

Oral delivery of insulin using chitosan capsules cross-linked with phytic acid

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Abstract. Phytic acid (PA) was used as a cross-linking agent for encapsulation of insulin in a chitosan matrix for oral delivery of insulin. PA–chitosan capsules were compared with tripolyphosphate (TPP)–chitosan capsules for stable oral delivery of insulin. During 2 h incubation in simulated gastric fluid, PA–chitosan capsules prepared using pH 6, 6% PA solutions showed better stability than TPP–chitosan capsules prepared using pH 7, 6% TTP solution. PA–chitosan capsules released less than 60% of their encapsulated insulin after 24 h incubation in simulated gastrointestinal fluids. TPP–chitosan capsules showed burst release and virtually the entire insulin content was released in 12 h. Both capsule types were tested *in vivo* via oral drug administration using diabetic mice. PA–chitosan capsules significantly decreased blood glucose levels while TPP–chitosan capsules caused a lesser reduction. The relative pharmacological bioactivity of PA–chitosan capsules prepared was 6.4% while that of TPP–chitosan capsules was 1.1%. PA–chitosan capsules appeared to have good potential for use in oral delivery of insulin for sustained control of the blood glucose level.

Keywords: Oral delivery, insulin, phytic acid, chitosan capsule, diabetic mice, bioavailability

1. Introduction

Oral delivery is a convenient and patient-friendly route for drug administration, however proteins and peptides are less suited to administer through oral route because of their susceptibility to enzymatic degradation in the gastrointestinal tract and low permeability across the intestinal epithelium [1]. Several strategies have been proposed to boost the bioavailability of these types of drugs, such as insulin, when administered orally. These strategies include use of permeation enhancers, enzyme inhibitors, encapsulation technology, hydrogels, microemulsions and liposomes [2,3]. Despite significant research, oral delivery of proteins still poses significant scientific challenges [4].

Encapsulation technology uses different types of capsules that protect proteins from enzymatic degradation in the intestine [5]. The ionic gelation method can be applied to form capsules via a rapid cross-linking reaction between multivalent cationic chitosan and anionic counter-ions as a cross-linking agent. Chitosan has been used in the pharmaceutical industry as a drug carrier owing to its non-toxicity, excellent biodegradability and biocompatibility [6]. Chitosan interacts with insulin and enhances the intestinal permeation and stability of insulin, hence, it is regarded as a potential vehicle for oral delivery of insu-

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lin [7]. This characteristic of chitosan can be ascribed to a combination of the effects of muco-adhesion and widening of the paracellular transport pathway [8]. Another advantage of using chitosan is a lack of mucosal damage associated with conventional permeation enhancers, such as fatty acids and bile salts [9].

Various studies have described the preparation of chitosan capsules using tripolyphosphate (TPP), as a cross-linking agent. TPP is a non-toxic polyanion that interacts with chitosan via electrostatic forces to form ionically cross-linked networks [6]. As a novel cross-linking agents, phytic acid (PA) is a naturally occurring substance in most legumes, including soybeans, wheat bran and nuts. PA, also called myo-inositol hexaphosphate, is composed of six phosphate molecules coupled to a myo-inositol ring via an ester bond. The number of anions in PA capable of reacting with cations of chitosan is two-fold higher than in TPP [10]. Although prior studies have shown the antioxidant and anticancer properties of phytic acid [11,12], its ability as a cross-linker for the encapsulation of insulin has yet to be evaluated.

The objective of this study was to develop PA–chitosan capsules using an ionic gelation method for insulin oral delivery to preserve the pharmacological bioactivity of insulin and to enhance its bioavailability. These capsules were tested *in vitro* for controlled release of insulin in gastrointestinal fluids. An *in vivo* study was also performed to evaluate the bioactivity of encapsulated insulin after oral administration to diabetic mice.

2. Materials and methods

2.1. Materials

Chitosan (degree of deacetylation 98%, MW 85.1 kDa) was obtained from Dongbang Chitobio Co. (Paju, Korea). In preparing chitosan, ground shells are deproteinated and demineralized by successive treatment with alkali and acid, after which the extracted chitin is deacetylated to chitosan by alkaline hydrolysis. Tripolyphosphate (TPP) (sodium tripolyphosphate pentabasic, FW 367.86), bovine insulin (MW 6 kDa), and streptozotocin were purchased from Sigma-Aldrich (St. Louis, MO, USA). A phytic acid (PA) solution (65%, FW 923.83) was purchased from Kanemi Soko Co. (Tokyo, Japan).

