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Peroral delivery of insulin using chitosan derivatives: A comparative study of polyelectrolyte nanocomplexes and nanoparticles

Anchalee Jintapattanakit[a,b], Varaporn B. Junyaprasert[a], Shirui Mao[b,c], Johannes Sitterberg[b], Udo Bakowsky[b], Thomas Kissel[b,*]

[a] *Department of Pharmacy, Faculty of Pharmacy, Mahidol University, 447 Sri-Ayutthaya, Bangkok 10400, Thailand*
[b] *Department of Pharmaceutics and Biopharmacy, Philipps-Universität Marburg, Ketzerbach 63, D-35032 Marburg, Germany*
[c] *School of Pharmacy, Shenyang Pharmaceutical University, Wenhua Road 103, 110016 Shenyang, China*

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Abstract

Polymeric delivery systems based on nanoparticles (NP) have emerged as a promising approach for peroral insulin delivery. Using a trimethyl chitosan (TMC) and a PEG-*graft*-TMC copolymer, polyelectrolyte complexes (PEC) and nanoparticles were prepared and their properties were compared. The amount of insulin was quantified by HPLC and the stability of PEC and NP upon exposure to simulated gastrointestinal (GI) fluid was monitored by dynamic laser light scattering. It was shown that polymer/insulin (+/−) charge ratio played an important role in PEC and NP formation. Stable, uniform, and spherical PEC/NP with high insulin association efficiency (AE) were formed at or close to optimized polymer/insulin (+/−) charge ratio, depending on polymer structure. All PEC were more stable in pH 6.8 simulated intestinal fluid (SIF) than NP. The PEC also appeared to play some role in protecting insulin from degradation at higher temperature and with proteolytic enzyme more efficiently than NP. On the basis of these results, polyelectrolyte complexation can be suggested as a potentially useful technique for generating insulin delivery systems for peroral administration.

Keywords: PEGylated trimethyl chitosan; Polyelectrolyte complexes; Nanoparticles; Insulin; Peroral delivery

1. Introduction

Insulin is a protein composed of two polypeptide chains which are covalently bound by disulfide bonds between cysteine residues. Repeated injections are generally required for the treatment of insulin-dependent diabetes mellitus. Although peroral application is considered as the most convenient route of drug administration, especially in long-term treatment, it is well known that the bioavailability of insulin after oral application is very low due to its instability in the gastrointestinal (GI) tract and its low permeability through the intestinal mucosa, requiring non-oral routes of delivery (Owens et al., 2003). New delivery approaches depend on protecting insulin against enzymatic degradation and enhancing their transport across the intestinal mucosa into the systemic circulation. Various approaches have been proposed to overcome barriers and to attain better oral bioavailability, including the use of surfactants, permeation enhancers, protease inhibitors, enteric coatings, carrier systems and chemical modifications of insulin (Morishita et al., 1992, 1993; Yamamoto, 1994; Carino et al., 2000; Nakashima et al., 2004). Amongst these, the use of colloidal polymeric particulate delivery systems, particularly mucoadhesive nanoparticles (NP) represents a promising concept (Ponchel and Irache, 1998; Takeuchi et al., 2001). NP have been shown to protect peptide drugs from degradation in the GI tract and hence improve their bioavailability (Lenaerts et al., 1990; Damagé et al., 1997). Moreover, mucoadhesive properties also play an important role in oral drug delivery system by prolonging the residence time of drug carriers and also increasing the intimacy of contact between drug and mucus membrane at the absorption sites, thus enhancing the permeability as well as reducing degradation of drugs.

Trimethyl chitosan (TMC) is a partially quarternised derivative of chitosan which is well soluble in a wide pH range (pH 1–9). Moreover TMC shows mucoadhesive properties (Snyman et al., 2003; van de Merwe et al., 2004; Sandri et al., 2005). TMC has been proven to be a potent intestinal absorption enhancer

* Corresponding author. Tel.: +49 6421 282 5881; fax: +49 6421 282 7016.
E-mail address: kissel@staff.uni-marburg.de (T. Kissel).

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of peptide and protein drugs, especially in neutral environments where chitosan is ineffective as an absorption enhancer (Thanou et al., 2000a,b). The best and maximum permeation-enhancing results are achieved with degree of quarternisation ca. 48% (Hamman et al., 2003). However, most of the studies were performed with TMC solution. It is well known that most peptides are unstable in the presence of water leading to the impracticality of solution administration. Thus recent studies have focused on the development of solid oral dosage forms (Polnok et al., 2004a; van de Merwe et al., 2004). With regard to chitosan-based formulations, nearly all insulin-loaded NP have been prepared by ionotropic gelation with tripolyphosphate (TPP) counterions (Dyer et al., 2002; Ma et al., 2002; Pan et al., 2002a,b; Grenha et al., 2005). To the best of our knowledge, no report has been published about insulin-loaded TMC NP. Therefore, TMC-TPP insulin NP were prepared in this study and influence of process parameters on the properties of NP was investigated. Furthermore, recently, polyelectrolyte complexes (PEC) of insulin and TMC/PEG-*graft*-TMC copolymers in the absence of TPP have been developed for intranasal delivery by our research group, with an insulin loading efficiency of up to 95% and the enhancement of insulin uptake in Caco-2 cells (Mao et al., 2005a, 2006). We are interested in the point how the TPP in NP formulation affect the properties of particles compared to PEC. In case of no significant difference, it would be better to fabricate the carriers with only polymer and insulin in order to avoid unforeseen adverse effects from the third component.

Therefore, the aims of the present work were, first, to develop nano-carrier systems based on TMC and PEG-*graft*-TMC copolymer using two techniques; polyelectrolyte complexation and ionotropic gelation with TPP counter ion as carriers for the oral administration of insulin and, second, to elucidate the influence of TPP in formulation on colloidal and insulin stability.

2. Materials and methods

2.1. Materials

Chitosan (400 kDa) was purchased from Fluka (Schnelldorf, Germany) with a degree of deacetylation of 84.7%. Depolymerization was carried out as described previously to obtain chitosan with molecular weight (MW) of ca. 100 kDa (Mao et al., 2004). TMC with quarternisation degree of 40% were prepared by reductive methylation of the parent chitosans based on one reaction step with two subsequent addition steps (Polnok et al., 2004b). PEGylated TMC copolymer, PEG(5k)$_{40}$-*g*-TMC(100) was synthesized by grafting polyethylene glycol (PEG) 5 kDa onto TMC 100 kDa according to the method described previously (Mao et al., 2005b). The following nomenclature was adopted for the copolymer: PEG(X)$_n$-*g*-TMC(100) where X denotes the MW of PEG in kDa and the subscript n represents the average number of PEG chains per TMC macromolecule of 100 kDa. The number of PEG chains grafted per TMC macromolecule (graft ratio) was calculated from integrals of the ^{1}H NMR signals for PEG blocks at ~3.35 ppm (–OCH_3) and TMC blocks at ~3.0 ppm (–$N(CH_3)_2$) and ~3.3 ppm (–$N^+CH_3)_3$). The graft ratio (wt%) was calculated according to the following equation:

$$\text{Graft ratio (wt\%)} = \frac{\text{MW}_{\text{PEG}} \times n}{(\text{MW}_{\text{PEG}} \times n) + \text{MW}_{\text{TMC}}}$$

where MW_{PEG} is the molecular weight of PEG, MW_{TMC} the molecular weight of TMC, and n is the average number of PEG chains per TMC macromolecule calculated by ^{1}H NMR. The properties of the polymers used in the present work are summarized in Table 1.

Human recombinant insulin powder (26.2 IU/mg) was obtained as a gift from Aventis Pharma AG (Frankfurt, Germany). TPP and *N*-Benzoyl-L-arginine ethyl ester (BAEE) were purchased from Fluka. Trypsin (1840 BAEE IU/mg) was obtained from Sigma (Steinheim, Germany). All other chemicals and solvents were of analytical grade.

