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Original Article

Effect of Tripolyphosphate on Physical and Enzymatic Stabilities of Insulin Loaded Nanoparticles of *N*-Trimethyl Chitosan

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Abstract The aim of the present work was to elucidate the influence of tripolyphosphate (TPP) on the colloidal and insulin stabilities. Using a trimethyl chitosan (TMC) with quaternization degree of 40%, insulin loaded nanoparticles were prepared by ionotropic gelation with TPP crosslinker. The nanoparticles were characterized for size, zeta potential, insulin loading, process yield, colloidal stability and the protection capability of insulin against enzymatic degradation of trypsin. The results showed that insulin nanoparticles were in the range of 200-260 nm with spherical or oval morphology. The highest insulin loading efficiency of nanoparticles with narrow size distribution was achieved when TPP:TMC:insulin mass ratio of 0.4:1:1 was used. The colloidal stability was TPP concentration dependent. The presence of TPP accelerated the degradation of free insulin and insulin loaded nanoparticles which increased with increasing TPP concentration. Therefore, the preparation of nanoparticles by ionotropic gelation with TPP would not be suitable for the development of insulin delivery system.

Keywords: insulin, nanoparticles, *N*-trimethyl chitosan, stability, tripolyphosphate, trypsin

INTRODUCTION

Currently, nanoparticles have been interestingly investigated as potential carriers for hydrophilic macromolecules such as proteins and vaccines. They are known to protect drug from degradation, to improve permeation/ penetration of the drugs across mucosal surface and also to control the release of the encapsulated or adsorbed drug.[1,2] Nanoparticles possess marked mucoadhesive properties that can prolong the residence time of drug carrier and also increase the intimacy of contact between drug and mucus membrane at the absorption sites. Regarding to insulin delivery, chitosan and its derivative, *N*-trimethyl chitosan (TMC) have been intensively used to develop insulin loaded nanoparticles.[3-11]

TMC, partially quaternized derivative of chitosan has been synthesized in an attempt to increase solubility of chitosan in water. It is well-soluble over a wide pH range (pH 1-14). Moreover, several studies have convincingly shown that soluble TMC has mucoadhesive properties[12-14] and excellent absorption enhancing effects for peptide and protein drugs, especially in neutral environments where chitosan is ineffective as an absorption enhancer.[15,16] There are several techniques available for preparing protein loaded TMC nanoparticles. One of the favorite techniques is ionotropic gelation of TMC with tripolyphosphate (TPP) counterion.[9,10,17,18] Recently,

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self-assembly polyelectrolyte nanocomplexes (PEC) of TMC and insulin have been developed without the use of TPP.[4,9,10] Nanocomplexes are formed utilizing the electrostatic interaction between positively charged TMC and negatively charged insulin as a driving force. Both techniques involve the mixing of two aqueous solutions at ambient temperature while stirring without using sonication and/or organic solvents which may be harmful to drug candidates during preparation. At present, few studies between insulin nanoparticles prepared by ionotropic gelation and polyelectrolyte complexation techniques have been reported.[9,10] These studies had paid attention only on physicochemical properties and stability of nanoparticles. However, the effect of TPP on the enzymatic stability of insulin against trypsin has not been elucidated.

Therefore, the aim of the present work was to explore the effect of TPP on the physical stability of nanoparticles and enzymatic stability of insulin. TMC nanoparticles were prepared by the ionotropic gelation technique and their potential to encapsulate the model insulin was studied. The effect of TPP on the colloidal stability against ionic strength of medium and on the stability of insulin against trypsin was evaluated using dynamic laser light scattering and HPLC, respectively.