2.2. Preparation of capsules

Insulin solution (3%, w/w) was prepared in distilled water and dispersed into chitosan solution (3%, w/w) prepared in 1% (v/v) acetic acid. 6% TPP solution was adjusted to pH 7 using 0.5 N HCl [13]. 6% PA solutions were adjusted to pH 6 using 2 N NaOH [14]. These parameters were selected after preliminary trials and the values giving maximum insulin encapsulation (90%) were chosen for this study. The chitosan and insulin mixture was dispersed as drops using disposable syringe with needle of 0.241 mm internal diameter into the cross-linking solutions at 25°C with gentle agitation. Capsules were cured for 20 min, separated, washed with distilled water and freeze dried at –20°C for 36 h.

2.3. Size and morphology observations

The size of freeze-dried capsules was determined using a microscope (TE 2000U, Nikon Corp., Tokyo, Japan) connected with a software program (Image-Pro Plus, v 5.1, Silver Spring, MD, USA). The surface and cross-sectional morphologies of the dried particles were examined using scanning electron microscope (JSM-6500F, Jeol Ltd, Tokyo, Japan). Specimens were loaded onto a specimen stub with two-sided adhesive tape and coated with gold using ion sputter (MCS101 + CC101, Cressington Scientific Instruments Ltd, Watford, UK).

2.4. FTIR spectrophotometry

In order to monitor the reaction of intermolecular complex, samples were mixed with potassium bromide powder and compressed into disc using a tablet presser (PerkinElmer, Waltham, MA, USA) and recorded by using FTIR spectrophotometer (IFS 66S, Bruker, Billerica, MA, USA).

2.5. *In vitro* release studies

Model drug release studies for capsules were carried out first in simulated gastric fluid (SGF) and then in simulated intestinal fluid (SIF). The SGF (pH 1.2) was prepared by dissolving 2 g of NaCl in 7 ml of 35% HCl and the mixture volume was adjusted to 1 l using distilled water. SIF was prepared by dissolving 250 ml of 0.2 M KH_2PO_4 in 118 ml of 0.2 N NaOH and the mixture volume was adjusted to 1 l using distilled water. The final pH of the SIF was 6.8 [15]. Capsules were first placed in SGF and incubated at 37°C for 2 h with shaking at 80 rpm in a shaking incubator (DS-310F, Dasol Scientific Co., Hwaseong, Korea). They were then transferred from SGF to SIF and incubated in shaking incubator at 37°C for more than 12 h to simulate the transit time through the small intestine and colon. SGF and SIF samples were collected periodically and the insulin release was determined by using a chromatographic method [16]. The collected release medium was passed through a 0.45 μm filter (17 mm PVDF syringe filter, National Scientific, TN, USA) and a sample of 20 μl was injected into a reverse phase HPLC column (4.6 mm \times 250 mm, Shiseido, Tokyo, Japan) equipped with a UV detector (340U, Dionex, Bavaria, Germany). The mobile phase consisted of a 0.1% solution of trifluoroacetic acid in water (A) and acetonitrile (B). The linear gradient allowed B to change from 20 to 50% in 20 min. The flow rate was maintained at 1 ml/min and detection was carried out at 280 nm. The percentage insulin release was calculated by dividing the amount of insulin in release medium by total insulin added to the test. The volume of the release medium was maintained at a constant volume by adding SGF or SIF equal to the amount withdrawn for analysis. The trials for insulin release were carried out in triplicates ($n = 3$).

2.6. *In vivo* evaluation of insulin bioavailability

Animal studies were performed according to protocols for the safe and humane treatment of animals approved by the Institutional Animal Use and Care Committee of Laboratory Animal Research Center at Yonsei University.

2.6.1. Preparation of diabetic mice

Streptozotocin (30 mg/ml) was dissolved in a 0.1 mM NaCl buffer and delivered at the rate of 65 mg/kg body weight through intra-peritoneal injections to BALB/c six-week old male mice [17]. Blood samples were collected from the tail vein and blood glucose levels were determined periodically using a Quantichrom™ plasma glucose assay (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's protocols and checking for glycemia. Mice were considered to be diabetic when the baseline glucose levels were greater than 470 mg/dl for 2 consecutive days. Prior to the start of each experiment, mice were fasted for 12 h, but provided with unlimited access to water [18].