2.2. Preparation of insulin PEC

Polymer–insulin PEC were prepared by self-assembly, utilizing the electrostatic interactions between the positively charged polymers and negatively charged insulin as a driving force (Mao et al., 2006). Briefly, PEC were prepared by mixing equal volumes of insulin and polymer solutions at the desired ratio (Table 2) under gentle magnetic stirring. The mixture was then incubated for 20 min at room temperature. The pH of polymer and insulin solutions was adjusted to pH 7.4.

2.3. Preparation of insulin NP

Polymer–insulin NP were prepared, based on the ionotropic gelation with TPP (Calvo et al., 1997). Briefly, polymer solutions at appropriate concentration were prepared by dissolving the dry polymer powder in 10 mM Tris buffer, pH 7.4. TPP was dissolved in purified water at various concentrations. The NP were spontaneously formed upon incorporation of equal volume

Table 1
Characteristics of TMC400 and PEG(5k)$_{40}$-*g*-TMC(100)

Polymers (kDa)	Substitution (%)[a]	TMC content (w/w%)	Molecular weight (kDa)[b]	[η] (dL/g)[c]
TMC400	39.0[d]	100	400	2.15
PEG(5k)$_{40}$-*g*-TMC(100)	6.4	32.8 ± 1.0	300	ND

ND, not determined.
[a] Calculation based on the primary amino group content in chitosan.
[b] Calculation based on the composition of the copolymer.
[c] Intrinsic viscosity in 2% acetic acid/0.2 M sodium acetate.
[d] Degree of quaternization.

Table 2
Formulation and characteristics of the polyelectrolyte complexes and nanoparticles with insulin

Formulation	Mass ratio		Charge ratio (Pol/Ins)[a]	Particle size (nm)	Polydispersity index	Zeta potential (mV)	Association efficiency (%)	Process yield (%)
	Pol/Ins	TPP/Pol						
TMC400 PEC-1	0.3:1[b]		1:1	181 ± 7	0.13 ± 0.03	24.7 ± 1.5	83 ± 5	78 ± 1
TMC400 PEC-2	1:1		15:1	142 ± 3*	0.33 ± 0.02[c]	29.2 ± 1.7*	19 ± 1*	34 ± 1*
TMC400 PEC-3	2:1		33:1	104 ± 4*	0.37 ± 0.03*	33.4 ± 2.0*	24 ± 1*	10 ± 1*
TMC400 NP-1	0.3:1[b]	0.6:1	1:1	443 ± 28**	0.27 ± 0.07**	12.3 ± 0.3**	86 ± 2	73 ± 4
TMC400 NP-2	1:1	0.4:1	15:1	227 ± 6**	0.14 ± 0.04**	17.1 ± 0.9**	87 ± 2**	67 ± 1**
TMC400 NP-3	2:1	0.6:1	33:1	228 ± 4**	0.11 ± 0.02**	8.6 ± 0.7**	87 ± 0**	33 ± 0**
PEG(5k)$_{40}$-*g*-TMC(100) PEC-1	1:1[b]		5:1	232 ± 6	0.28 ± 0.06	24.3 ± 2.2	93 ± 0	45 ± 1
PEG(5k)$_{40}$-*g*-TMC(100) PEC-2	2:1		9:1	159 ± 5*	0.29 ± 0.03	27.5 ± 1.5*	36 ± 3*	24 ± 2*
PEG(5k)$_{40}$-*g*-TMC(100) NP-2	2:1	0.1:1	9:1	317 ± 11**	0.18 ± 0.05**	21.0 ± 1.4**	93 ± 0**	50 ± 4**

The initial concentration of insulin was 1 mg/ml in all the formulations. Mean ± S.D.
[a] Calculation based on the polymer/insulin mass ratio.
[b] Optimized polymer/insulin mass ratio for preparation of polyelectrolyte complexes.
* Statistically significant differences ($p < 0.05$) compared with that of PEC at optimal polymer/insulin mass ratio.
** Statistically significant differences ($p < 0.05$) compared with that of PEC at the same polymer/insulin mass ratio.

of TPP solution in the polymer solution under gentle magnetic stirring at room temperature. Insulin solution (1 mg/ml, pH 7.4) was premixed with equal volume of polymer solution before the addition of TPP solution. The final pH values were in the range of 7.4–7.7.

2.4. Characterization of insulin PEC/NP

Measurement of particle size and average count number (ACN) were performed on freshly prepared samples by photon correlation spectroscopy (PCS) using a Autosizer Lo-C (Malvern Instruments, Herrenberg, Germany) equipped with a 10 mW HeNe laser (633 nm) at 90° angle at the temperature of 25 °C. The ACN, measuring in term of kcps (kilo count per second), reflects the signal intensity, which is a measurement of particle concentration in a sample. Average values of particle size were calculated from the data of 10 runs.

The zeta potential of PEC and NP were obtained by laser Doppler velocimetry (LDV) using a Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany) at 25 °C in 10 mM Tris buffer pH 7.4. Zeta potential of samples are expressed as mean ± S.D. ($n = 10$).

To determine the association efficiency (AE) and process yield (PY), triplicate batches of PEC and NP were centrifuged at 14,000 rpm for 30 min at room temperature, and the insulin content in the supernatant was determined by RP-HPLC as described elsewhere (Simon et al., 2004). The pellet was then lyophilized and weighed. The AE and PY were calculated as follows:

$$\text{AE}\,(\%) = \frac{\text{total insulin amount} - \text{free insulin amount}}{\text{total insulin amount}} \times 100$$

$$\text{PY}\,(\%) = \frac{\text{particles weight}}{\text{total solids (polymer + insulin + TPP) weight}} \times 100$$

The morphological examination of PEC and NP were conducted by atomic force microscopy (AFM) (NanoWizard™, JPK Instruments, Berlin, Germany). The samples were diluted with ultra pure water and 10 μl of the diluted sample was applied to a freshly cleaved mica surface and allowed to adhere to the surface for a few minutes. The supernatant was removed and the samples were allowed to air-dry (ca. 10 min). Commercially available silicon tips attached to I-type silicon cantilevers with a length of 230 μm, a resonance frequency of about 170 kHz and a scan frequency of 0.8–1.1 Hz were used. All measurements were performed in tapping mode in order to avoid damage of sample surface (Shi et al., 2003).

2.5. Stability of PEC and NP in simulated GI fluids

In order to investigate the stability of PEC and NP in GI fluid, PEC and NP were mixed with pH 1.2 simulated gastric fluid (SGF) or pH 6.8 simulated intestinal fluid (SIF) without enzymes in different ratios. The integrity of the PEC and NP was monitored by dynamic laser light scattering.

2.6. Stability of insulin in PEC and NP

2.6.1. Temperature stability studies

Two milliliters of PEC or NP suspensions, containing 500 μg/ml insulin, were incubated at room temperature, 37 °C, and 50 °C. At predetermined time intervals (0, 15, 60, 145, 360 min), 100 μl aliquot was withdrawn and then 50 μl of 0.25% acetic acid solution was added to dissolve the particles followed by dilution with 10 mM Tris buffer to 1 ml. The insulin content was determined by HPLC. In addition, the stability of pure insulin at different temperatures was assessed under the same conditions. All samples were prepared in triplicate.