MATERIALS AND METHODS

Materials

TMC with quarternization degree of 40% was obtained from a chitosan 84.7% deacetylated (MW 400 kDa) (Fluka, Schnelldorf, Germany) via methylation procedure as previously described.[19]

Human recombinant insulin powder (28.5 IU/mg), trypsin from procine (1060 BAEE unit/mg) and TPP were purchased from Sigma (Saint Louis, Missouri, USA). *N*-Benzoyl-L-arginine ethyl ester (BAEE) was supplied from Fluka (Schnelldorf, Germany). All other chemicals used were of analytical purity, except those for HPLC assay which were of HPLC purity.

Preparation of TMC Nanoparticles

Insulin loaded TMC nanoparticles were prepared by ionic crosslinking of TMC solution with TPP at ambient temperature.[9] To prepare insulin solution, insulin was dissolved in 87% (v/v) of 0.01 N HCl and 13% (v/v) of 0.1 M Tris was subsequently added, resulting in a clear insulin solution in 10 mM Tris buffer at pH 7.4. TMC solution was prepared by dissolving the dry TMC powder in 10 mM Tris buffer, pH 7.4. TPP was dissolved in purified water at various concentrations. The nanoparticles were spontaneously formed upon incorporation of equal volume of TPP solution in the polymer solution under gentle mild magnetic stirring. Insulin solution was premixed with equal volume of polymer solution before the addition of TPP solution. The final pH was in the range of 7.4-7.7. Freshly prepared solutions were used in each experiment.

Characterization of TMC Nanoparticles

The nanoparticles were characterized for their size with photon correlation spectroscopy (PCS) using an 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 particle size distribution of the nanoparticles was reported as a polydisperse index (PDI). The zeta potential values of nanoparticles were obtained by laser Doppler velocimetry (LDV) using a Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany). Morphological examination of the nanoparticles was performed by atomic force microscopy (AFM) (NanoWizard™, JPK Instruments, Berlin, Germany). All measurements were performed in tapping mode to avoid damage of the sample surface.

Loading Efficiency and Process Yield of Insulin Loaded Nanoparticles

The amount of insulin entrapped in the nanoparticles was calculated from the difference between the total amount added to the loading solution and the amount of non-entrapped insulin remaining in the supernatant. Triplicate batches of nanoparticles were centrifuged at 14,000 rpm for

30 min at room temperature, and the insulin content in the supernatant was determined using a Shimadzu HPLC (Shimadzu, Japan) system equipped with a UV detector at 230 nm. A Vydac™ C4 column (5 μm, 4.6 × 250 nm) (Hesperia, CA, USA) was employed with a flow rate of 1 ml/min using 30:70 acetonitrile:H_2O containing 0.1% trifluoroacetic acid (TFA) as mobile phase. Loading efficiency (LE) and process yield (PY) were calculated as follows:

$$\text{LE} = \frac{\text{Total insulin amount} - \text{Free insulin amount}}{\text{Total insulin amount}} \times 100\% \quad (1)$$

$$\text{PY} = \frac{\text{Particle weight}}{\text{Total solids (polymer + insulin + TPP) weight}} \times 100\% \quad (2)$$

Colloidal Stability of Insulin Loaded Nanoparticles

Insulin loaded nanoparticles were mixed with various concentrations of sodium chloride solutions. The integrity of particles in terms of size and number of particle formed was immediately monitored by dynamic laser light scattering.

Insulin Degradation Study by Trypsin

Trypsin was dissolved in 10 mM Tris buffer pH 7.4 and the concentration was adjusted to 3000 BAEE IU/ml. Proteolysis of insulin was initiated by the addition of 100 μl trypsin solution in 900 μl of insulin solution and nanoparticles suspension containing 500 μg/ml. Three vials of mixture were taken out at predetermined time points and the enzymatic reaction was stopped by an addition of 0.1% trifluoroacetic acid solution. The undegraded insulin concentration was then quantified by HPLC.