2.6.2. Determination of insulin bioactivity in different formulations

Diabetic BALB/c male mice each weighing approximately 50 g were randomly assigned to four groups with eight mice ($n = 8$) in each group. Distinctive formulations were administered to these groups. All capsules were administered at a dosage of 40 IU/kg body weight while the insulin solution

was administered at 1 IU/kg. The control solution and insulin were administered via subcutaneous injection while capsule formulations were delivered orally by capsule injector (Jeung Do Bio & Plant Co., Seoul, Korea) to place the capsule deep enough to trigger the swallowing reflex [19]. In addition to each dose, 50 μ l of normal saline was also administered. Treated mice were maintained in restraining cages with access to water but no food was provided. Blood samples were collected periodically for 48 h from the tail vein and glucose levels were determined immediately using a plasma glucose assay.

2.7. Pharmacodynamic analysis

The mean glucose level in blood samples collected prior to administration of the drug formulations was used as a base line. Percentage reductions in glucose levels after drug administration were evaluated and plotted against time. The efficiency of each formulation was evaluated in terms of the area above the blood glucose level–time curve for 48 h (AAC_{0-48}), the maximum percentage decrease in blood glucose levels (C_{max}) and the time (t_{max}) required to achieve C_{max} [20]. Since a drop in the blood glucose level is directly proportional to insulin, the AAC for the blood glucose level–time curve was proportional to the AAC for the insulin level–time curve [21]. Therefore, the AAC value was an estimate of the formulation efficacy based on a pharmacokinetic–pharmacodynamic correlation. AAC values were calculated using the linear trapezoidal rule [22]. C_{max} was calculated as follows [23]:

$$C_{max} = 100 - \text{lowest glucose level.}$$

The AAC, C_{max} and t_{max} values were represented as the mean \pm SD and the relative pharmacological bioactivity (F) was calculated by comparing the AAC values for oral and subcutaneous (SC) doses as determined using the following equation [24]:

$$F = (AAC_{\text{Oral}} \times \text{SC-dose}) / (AAC_{\text{SC}} \times \text{Oral-dose}) \times 100.$$

2.8. Data analysis

The properly replicated data was analyzed statistically through analysis of variance and Duncan's multiple range tests using Statistical Analysis System (SAS 9.2, SAS Institute, Cary, NC, USA). Significance was defined at $p < 0.05$.

3. Results

3.1. Size and morphology of capsules

The average capsule sizes of PA–chitosan and TPP–chitosan capsules were 1.68 ± 0.16 and 1.62 ± 0.13 mm, respectively. The morphological characteristics of these capsules, studied using SEM, are presented in Fig. 1. The inner porous structure of TPP–chitosan capsules (Fig. 1D) may facilitate the diffusion of insulin through the capsules which can decrease the entrapment and increase the drug release. However, PA–chitosan capsules (Fig. 1B) showed much closer and denser cross-linking structure enough to increase insulin entrapment efficiency. Also, their shells were harder and thicker than TPP–chitosan capsules to protect insulin from harsh gastric condition.

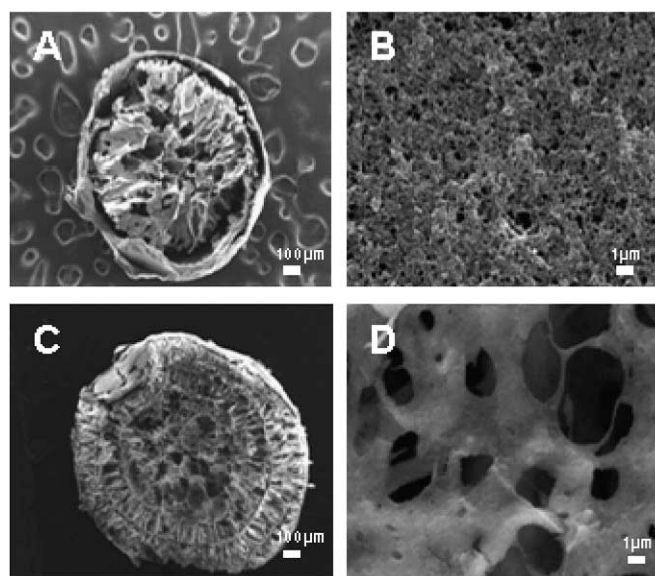


Fig. 1. SEM of micrographs of insulin-loaded chitosan capsules: (A) cross-sectional morphology ($\times 40$) of PA–chitosan capsules, (B) surface morphology ($\times 5000$) of PA–chitosan capsules, (C) cross-sectional morphology ($\times 40$) of the TPP–chitosan capsules, (D) surface morphology ($\times 5000$) of the TPP–chitosan capsules.