2.6.2. Enzymatic stability studies with trypsin

First, trypsin was dissolved in 10 mM Tris buffer pH 7.4, and the concentration was adjusted to 3000 BAEE IU/ml. One hundred microliter of the solution was then added to 900 μl of

insulin solution, PEC and NP suspensions containing 500 μg/ml of insulin. The initial concentrations of insulin and trypsin in the sample solution (1 ml) were 450 μg/ml and 300 BAEE IU/ml, respectively. Three vials of mixture were taken out at predetermined time points and the enzymatic reaction was stopped by the addition of 1 ml of acetonitrile/purified water mixture (3/1, v/v) containing 0.1% trifluoroacetic acid. The insulin concentration was then quantified by HPLC.

In vitro evaluation of trypsin activity in the presence of polymers was also investigated using the method described previously (Sakuma et al., 1997). Briefly, 900 μl of polymer solution corresponding to the polymer concentration in PEC or NP suspension was mixed with 100 μl of trypsin solution (3000 BAEE IU/ml in 10 mM Tris buffer pH 6.8). Subsequently the mixtures were incubated at 37 °C for 30 min. Afterward the samples were centrifuged at 14,000 rpm for 30 min. The enzymatic activity in the supernatant was determined from the change of the absorbance at 253 nm/min, using BAEE as the substrate. Two hundred microliter of supernatant was pipetted into a 1-cm cell. After adding 25.71 μg of BAEE dissolved in 3 ml of 10 mM Tris buffer (pH 6.8), the increase in absorbance (ΔA 253 nm) caused by the hydrolysis of this substrate to *N*-α-benzoylarginine (BA) was recorded at 1 min intervals for 5 min using UV/Vis spectrophotometer (UV-160, Shimadzu). In addition, the enzymatic activity in the absence of polymer was measured under the same condition.

2.7. Calculations and statistics

Results are depicted as mean ± S.D. from at least three measurements. The *t*-test or one-way ANOVA with the Scheffe test applied post hoc for paired comparisons were performed to compare two or multiple groups, respectively. All analyses were determined using the SPSS program (SPPS 9.0 for windows) and differences were considered to be significant at a level of $p < 0.05$.

3. Results and discussion

The aim of the present study was to investigate the influence of TPP in the formulation on the physical stability of particles and that of insulin by using 40%DQ TMC 400 kDa with two techniques, polyelectrolyte complexation and ionotropic gelation with TPP. Previously, it has been shown that PEG(5k)$_{40}$-*g*-TMC(100) could stabilize insulin in PEC more efficiently than chitosan 100 kDa due to hydrophilic PEG chains (Mao et al., 2006). Therefore, it was used here to examine whether it exhibited in the same trend with NP.

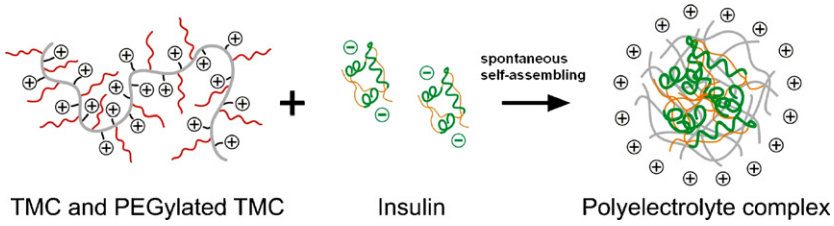
3.1. Preparation and characterization of insulin PEC

Self-assembled insulin PEC were prepared by electrostatic interactions between positively charged polymer and negatively charged insulin as a driving force (Scheme 1). Stable, uniform and nano-sized PEC can be formed only at ≥optimal polymer/insulin (+/−) charge ratio with a particle size in the range of 100–320 nm, with a positive surface charge. The optimal (+/−) charge ratio between polymer and insulin was polymer structure dependent: it was 1:1 for TMC400, compared to 5:1 for PEG(5k)$_{40}$-*g*-TMC(100) (Table 2). The PEC were spherical or almost spherical as revealed by their AFM images (Fig. 1a, b and d). However, excessive TMC400 chains are observed in PEC at polymer/insulin (+/−) charge ratio of 15:1 (Fig. 1b).

As shown in Table 2, soluble insulin PEC prepared at optimal polymer/insulin (+/−) charge ratio displayed high insulin AE and PY with low polydispersity index (PDI). On the contrary, the particle size, AE and PY of PEC decreased while the zeta potential and PDI increased with the increased (+/−) charge ratio of polymer to insulin ($p < 0.05$). These results agree well with the results reported by Fredheim and Christensen (2003) who found that the maximum yield of lignosulfonate–chitosan complexes was performed at optimal ligonosulfonate/chitosan (w/w) mixing ratio. The precipitated yield declined when increasing ratio of chitosan. These finding can probably be explained by the conformation of polymer (Snyman et al., 2004). When polymer concentration increased, the charge density and sterical hindrances between the pendant groups (methyl groups and PEG segments) increased, resulting in low flexibility of polymer chains. These hinder insulin to interact with polymer chains, causing low AE and PY. Additionally, a decrease of associated insulin amount in PEC and an increase of positively charged polymer chains towards the external aqueous medium could promote complex condensation, consequently a decreased particle size and high zeta potential PEC were obtained.

3.2. Preparation and characterization of insulin NP

Insulin NP were prepared by ionotropic gelation in a two-step procedure: (a) the complex formation between the two oppositely charged polyelectrolytes, polymer and insulin, and (b) cross-linking with TPP anions as depicted in Scheme 2.



Scheme 1. Schematic representation of insulin polyelectrolyte complex formation.

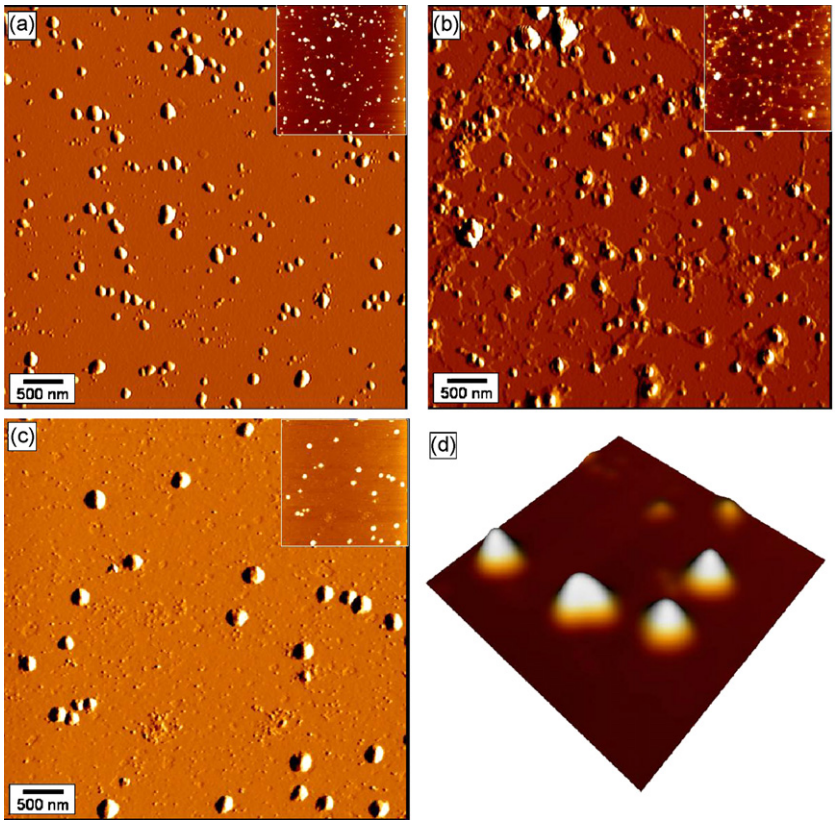
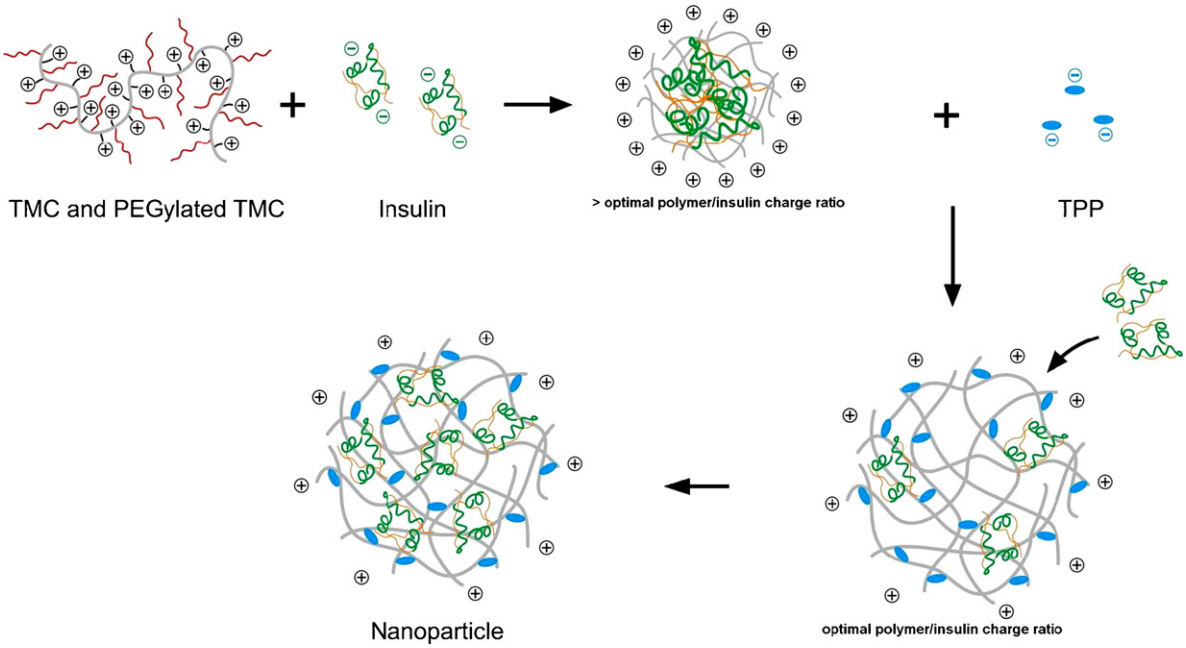