Statistical Analysis

The results were expressed as mean ± S.D. Statistical significance was measured by means of one-way analysis of variance (ANOVA), followed by Scheffe *post hoc* for individual group comparisons with SPSS software version 11.5. Probability values of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Preparation and Characterization of Insulin Loaded TMC Nanoparticles

According to a previous study,[9] the optimal amount of TPP used in preparing insulin nanoparticles depended on the (+/–) charge ratio of polymer/insulin. When considering TMC with quarternization degree of 40%, the least TMC/insulin (+/–) charge ratio which formed nanocomplexes was 2:1 (TMC/insulin mass ratio = 0.3:1). At that ratio, an incorporation of TPP in the formula resulted in the aggregation of particles. To investigate the effect of TPP on the physicochemical properties of insulin loaded nanoparticles, the nanoparticles were prepared at TMC/insulin (+/–) charge ratio of 6:1 (TMC/insulin mass ratio = 1:1) with different TPP/TMC mass ratio.

Figure 1 represents the effect of TPP on the particle size, zeta potential, PDI, insulin LE and PY of insulin nanoparticles. Insulin nanocomplexes (without TPP) displayed a size of 142 nm with board size distribution and high positive zeta potential. The association of insulin to nanocomplexes and PY were relatively low, consistent with the observation as a clear solution. The incorporation of TPP with respect to TMC led to a significant increase in particle size as seen in Figure 1a ($p < 0.05$). The zeta potential of nanoparticles linearly decreased with increasing TPP:TMC mass ratio (Figure 1b) which may be resulted from binding of negatively charged TPP and insulin with the positive charged polymers. As shown in Figure 1c, PDI of the nanoparticles decreased with increasing TPP:TMC mass ratio and reached a minimum value at TPP:TMC mass ratio of 0.4:1. Moreover, it was found that the LE and PY of insulin nanoparticles increased with increasing TPP:TMC mass ratio and reached a maximum at 0.4:1 TPP:TMC as shown in Figure 1d. As previously reported, the polymer/insulin (+/–) charge ratio played an important role in nanocomplex and

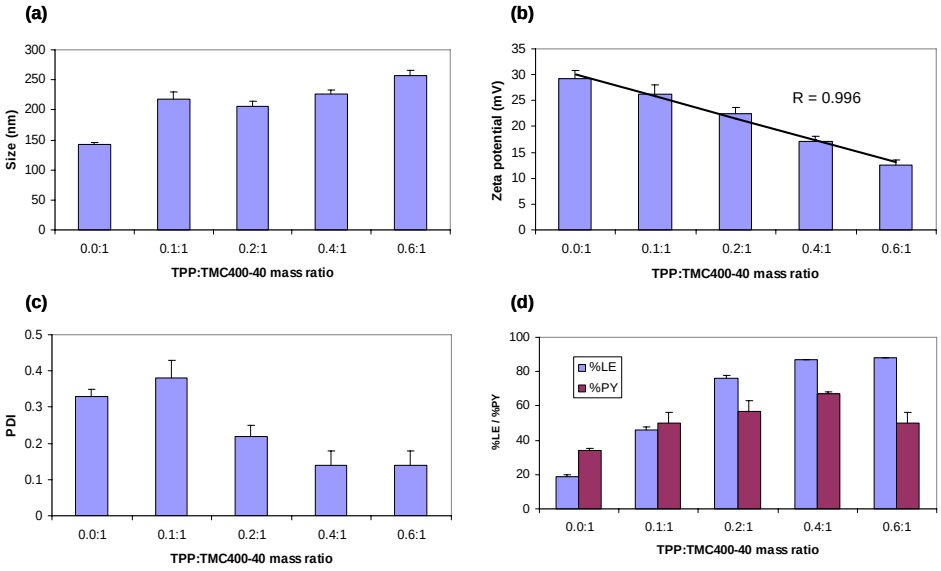

Figure 1. Effect of TPP on (a) particle size, (b) zeta potential, (c) polydispersity index and (d) insulin association efficiency and process yield of insulin nanoparticles. Each value represents the mean ± S.D. of three experiments.

nanoparticle formation.[9] Stable and uniform nanocomplexes / nanoparticles with high insulin LE could be formed at optimized polymer/insulin (+/–) charge ratio. This implies that at the TMC/insulin mass ratio of 1:1, 0.4:1 TPP:TMC mass ratio could reduce the TMC/insulin (+/–) charge ratio of system close to optimal TMC/insulin (+/–) charge ratio.