3.2. Cross-linking mechanism

The molecular structure and pKa values of PA and TPP are presented in Fig. 2A and B, respectively. The pKa values of both types of molecules were calculated using the Marvin program [10]. The structure of chitosan is also shown in Fig. 2C. In order to study the cross-linking mechanism, FTIR spectra of chitosan, PA–chitosan capsules and TPP–chitosan capsules were taken, as presented in Fig. 3. A characteristic peak appeared at the frequency of 1150 cm^{-1} indicating the presence of P=O group which is supposed to be due to ionic cross-linking of chitosan with PA or TPP [25]. Peak at 3434 cm^{-1} has been attributed previously to ^-OH stretching vibration in chitosan matrix [26]. In chitosan capsules, the peak frequency is shifted from 3434 to 3399 cm^{-1} and the peak becomes wider indicating that the hydrogen bonding is enhanced. When chitosan is cross-linked with either PA or TPP, the shoulder amide peak at 1644 cm^{-1} disappeared and shifted to a new peak at 1630 cm^{-1} [27]. The peak at 1630 cm^{-1} reflects a stronger intensity in PA–chitosan capsules than TPP–chitosan capsules. The peak at 1602 cm^{-1} due to protonated amine group bending vibration also developed to 1534 cm^{-1} indicating that an ionic cross-linking has occurred at the amine group on chitosan [28]. This interaction could represent the binding of phosphate on this site.

3.3. Release of insulin in simulated gastrointestinal fluid

Controlled studies of insulin release, from PA–chitosan and TPP–chitosan capsules prepared using 6%, pH 6 PA solution and 6%, pH 7 TTP solution, respectively, were carried out in SGF (pH 1.2) and SIF (pH 6.8) and results are presented in Fig. 4. Insulin release was predominantly dependent on the pH of the release medium. The influence of the cross-linking agent on the capsule stability and the *in vitro* drug release behavior was evaluated. During incubation for 2 h at pH 1.2 in SGF, TPP–chitosan capsules showed a higher insulin release. However, PA–chitosan capsules released considerably lower

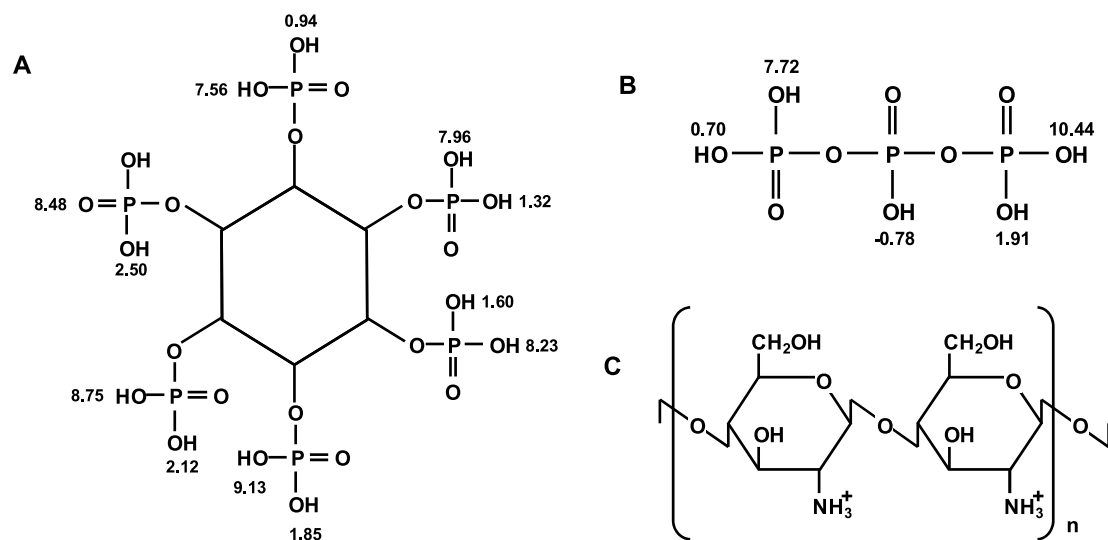


Fig. 2. Chemical structure and pKa values of phytic acid (A) and tripolyphosphate (B); structure of chitosan (C).