Fig. 1. Atomic force microscopy images (5 μm × 5 μm) of: (a) TMC400-insulin PEC at optimal polymer/insulin mass ratio of 0.3:1, (b) TMC400-insulin PEC at polymer/insulin mass ratio of 1:1, (c) TMC400-insulin NP at polymer/insulin/TPP mass ratio of 1:1:0.4, and (d) three-dimensional image of 0.3:1 TMC400-insulin PEC. The inserts are height mode of each image.

3.2.1. Optimal ratio of TPP and polymer in NP

Many studies have reported that the quantity of TPP in a given formulation has a significant effect on the protein encapsulation and characteristic of NP (Pan et al., 2002a,b; Janes and Alonso, 2003; Grenha et al., 2005). Therefore, the optimal amount of TPP in formulation was investigated in detail.

The optimal condition at which NP were formed was established using dynamic light laser scattering as previously



Scheme 2. Schematic representation of insulin nanoparticle formation.

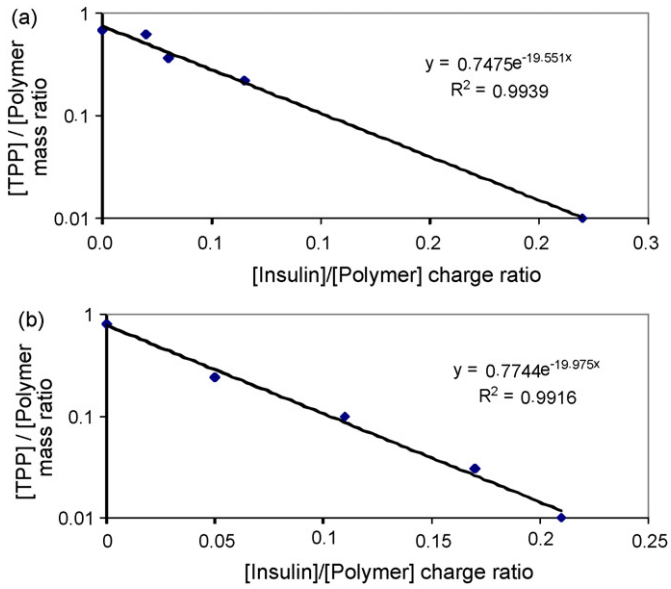


Fig. 2. Correlation between insulin/polymer (+/−) charge ratio and TPP/polymer mass ratio for nanoparticle formation of (a) 40%DQ TMC 400 kDa, (b) PEG(5k)40-*g*-TMC(100).

described (Mao et al., 2006). Mixtures of polymer and insulin with different polymer/insulin (+/−) charge ratio were titrated against TPP solution, and particle size and kcps values were measured. The points at which the kcps values reached a maximum or plateau were denoted as the end point of titration. When adding the TPP solution into polymer–insulin mixtures, kcps values of mixture increased until a plateau, referred to a TPP amount independent region which was reached at a specific TPP volume. After this point, the addition of an excess amount of TPP led to a drop in the kcps values together with considerable increase of particle size. A linear relationship was observed between the logarithm of optimal TPP/polymer mass ratio and insulin/polymer (+/−) charge ratio with correlation coefficient of 0.9939 for TMC400 and 0.9916 for PEG(5k)$_{40}$-*g*-TMC(100) as shown in Fig. 2.

Effect of TPP amount on the properties of NP was also evaluated using TMC400 as an example. In case of NP prepared at optimal TMC400/insulin (+/−) charge ratio of 1:1, no difference in AE and PY between TMC400-insulin NP and PEC prepared at the same polymer/insulin (+/−) charge ratio was observed (Table 2), but particle size of NP was significant larger than that of the PEC and flocculation occurred in a short period of time. However, in case of NP prepared at >polymer/insulin (+/−) charge ratio, compared to PEC at the same 15:1 TMC400/insulin (+/−) charge ratio, incorporation of increasing amounts of TPP with respect to TMC400 led to a significant increase in the particle size, AE and PY ($p < 0.05$) and led to a significant decreased ($p < 0.05$) zeta potential and PDI. Equilibrium was reached at the TPP/TMC400 mass ratio of 0.4:1, as shown in Table 3. Additionally, the results showed linear relationship as a function of the TPP amount with correlation coefficients of 0.9845, 0.9926, 0.9153 and 0.8770 for ACN, zeta potential, PY and AE, respectively.

Compared to insulin, TPP has a much smaller molecular with a higher negative charge density. It can dominate interaction of insulin with positively charged polymers causing a reduction in the positive charge density of polymers, and also in (+/−) charge ratio between polymer and insulin which can be seen from a reduction of zeta potential of particles when increasing TPP amount. The structure of particle is then loose, leading to a larger size and it is very likely that this structure allows them to capture more insulin (Scheme 2). Similar result was found by Grenha et al. (2005) who observed that insulin AE of chitosan NP increased with increasing TPP concentration. Recently, Boonsongrit et al. (2006) reported that adding TPP did not affect the entrapment efficiency of insulin–chitosan microparticles when microparticles were formed at optimal chitosan/insulin mass of 1.25:1. Therefore, it is reasonable to assume that polymer/insulin (+/−) charge ratio plays an important role in PEC and NP formation. The highest insulin AE of NP with a narrow size distribution could be achieved when polymer/insulin (+/−) charge ratio was close to optimal ratio by using specific TPP amount. Fig. 1c displays the AFM image of fresh TMC400-insulin NP prepared at the optimal condition.