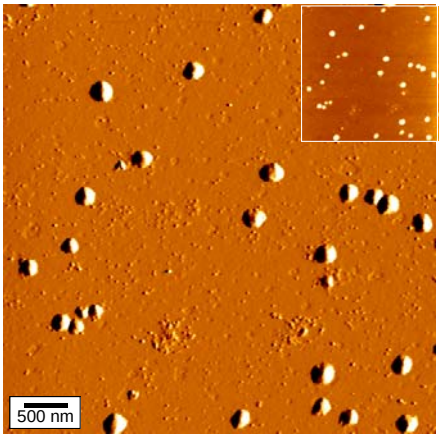

Figure 2. Atomic force microscopy image (5μm × 5 μm) of insulin loaded TMC nanoparticles at TPP:TMC:insulin mass ratio of 0.4:1:1. The insert is height mode of image.

Colloidal Stability

Figure 3 presents the change of Kcps (Kilo count per second) value, an index of number of particles formed, of insulin nanoparticles at different ionic strength solution. In case of insulin nanocomplexes prepared in an absence of TPP, no apparent change in Kcps values was observed even at ionic strength of 75 mM. On the other hand, dissociation of insulin nanoparticles increased with increasing ionic strength. Influence of TPP on the dissociation of particles was obviously observed when the ionic strength was higher than 50 mM. This phenomenon is probably the results of reduced attraction between the oppositely charged polyelectrolytes contributed by the presence of TPP in formulation and the counter-ion environment.[20]

Stability of Insulin against Trypsin

In order to evaluate the potential role of nanoparticles in protecting insulin from proteolytic enzyme, the enzymatic stability of insulin was investigated in the presence of trypsin. The effect of TPP on the insulin degradation was also established.

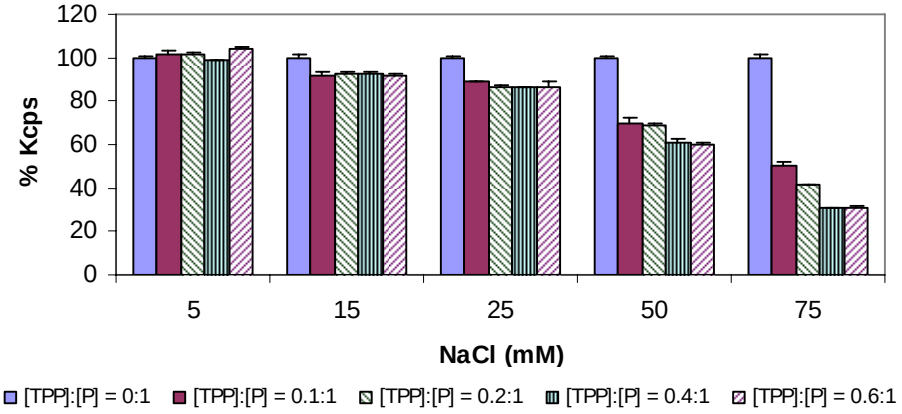

Figure 3. Effect of ionic strength on the dissociation of insulin nanoparticles prepared at different TPP:TMC ratio.