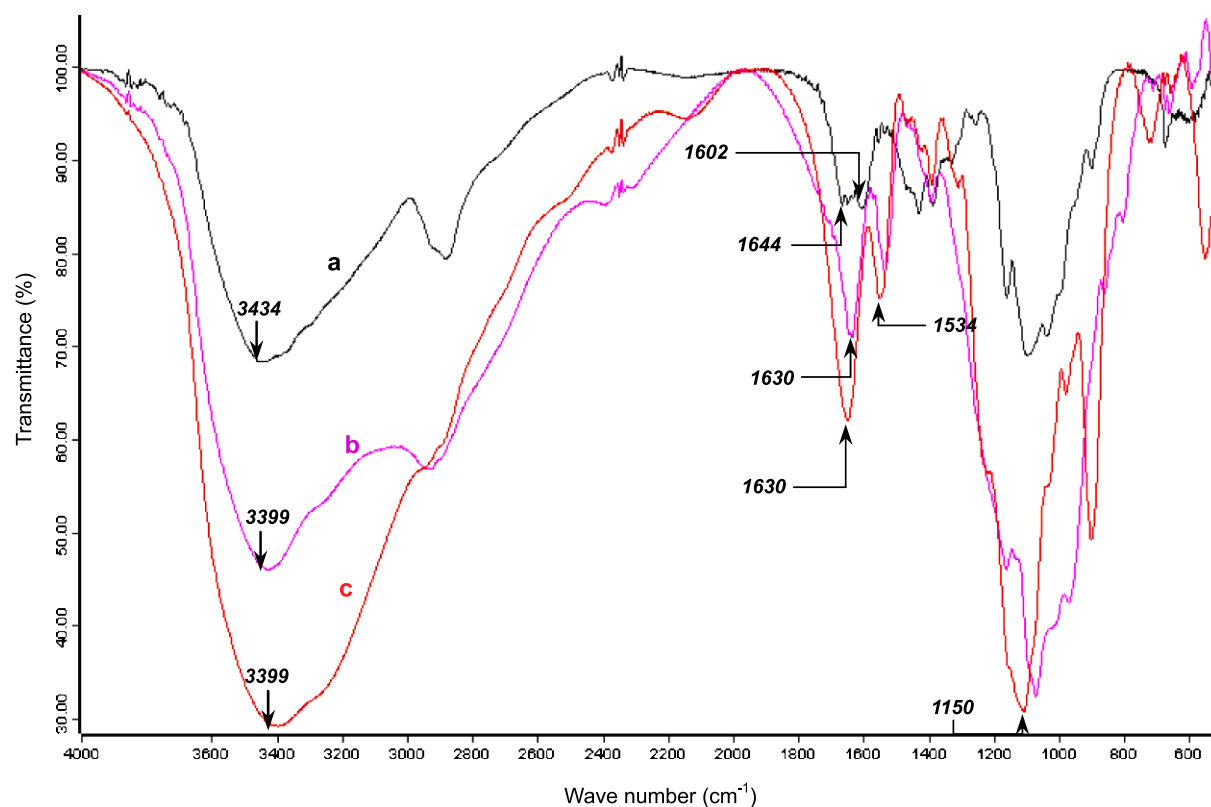


Fig. 3. The FTIR spectra: (A) chitosan, (B) TPP–chitosan capsules, (C) PA–chitosan capsules. (Colors are visible in the online version of the article; <http://dx.doi.org/10.3233/BME-2011-0654>.)