3.2.2. *Effect of order of mixing*

In some cases, the mixing order influenced PEC or NP formation (MacLaughlin et al., 1998; Mao et al., 2006). In order to investigate whether the order of mixing affects the physicochem-

Table 3
Influence of TPP/polymer mass ratio on the properties of insulin nanoparticles[a]

Mass ratio (TPP:Pol)	Average count number (kcps)[b]	Particle size (nm)	Polydispersity index	Zeta potential (mV)	Process yield (%)	Association efficiency (%)
0.0:1	283 ± 18	142 ± 3	0.33 ± 0.02	29.2 ± 1.7	34 ± 1	19 ± 1
0.1:1	886 ± 95	217 ± 13	0.38 ± 0.05	26.3 ± 1.8	50 ± 6	46 ± 2
0.2:1 (1)[a]	1257 ± 113	205 ± 10	0.22 ± 0.03	22.5 ± 1.1	57 ± 6	76 ± 2
0.2:1 (2)[c]	1081 ± 86*	184 ± 6*	0.21 ± 0.02	22.6 ± 0.9	58 ± 4	70 ± 1*
0.4:1	2078 ± 147	227 ± 6	0.14 ± 0.04	17.1 ± 0.9	67 ± 1	87 ± 0
0.6:1	2641 ± 152	257 ± 9	0.14 ± 0.04	12.6 ± 1.0	50 ± 6	88 ± 0

The concentration of insulin and TMC are 1 mg/ml in all formulations. $n = 3$. Mean ± S.D.
[a] Nanoparticles were obtained by premixing insulin with polymer solution, prior to nanoparticle formation.
[b] Measured at 90° angle through a 100-μm pin hole.
[c] Nanoparticles were obtained by premixing insulin with TPP solution, prior to nanoparticle formation.
* Statistically significant differences ($p < 0.05$) compared with that of TMC400-insulin premixed NP.

ical properties of NP, taking 0.2:1 TPP/TMC400 mass ratio as an example, insulin was premixed with either the TPP solution or the polymer solution prior to NP formation. As shown in Table 3, no difference of PY was found between TMC400-insulin premixed and TPP-insulin premixed NP ($p > 0.05$), while bigger size with higher ACN and AE were observed with TMC400-insulin premixed NP ($p < 0.05$).

The results are inconsistent with previous reports by Ma et al. (2002) in which no influence of mixing order on the size and ACN of chitosan–insulin NP was observed. However, they did not investigate effect of mixing order on the insulin AE. We found that such effect influenced insulin AE of TMC400 NP. The higher AE were obtained with TMC400-insulin premixed NP. Similar results were also observed with PEG(5k)$_{40}$-*g*-TMC(100) NP (data not shown). This finding can probably be explained by competition between insulin and TPP to interact with polymers.

3.3. Colloidal stability in simulated GI fluids

In order to evaluate the stability of the PEC and NP in the GI fluids, various ratios of PEC and NP were diluted in either SGF or SIF and the integrity of particles was measured. It was found that all PEC and NP were not stable in SGF and dissociation appeared immediately after adding SGF. This is probably due to the electrostatic repulsion between polymer and insulin at the pH of 1.2 where they exhibit positive charges (the pK_a of TMC is approximately 6.0 (Kotźe et al., 1999) and apparent isoelectronic point of insulin is 6.4).

With regard to dilution with SIF, all PEC appeared to be stable when volume ratio of PEC/SIF was <1:2 with no apparent change in kcps values and particle size. 26% TMC400 PEC and 87% PEG(5k)$_{40}$-*g*-TMC(100) PEC dissociated when SIF was increased to the 1:5 PEC/SIF volume ratio. In contrast to NP, 30% dissociation NP immediately occurred at NP/SIF volume ratio of 1:1, irrespective to the NP structures and dissociating clearly increased with increasing volume of SIF. A linear correlation between the percent of kcps remained and the added volume of SIF was observed with regression coefficient of 0.996 for TMC400 and 1.000 for PEG(5k)$_{40}$-*g*-TMC(100).

Since we found no apparent change in particle size, only the evolution of kcps values are presented in Fig. 3 which clearly showed that the percentage kcps of NP was much less than that of PEC at the same dilution ratio. We hypothesized that the stability of PEC and NP depended on the electrostatic interaction intensity between polymer and insulin which decreased by shielding of counter ion with increasing ionic strength (increasing counter-ion concentration) (Knaul et al., 1999; Mao et al., 2006). Additionally, the attraction between polymer and insulin was reduced by TPP leading to a decrease in physical stability of NP compared to PEC prepared at the same pH. These finding were comparable to the results report previously by Boonsongrit et al. (2006) who found that most of insulin was released within 10 min from the chitosan–insulin microspheres in the pH 7.4 phosphate buffer and in the pH 3 HCl solution indicating the dissociation of microspheres. Possible explanation for the PEC and NP dissociation is that the system pH was close to pH 6.8 of SIF when increasing ratio of SIF. As suggested by Mao et al. (2006), the equivalent pH values for TMC and PEGlyated TMC-insulin PEC are approximately 7.3. We also evaluated the influence of pH on the NP formation and found that the equivalent pH value for TMC400-insulin NP was also approximately 7.3. At lower pH, the particle size increased with decreased system pH (data not shown).

3.4. Effect of ionic strength of the medium on the stability of PEC and NP

In order to confirm whether the electrostatic interaction between polymer and insulin influenced the stability of PEC and NP, TMC400 NP were prepared at different pH of 5.0, 6.8 and 7.4 and their stabilities in a series of concentrated sodium chloride solutions were evaluated. In general, the presence of sodium chloride reduces the electrostatic attraction between the oppositely charged of polymer and insulin by contributing to the counter-ion environment (Berger et al., 2004). It is well known that the charge density of insulin was pH dependent. At the pH of 5.0 where insulin exhibits weakly negatively charged, NP with particle size of 860 nm were obtained indicating a weak interaction between polymer and insulin. Thirty percent of NP dissociation was observed when ionic strength of the solution was 5 mM and 60% NP dissociation at ionic strength of the solution of 15 mM with no apparent change in particle size. On the other hand, NP with particles of 511 and 306 nm were obtained when preparing at pH 6.8 and 7.4, respectively. Only 20% dissociation of NP was found at the ionic strength of 25 mM. In the case of PEC, at pH 7.4, no apparent changes in kcps values and size of TMC400 and PEG(5k)40-*g*-TMC(100)-insulin PEC were observed when the ionic strength of medium is <25 mM.

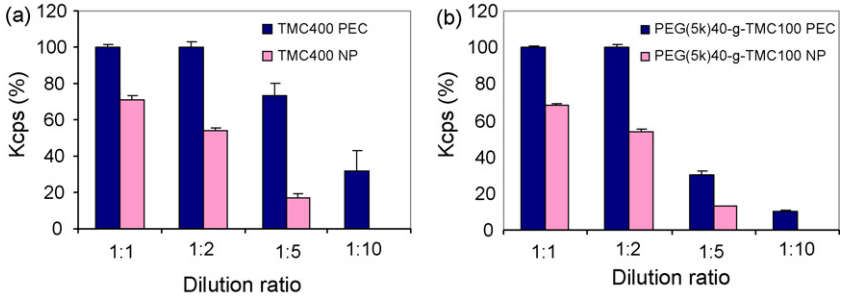


Fig. 3. Colloidal stability of: (a) 40%DQ TMC 400 kDa and (b) PEG(5k)$_{40}$-*g*-TMC(100) after diluting with pH 6.8 simulated intestinal fluid without enzymes. Each value represents the mean ± S.D. of three experiments.

Therefore, polyelectrolyte complexation is an efficient way to prepare nano-carrier systems.