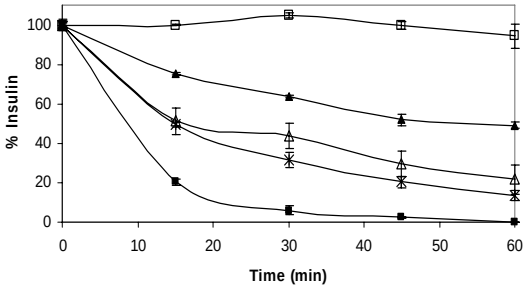

Figure 4. Effect of TPP on the enzymatic degradation of insulin by trypsin. Each value represents the mean ± S.D. of three experiments. The initial concentrations of insulin and trypsin were 450 μg/ml and 300 BAEE IU/ml, respectively. –✳– free insulin (w/ trypsin); –□– free insulin + TPP (w/o trypsin); –■– free insulin + TPP (w/ trypsin); –△– insulin nanoparticles with TPP:TMC:insulin mass ratio of 0.2:1:1 (w/ trypsin); –▲– insulin nanocomplexes with TMC:insulin mass ratio of 1:1 (w/ trypsin).

Each value represents the mean ± S.D. of three experiments. As demonstrated in Figure 4, under the experimental conditions, about 86.7 ± 2.5% of free insulin control solution was degraded within 60 min. In an absence of trypsin, free insulin was not degraded by TPP. Surprisingly, TPP accelerated the degradation of free insulin by trypsin which can be seen from a dramatically decreasing in residue amount of insulin in the presence of TPP. In general, insulin molecule is composed of 2 chains: A-chain with 21 amino acids and B-chain with 30 amino acids, linked by disulfide bridges between cysteine residues. Trypsin cleaves insulin initially at only two sites, at the carboxyl side of residues B29-Lys and B22-Arg.[21] Since the bonds susceptible to tryptic cleavage are located at the hydrophobic domain of carboxyl terminus of the B-chain, it is possible that TPP affects the conformation or secondary structure of insulin, resulting in such segment is easily attacked by trypsin. However, this hypothesis needs to be further studied in more detail.

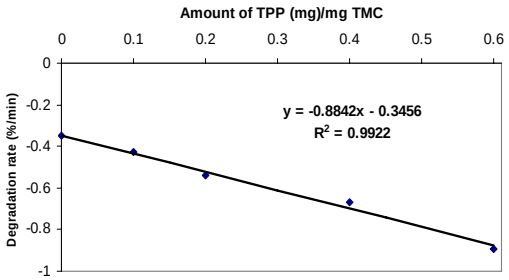

Figure 5. Relationship between TPP:TMC mass ratio and degradation rate of insulin.

It was observed that preparing insulin in form of nanoparticles with TMC could protect insulin from trypsin digesting (Figure 4). However, the protective effect of nanoparticles was still lower than insulin nanocomplexes prepared without the use of TPP. Furthermore, the degradation of insulin loaded nanoparticles increased with

increasing TPP. A linear relationship between amount of TPP and insulin degradation rate was established as presented in Figure 5. As reported by Akiyoshi *et al*.,[22] insulin can be protected from α-crymotrypsin digestion by forming complexes with cholesterol-bearing pullulan (CHP). Malkov *et al.* also observed that binding of insulin with *N*-[8-(2-hydroxy-benzyol)amino]caprylate (SNAC) prevented insulin degradation from trypsin.[23] Therefore, the less protective effect of nanoparticles could be explained by the loose interaction of TMC and insulin by TPP together with the accelerating effect of TPP on the degradation of insulin by trypsin.

CONCLUSION

In this study, insulin loaded TMC nanoparticles were prepared under mild conditions using TPP as a crosslinker. Soluble insulin nanoparticles in the size range of 200-260 nm with spherical or oval morphology were obtained. The highest insulin LE of nanoparticles with narrow size distribution was achieved at TPP:TMC: insulin mass ratio of 0.4:1:1. The colloidal stability was TPP concentration dependent. The presence of TPP accelerated the degradation of free insulin and insulin loaded nanoparticles which increased with increasing TPP concentration. In conclusion, nanoparticles prepared by ionotropic gelation with TPP would not be a useful carrier for insulin delivery. The influence of TPP on the secondary structure of insulin should be further studies.

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