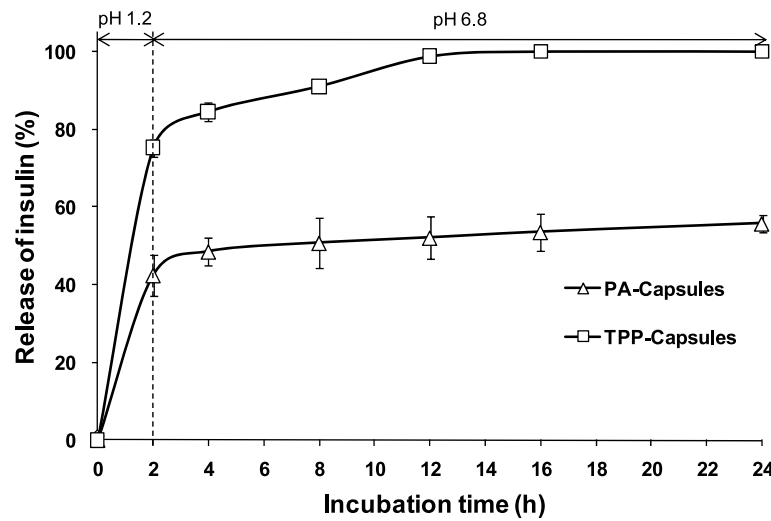


Fig. 4. Insulin release from chitosan capsules cross-linked using pH 6, 6% PA solutions and pH 7, 6% TPP solutions during incubation for 2 h in simulated gastric fluid (pH 1.2) and 22 h in simulated intestinal fluid (pH 6.8). Bars represent the standard error of the mean ($n = 3$).

insulin than TPP–chitosan capsules. Fast insulin release occurred from TPP–chitosan capsules in SIF with all insulin released till 12 h incubation. However, PA–chitosan capsules prepared using pH 6, 6% PA solutions were much more stable in SIF and showed significantly ($p < 0.05$) lower (<60% of the total) insulin release until 24 h of incubation in gastrointestinal fluid.

3.4. Hypoglycemic effects of encapsulated insulin

In vivo evaluation of PA–chitosan capsules for oral delivery of insulin was carried out using diabetic BALB-c mice and compared with results for TPP–chitosan capsules. In addition, insulin was also administered to mice through SC injection. The effects of different insulin formulations and normal saline on the blood glucose levels of the 4 groups of distinctively treated diabetic mice of similar biological characteristics are illustrated in Fig. 5. Injections of normal saline and insulin were administered at dose rates of 1 IU/kg, whereas capsules were administered 40 IU/kg body weight. In our preliminary studies, capsules without insulin were tested in animals to clarify that phytic acid has no dietary effects on hypoglycemia (data was not shown). Mice receiving normal saline without insulin (group 1) did not show any change in blood glucose levels. SC injection of insulin (group 4) resulted in a sharp reduction (84.3%) in the blood glucose level within 1 h. However, the level increased gradually and the insulin effect vanished until 12 h after injection. Group 2 mice, treated with PA–chitosan capsules, showed significant ($p < 0.05$) decreases (>50%) in blood glucose levels that lasted more than 24 h, and the effect did not vanish until 48 h after treatment. The maximum (58.9%) reductions in glucose was observed in group 2 mice at 6 h. Group 3 mice treated with TPP–chitosan capsules showed little hypoglycemic effect with a less than 20% decrease in the blood glucose level that lasted for less than 12 h.

3.5. Pharmacological bioavailability of encapsulated insulin

The main pharmacodynamic parameters associated with both oral insulin administration using chitosan capsules and SC injection is listed in Table 1. The equivalency of each formulation was evaluated