3.5. Effect of temperature on the stability of insulin

Some studies indicated that elevated temperature facilitates PEC and NP formation with the low aggregation tendency (Mao et al., 2001, 2005a). Proteins are very labile molecules sensitive to thermal stress (Brange and Langkjaer, 1992; van de Weert et al., 2000; Frokjaer and Otzen, 2005). Therefore, stability of insulin in the PEC and NP at different temperatures was investigated and compared with that of free insulin solution.

The results of insulin stability at four different temperatures are shown in Fig. 4. It was demonstrated that the PEC and NP could protect insulin from degradation even at higher temperature. At temperature <50 °C, insulin itself was quite stable for at least 2.5 h and degradation was observed at 6 h even at room temperature.

In the case of PEC, they could protect insulin from degradation for at least 6 h even at temperature of 50 °C while approximately 50% of free insulin was degraded. Furthermore, no difference in results was observed between TMC400 and PEG(5k)$_{40}$-*g*-TMC(100) PEC. In the case of NP, they also could protect insulin at least 6 h even at 50 °C and their protecting effect at 50 °C was higher than that at 20 °C ($p < 0.05$). Akiyoshi et al. (1998) also observed this phenomenon. This is probably due to the facilitation of NP formation and compaction at elevated temperature which can be seen from the increased kcps values (ca. 10–20%) with a slight decrease in particle size (ca. 10%), compared to the value at 20 °C (data not shown). Generally, an increase in temperature increases entropy of system which is associated with the release of small counterions initially bond to the polymers, resulting in compaction of particles (Tsuchida and Takeoka, 1994; Fredheim and Christensen, 2003).

With regard to TMC400, the protecting effect of PEC was higher than that of NP, especially at 6 h ($p < 0.05$). These results could be explained from insulin association mechanism. PEC were formed by only columbic interactions between negatively charged insulin and positively charged polymer, which increased with increasing temperature resulting in more PEC (Mao et al., 2006). On the contrary, the ionic gelation method, cross-linked polymer chains by TPP to form reticular structure in which insulin could be captured and electrostatically interacted with remained positively charged polymers. That means the interaction between polymer and insulin could be impaired by TPP molecules. Therefore, the polyelectrolyte complexation is an efficient way to improve the stability of insulin.

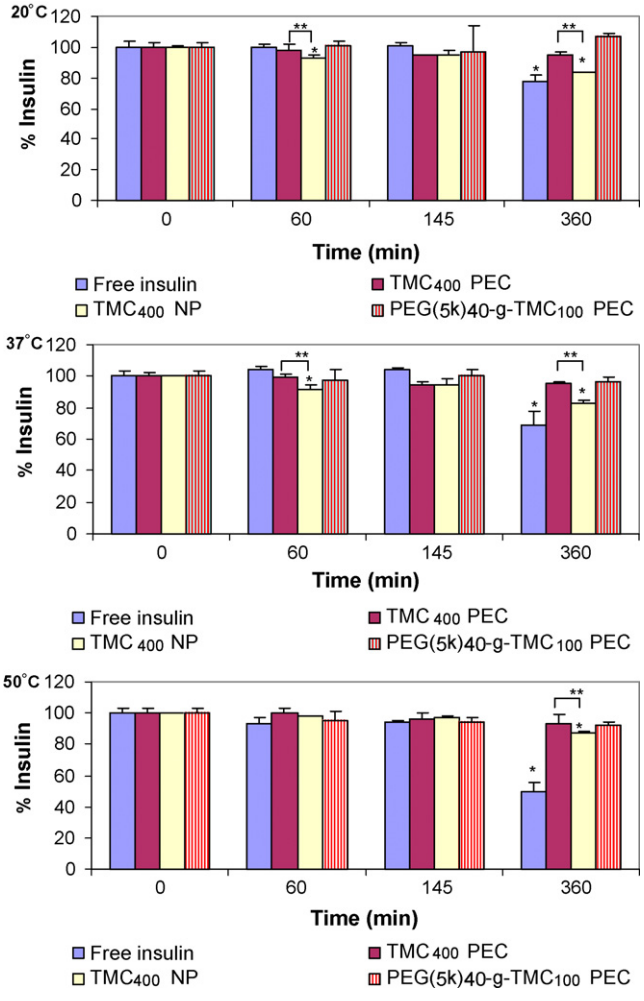


Fig. 4. Stability of insulin in TMC400 PEC, TMC400 NP and PEG(5k)40-*g*-TMC(100) PEC at different temperatures. *Statistically significant differences from the values of at 0 min ($p < 0.05$). **Statistically significant differences from the values of TMC400 PEC ($p < 0.05$).

3.6. Insulin protection from enzymatic degradation

To evaluate the potential role of PEC and NP in protecting insulin from enzyme presence in the digestive tract, the enzymatic stability of insulin was investigated in the presence of serine protease, trypsin. Fig. 5 depicts the residual amount of insulin after incubation of insulin alone and insulin associated PEC and NP with trypsin.

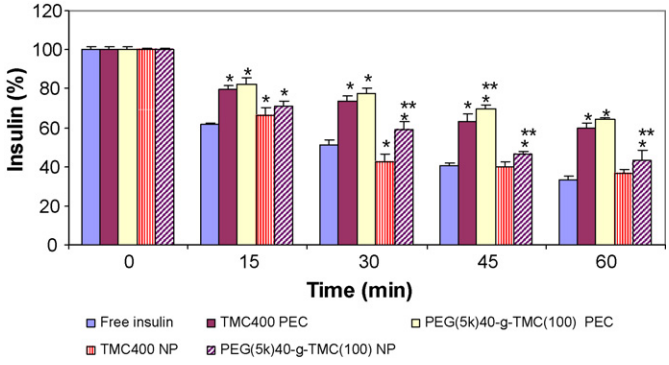


Fig. 5. Enzymatic degradation of insulin by trypsin. Each value represents the mean ± S.D. of three experiments. The initial concentrations of insulin and trypsin were 450 μg/ml and 300 BAEE IU/ml, respectively. TMC400 PEC = [P]/[Ins] of 0.3:1, TMC400 NP = [P]/[Ins]/[TPP] of 1:1:0.2, PEG(5k)$_{40}$-*g*-TMC(100) PEC = [P]/[Ins] of 1:1, PEG(5k)$_{40}$-*g*-TMC(100) NP = [P]/[Ins]/[TPP] of 2:1:0.2. *Statistically significant differences from the values of free insulin ($p < 0.05$). **Statistically significant differences from the values of TMC400 ($p < 0.05$).

Compared to free insulin solution, partial protection of insulin from trypsin digestion was observed with PEC ($p < 0.05$) and the protective effect of PEG(5k)$_{40}$-*g*-TMC(100) PEC was higher than TMC400 PEC ($p < 0.05$). With NP, it seemed that TMC400 NP did not protect insulin from trypsin digestion under similar conditions ($p > 0.05$) and the presence of TPP accelerated the degradation of insulin which can be seen from an increasing in degraded insulin with increased TPP concentration (data not shown). A linear relationship was observed between TPP concentration and degradation rate of insulin with correlation coefficient of 0.9856. Although PEG(5k)$_{40}$-*g*-TMC(100) NP could protect insulin from trypsin digestion ($p < 0.05$), the protective effect was much lower compared to its PEC ($p < 0.05$), probably as a consequence of the loose interaction of polymer and insulin by TPP. Bernkop-Schnürch and Dundalek (1996) observed that trypsin penetrated and digested proteins in NP.

