apical and the basolateral compartment with the free-soluble polymer indicating that the permeation of insulin in the presence of free-soluble polymer is predominantly transported via opening the tight junction and not through the intracellular pathway. Degradation of insulin cannot be fully ruled out as it can count for some of the insulin missing. However, as the missing amount with free insulin is only 0.5%, for the free-soluble polymers between 2.9% and 12% and for nanoparticles it is about 19–27%, it can be assumed that with nanoparticles a substantial amount of insulin (intactly or degraded) is entrapped within the Caco-2 cells.

3.6. Cytotoxicity studies

To determine the integrity of the cytoplasmic membrane after application of the polymers, trypan blue staining was used. Staining the Caco-2 cells with 0.1% trypan blue, revealed a less than 1% intracellular dye uptake by the cells [data not presented]. This demonstrates that the Caco-2 cells were still viable after the completion of the transport experiments in the presence of polymers. Hence, it can be stated that the polymers were not cytotoxic at the concentration used in this experiment.

4. Conclusions

In this study four chitosan derivatives TMC, DMEC, DEMC and TEC were synthesized and tested for their effect on the intestinal permeation of insulin in comparison to chitosan itself. Moreover, these derivatives were used to make nanoparticle systems by the polyelectrolyte complexation method. The transport efficiency of insulin in free form, in the presence of free-soluble polymers, across the Caco-2 cell monolayer, was shown to be higher than its loaded form in nanoparticles prepared with the same chitosan derivatives. Our study clearly shows that the chitosan derivatives in free-soluble form have higher positive surface charge and can be consequently considered as good permeation enhancers for hydrophilic drugs through the paracellular route in suitable drug delivery systems. The chitosan derivatives in nanoparticle form have less positive surface charge and their interactions with tight junction are limited and hence the drug transport across the monolayer is more likely through the transcellular pathway rather than by tight junction opening. However, further studies are required to prove the mechanism by which the nanoparticles are able to transport peptide drugs across the intestinal membrane. Mass balance studies of the insulin from the apical to the basolateral side of the Caco-2 cells have shown that a significant amount of the nanoparticles must be entrapped within the Caco-2 cell monolayers. In conclusion, it can be stated that for oral drug delivery of hydrophilic compounds like insulin and other peptides targeted to the intestinal tract, the chitosan derivatives in the free-soluble forms are more suitable as drug enhancers than in their nanoparticle forms loaded with insulin.

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