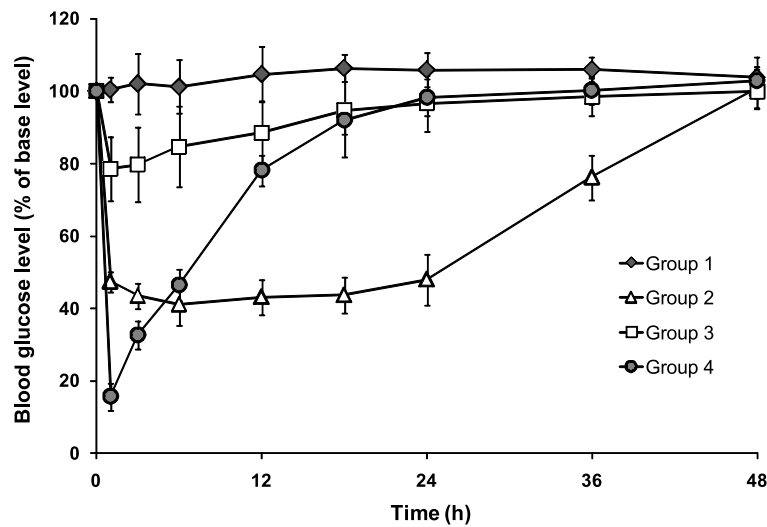


Fig. 5. Changes in the blood glucose level in response to oral delivery of encapsulated insulin and subcutaneous injections of normal saline and insulin solution in diabetic mice. Group 1 – normal saline solution as a control; group 2 – PA–chitosan capsules (prepared using a pH 6, 6% PA solution); group 3 – TPP–chitosan capsules (prepared using a pH 7, 6% TPP solution) and group 4 – an insulin solution. Bars represent the standard error of the mean ($n = 8$).

Table 1

Pharmacodynamic parameters after oral administration of PA and TPP–chitosan capsules and subcutaneous injection of insulin in diabetic mice (mean \pm SD, $n = 8$)^a

Formulations	AAC _{0–48} ^b	C_{\max} (%) ^c	t_{\max} (h) ^d	F (%) ^e
PA–chitosan capsules (pH 6, 6% PA solution)	1942.5 \pm 67.4 ^a	58.9 \pm 5.8 ^b	6	6.4 \pm 0.6 ^b
TPP–chitosan capsules (pH 7, 6% TPP solution)	327.5 \pm 23.1 ^b	21.4 \pm 8.0 ^c	1	1.1 \pm 0.3 ^c
Subcutaneous injections	749.5 \pm 37.7 ^c	84.3 \pm 3.6 ^a	1	100 ^a

Notes: ^aMeans within same columns which have no common letters superscripts are significantly different ($p < 0.05$).

^bAAC_{0–48} represents the area above the blood glucose level–time curve, values were calculated using the linear trapezoidal rule. ^c C_{\max} represents the maximum decrease in blood glucose levels ($C_{\max} = 100 - \text{lowest \% blood glucose}$). ^d t_{\max} is the time required to attain C_{\max} . ^e F indicates the relative pharmacological bioactivity.

in terms of the area above the blood glucose level–time curve (AAC) for 48 h, the maximum decrease in the blood glucose level (C_{\max}), the time required to achieve C_{\max} (t_{\max}), and the relative pharmacological bioactivity (F) [24]. The values of AAC_{0–48}, C_{\max} and t_{\max} for PA–chitosan capsules were significantly higher than those for TPP–chitosan capsules. These values can be used to calculate the relative pharmacological availability, F (%). The F (%) of PA–chitosan capsules prepared using both pH 6, 6% PA solutions was 6.4% whereas that of TPP–chitosan capsules was only 1.1% showing that PA–chitosan capsules were six folds more effective than TPP–chitosan capsules for oral delivery of insulin. PA–chitosan capsules released insulin in its active form *in vivo* and they improved the intestinal absorption of insulin to a greater extent than TPP–chitosan capsules.

4. Discussion

Insulin and other protein drugs are generally delivered through injections as the oral delivery results in their digestion into constituent amino acids by various enzymes located throughout the gastrointestinal tract. These constituent amino acids are absorbed by the epithelium but do not have the biological activity of original protein or polypeptide [9]. Subcutaneous injection of insulin has various disadvantages such as hyperinsulinemia, pain, allergic reactions and low patient compliance. Insulin injected via SC depot seeps into the general circulation and exposes all tissues to an equal concentration of insulin, while the liver receives only a fraction of the injected dose. Muscle cells and adipocytes can, therefore, respond to the injected dose without the insulin supply being subject to monitoring by the liver [5]. Therefore, development of suitable oral delivery system can be an attractive alternative to injection for the administration of proteins and peptides. The objective of our study was to evaluate PA as a cross-linking agent in improving the stability of chitosan capsules for the oral delivery of insulin aiming at targeted and sustained insulin release in intestine as elaborated in Fig. 6.