We also examined the possible inhibitory effects of polymers on trypsin. At the polymer concentration corresponding to the concentration in PEC and NP, all polymers did not affect the trypsin activity (data not shown) which agrees well with previously reports (Kotźe et al., 1997; Leuβen et al., 1997). This indicated that the protective effect is unlikely due to inhibition of trypsin's activity but probably due to a shielding effect of polymer on insulin. This shielding effect is achieved through polymer/insulin interaction. Similarly, Malkov et al. (2005) reported insulin could be protected from trypsin digestion by binding of *N*-[8-(2-hydroxybenzoyl)amino]caprylate (SNAC) to insulin. Akiyoshi et al. (1998) also found the complexed insulin with cholesterol-bearing pullulan (CHP) was significantly protected from enzymatic degradation by α-crymotrypsin. Attack of the enzyme was effectively prevented because insulin was tightly complexed to the CHP self-aggregate.

When considering polymer structures, The protective effect of PEG(5k)$_{40}$-*g*-TMC(100) was higher than that of TMC400 both in PEC and NP ($p < 0.05$), probably due to a consequence of the steric effect of polyethylene glycol segments that hinders the enzyme access to the protein (Iwanaga et al., 1997; Yeh, 2000). These results are in agreement with observation made by Mao et al. (2006), suggesting that PEGylated TMC copolymers could improve the stability of insulin in PEC due to hydrophilic PEG chains. Taking all the above results into consideration, complexation with PEGylated TMC copolymers are a promising strategy for insulin carriers.

4. Conclusions

Self-assembled insulin PEC and NP were formed using TMC and PEGylated TMC copolymer. NP exhibited a bigger particle size and lower zeta potential than PEC at the same polymer/insulin mass ratio. The highest AE of NP with narrow size distribution was achieved at specific TPP amount depending on the polymer structure and the polymer/insulin (+/−) charge ratio. PEC showed higher stability in pH 6.8 simulated intestinal fluid than NP prepared at the same condition. PEC also protected associated insulin from degradation even at 50 °C and in the presence of trypsin more efficiently than NP. In addition, it was found that all polymers did not affect the activity of trypsin. Based on the results obtained, it was reasonable to assume that polymer/insulin (+/−) charge ratio played an important role in forming PEC and NP. Stable, uniform, spherical PEC and NP with high insulin AE could be formed at or close to optimal polymer/insulin (+/−) charge ratio, depending on the polymer structure. Physical stability of particles and associated insulin in PEC and NP depended on the electrostatic interactions between positively charged polymers and negatively charged insulin. These studies have contributed much to the understanding of PEC and NP formation with insulin.

In summary, polyelectrolyte complexation seems to be a potentially useful technique for fabricating insulin delivery systems for peroral administration. The mucoadhesive properties of PEC will be further studied.

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References

Akiyoshi, K., Kobayashi, S., Shichibe, S., Mix, D., Baudys, M., Kim, S.W., Sunamoto, J., 1998. Self-assembled hydrogel nanoparticle of cholesterol-bearing pullulan as a carrier of protein drugs: complexation and stabilization of insulin. J. Controlled Release 54, 313–320.

Berger, J., Reist, M., Mayer, J.M., Felt, O., Gurny, R., 2004. Structure and interactions in chitosan hydrogels formed by complexation or aggregation for biomedical applications. Eur. J. Pharm. Biopharm. 57, 35–52.

Bernkop-Schnürch, A., Dundalek, K., 1996. Novel bioadhesive drug delivery system protecting (poly)peptides from gastric enzymatic degradation. Int. J. Pharm. 138, 75–83.

Boonsongrit, Y., Mitrevej, A., Mueller, B.W., 2006. Chitosan drug binding by ionic interaction. Eur. J. Pharm. Biopharm. 62, 267–274.

Brange, J., Langkjaer, L., 1992. Chemical stability of insulin. 3: Influence of excipients, formulation, and pH. Acta Pharm. Nord. 4, 149–158.

Calvo, P., Remunan-Lopez, C., Vila-Jato, J.L., Alonso, M.J., 1997. Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers. J. Appl. Polym. Sci. 63, 125–132.

Carino, G.P., Jacob, J.S., Mathiauty, E., 2000. Nanosphere based on oral insulin delivery. J. Controlled Release 65, 261–269.

Damagé, C., Vonderscher, J., Marbach, P., Pinget, M., 1997. Poly(alkyl-cyanoacrylate) nanocapsules as a delivery system in the rat for octreotide, a long-acting somatostatin analogue. J. Pharm. Pharmacol. 49, 949–954.

Dyer, A.M., Hinchcliffe, M., Watts, P., Castile, J., Jabbal-Gill, I., Nankervis, R., Smith, A., Illum, L., 2002. Nasal delivery of insulin using novel chitosan based formulations: a comparative study in two animal models between simple chitosan formulations and chitosan nanoparticles. Pharm. Res. 19, 998–1008.

Fredheim, G.E., Christensen, B.E., 2003. Polyelectrolyte complexes: interactions between lignosulfonate and chitosan. Biomacromolecules 4, 232–239.

Frokjaer, S., Otzen, D.E., 2005. Protein drug stability: a formulation challenge. Nat. Rev. Drug Disc. 4, 298–306.

Grenha, A., Seijo, B., Remuñán-López, C., 2005. Microencapsulated chitosan nanoparticles for lung protein delivery. Eur. J. Pharm. Sci. 25, 427–437.

Hamman, J.H., Schultz, C.M., Kotźe, A.F., 2003. *N*-trimethyl chitosan chloride: optimum degree of quaternization for drug absorption enhancement across epithelial cells. Drug Dev. Ind. Pharm. 29, 161–172.

Iwanaga, K., Ono, S., Narioka, K., Morimoto, K., Masawo, K., Yamasita, S., Nange, M., Oku, N., 1997. Oral delivery of insulin by using surface coating

liposomes: improvement of stability of insulin in GI tract. Int. J. Pharm. 157, 73–80.

Janes, K.A., Alonso, M.J., 2003. Depolymerized chitosan nanoparticles for protein delivery: preparation and characterization. J. Appl. Polym. Sci. 88, 2769–2776.

Knaul, J.Z., Hudson, S.M., Creber, K.A.M., 1999. Improved mechanical properties of chitosan fibers. J. Appl. Polym. Sci. 72, 1721–1732.

Kotźe, A.F., de Leeuw, B.J., Leuβen, H.L., de Boer, A.G., Verhoef, J.C., Junginger, H.E., 1997. Chitosans for enhanced delivery of therapeutic peptides across intestinal epithelial: in vitro evaluation in Caco-2 cell monolayers. Int. J. Pharm. 159, 243–253.

Kotźe, A.F., Thanou, M.M., Lueßen, H.L., de Boer, B.G., Verhoef, J.C., Junginger, H.E., 1999. Effect of the degree of quaternization of *N*-trimethyl chitosan chloride on the permeability if intestinal epithelial cells (Caco-2). Eur. J. Pharm. Biopharm. 47, 269–274.

Lenaerts, V., Couvreur, P., Grislain, L., Maincent, P., 1990. Nanoparticles as a gastroadhesive drug delivery system. In: Lenaerts, V., Gurny, R. (Eds.), Bioadhesive Drug Delivery Systems. CRC Press, Florida, pp. 93–104.

Leuβen, H.L., Rentel, C.O., Kotźe, A.F., Lehr, C.M., de Boer, A.G., Verhoef, J.C., Junginger, H.E., 1997. Mucoadhesive polymers in peroral peptide drug delivery. IV: Polycarbophil and chitosan are potent enhancers of peptide transport across intestinal mucosae in vitro. J. Controlled Release 45, 14–23.

Ma, Z., Yeoh, H.K., Lim, L.-Y., 2002. Formulation pH modulated the interaction of insulin with chitosan nanoparticles. J. Pharm. Sci. 91, 1396–1404.

MacLaughlin, F.C., Mumper, R.J., Wang, J., Tagliaferri, J.M., Gill, I., Hinchcliffe, M., Rolland, A.P., 1998. Chitosan and depolymerized chitosan oligomers as condensing carriers for in vivo plasmid delivery. J. Controlled Release 56, 259–272.

Malkov, D., Angelo, R., Wang, H., Flanders, E., Tang, H., Gomez-Orellana, I., 2005. Oral delivery of insulin with the eligen® technology: mechanistic studies. Curr. Drug Deliv. 2, 191–197.

Mao, H., Roy, K., Troung-Le, V.L., Janes, K.A., Lin, K.Y., Wang, Y., August, J.T., Leong, K.W., 2001. Chitosan-DNA naoparticles as gene carriers: synthesis, characterization and transfection efficiency. J. Controlled Release 70, 399–421.

Mao, S., Shuai, X., Unger, F., Simon, M., Bi, D., Kissel, T., 2004. The depolymerization of chitosan: effects on physicochemical and biological properties. Int. J. Pharm. 281, 45–54.

Mao, S., Germershaus, O., Fischer, D., Linn, T., Schnepf, R., Kissel, T., 2005a. Uptake and transport of PEG-*graft*-trimethyl-chitosan copolymer-insulin nanocomplexes by epithelial cells. Pharm. Res. 22, 2058–2068.

Mao, S., Shuai, X., Unger, F., Wittmar, M., Xie, X., Kissel, T., 2005b. Synthesis, characterization and cytotoxicity of poly(ethylene glycol)-*graft*-trimethyl chitosan block copolymer. Biomaterials 26, 6343–6356.

Mao, S., Bakowsky, U., Jintapattanakit, A., Kissel, T., 2006. Self-assembled polyelectrolyte nanocomplexes between chitosan derivatives and insulin. J. Pharm. Sci. 95, 1035–1048.

Morishita, M., Norishita, I., Takayama, K., Machida, Y., Nagai, T., 1992. Novel oral microspheres of insulin with protease inhibitor protecting from enzymatic degradation. Int. J. Pharm. 78, 1–7.

Morishita, M., Morishita, I., Takayama, K., Machida, Y., Nagai, T., 1993. Site-dependent effect of aprotinin, sodium caprate, Na_2EDTA and sodium glycocholate on the intestinal absorption of insulin. Biol. Pharm. Bull. 16, 68–72.

Nakashima, K., Miyagi, M., Goto, K., Matsumolto, Y., Ueoka, R., 2004. Enzymatic and hyperglycemia stability of chemically modified insulins with hydrophobic acyl groups. Bioorg. Med. Chem. Lett. 14, 481–483.

Owens, D.R., Zinman, B., Bolli, G., 2003. Alternative routes of insulin delivery. Diab. Med. 20, 886–898.

Pan, Y., Li, Y., Zhao, H., Zheng, J., Xu, H., Wei, G., Hao, J., Cui, F., 2002a. Bioadhesive polysaccharide in protein delivery system: chitosan nanoparticles improve the intestinal absorption of insulin in vivo. Int. J. Pharm. 249, 139–147.

Pan, Y., Zheng, J., Zhao, H., Li, Y., Xu, H., Wei, G., 2002b. Relationship between drug effects and particle size of insulin-loaded bioadhesive microspheres. Acta. Pharmacol. Sin. 23, 1051–1056.

Polnok, A., Verhoef, J.C., Borchard, G., Sarisuta, N., Junginger, H.E., 2004a. In vitro evaluation of intestinal absorption of desmopressin using drug-delivery systems based on superporous hydrogels. Int. J. Pharm. 269, 303–310.

Polnok, A., Verhoef, J.C., Sarisuta, N., Junginger, H.E., 2004b. Influence of methylation process on the degree of quaternization of *N*-trimethyl chitosan chloride. Eur. J. Pharm. Biopharm. 57, 77–83.

Ponchel, G., Irache, J.-M., 1998. Specific and non-specific bioadhesive particulate systems for oral delivery to the gastrointestinal tract. Adv. Drug Deliv. Rev. 34, 191–219.

Sakuma, S., Ishida, Y., Sudo, R., Suzuki, N., Kikuchi, H., Hiwatari, K., Kishida, A., Akashi, M., Hayashi, M., 1997. Stabilization of salmon calcitonin by polystyrene nanoparticles having surface hydrophilic chains, against enzymatic degradation. Int. J. Pharm. 159, 181–189.

Sandri, G., Rossi, S., Bonferoni, M.C., Ferrari, F., Zambito, Y., Di Colo, G., Caramella, C., 2005. Buccal penetration enhancement properties of *N*-trimethyl chitosan: Influence of quaternization degree on absorption of a high molecular weight molecule. Int. J. Pharm. 297, 146–155.

Shi, H.G., Farber, L., Michaels, J.N., Dickey, A., Thompson, K.C., Shelukar, S.D., Hurter, P.N., Reynolds, S.D., Kaufman, M.J., 2003. Characterization of crystalline drug nanoparticles using atomic force microscopy and complementary techniques. Pharm. Res. 20, 479–484.

Simon, A., Wittmar, M., Bakowsky, U., Kissel, T., 2004. Self-assembling nanocomplexes from insulin and water-soluble branched polyesters, poly[(vinyl-2-(diethylamino)-propylcarbamate-*co*-(vinyl acetate)-*co*-vinyl alcohol)]-*graft*-poly(L-lactic acid): a novel carrier for transmucosal delivery of peptides. Bioconjug. Chem. 15, 841–849.

Snyman, D., Hamman, J.H., Kotzé, A.F., 2003. Evaluation of the mucoadhesive properties of *N*-trimethyl chitosan chloride. Drug Dev. Ind. Pharm. 29, 61–69.

Snyman, D., Kotzé, A.F., Walls, T.H., Govender, T., Lachmann, G., 2004. Conformational characterization of quaternised chitosan polymers. Proc. Int. Symp. Controlled Release Bioact. Mater. 31, 211.

Takeuchi, H., Yamamoto, H., Kawashima, Y., 2001. Mucoadhesive nanoparticulate systems for peptide drug delivery. Adv. Drug Deliv. Rev. 47, 39–54.

Thanou, M., Florea, B.I., Langemeyer, M.W., Verhoef, J.C., Junginger, H.E., 2000a. *N*-trimethylated chitosan chloride (TMC) improves the intestinal permeation of the peptide drug buserelin in vitro (Caco-2 cells) and in vivo (rats). Pharm. Res. 17, 27–31.

Thanou, M., Verhoef, J.C., Marbach, P., Junginger, H.E., 2000b. Intestinal absorption of octreotide: *N*-trimethyl chitosan chloride (TMC) ameliorates the permeability and absorption properties of the somatostatin analogue in vitro and in vivo. J. Pharm. Sci. 89, 951–957.

Tsuchida, E., Takeoka, S., 1994. Interpolymer Complexes and their Ionconduction, Macromolecule Complexes in Chemistry and Biology. Springer-Verlag, Berlin/Heidelberg.

van de Merwe, S.M., Verhoef, J.C., Kotzé, A.F., Junginger, H.E., 2004. *N*-trimethyl chitosan chloride as absorption enhancer in oral peptide drug delivery. Development and characterization of minitablet and granule formulations. Eur. J. Pharm. Biopharm. 57, 85–91.

van de Weert, M., Hennink, W.E., Jiskoot, W., 2000. Protein instability in Poly(lactic-*co*-glycolic acid) microparticles. Pharm. Res. 17, 1159–1167.

Yamamoto, A., 1994. Effects of various protease inhibitors on the intestinal absorption and degradation of insulin in rats. Pharm. Res. 11, 1496–1500.

Yeh, M., 2000. The stability of insulin in biodegradable microspheres based on blends of lactide polymers and poly (ethylene glycol). J. Microencapsul. 17, 743–756.