Molecular charge density is an important factor in electrostatic interactions and sufficient charge number is necessary for anions to cross-link with chitosan through ionic interactions [28]. The difference of cross-linking activity between PA and TPP was evaluated by examination of entrapment efficiency. PA–chitosan capsules showed a much higher entrapment efficiency than TPP–chitosan capsules (data was not shown). TPP is the most commonly used counter-ion, containing a maximum of 5 negative charges

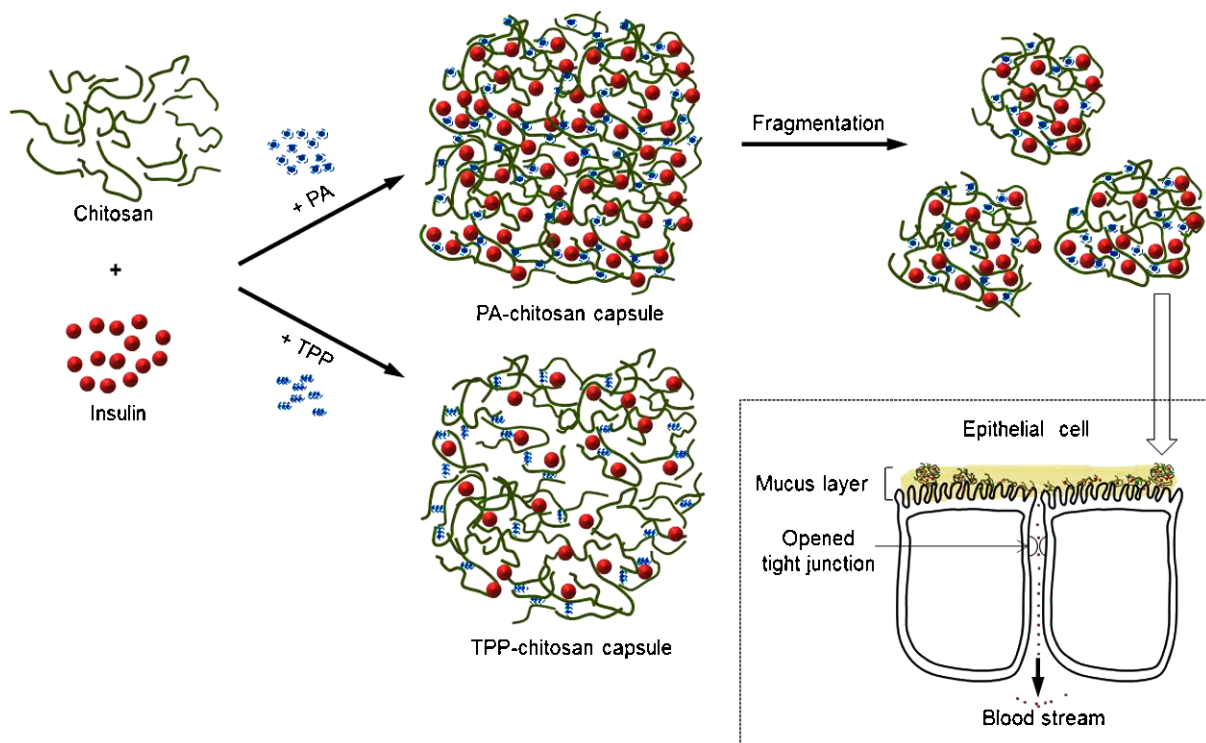


Fig. 6. Schematic presentation of insulin-loaded chitosan capsules cross-linked with PA or TPP and their presumed mechanism of cellular transport of insulin from chitosan capsules. (Colors are visible in the online version of the article; <http://dx.doi.org/10.3233/BME-2011-0654>.)

(Fig. 2A). PA is a multivalent anion that carries 12 negative charges (Fig. 2B); more than twice the charge number of TPP. The porous structure of TPP–chitosan capsules (Fig. 1D) may facilitate the diffusion of insulin through the hydrogel matrix, which can decrease the entrapment efficiency and increase the drug release in acidic condition. However, cross-linking through ionic gelation of PA–chitosan capsules showed closer and denser network and capsule shell was harder and thicker than TPP–chitosan capsules (Fig. 1).

The cross-linking of chitosan capsules could be characterized by FTIR. It is observed that spectrum of chitosan capsules by cross-linking with PA or TPP was different, particularly in the wave number range from 1100 to 1700 cm^{-1} . When chitosan is cross-linked with either PA or TPP, the shoulder amide peak at 1644 cm^{-1} disappeared and developed a new sharp peak at 1630 cm^{-1} [27]. This is very important evidence, as it is argued that the appearance of this band coincides with cross-linking of chitosan with PA or TPP. And the peak of 1630 cm^{-1} shows a stronger intensity in PA–chitosan capsules than TPP–chitosan capsules, which corresponds to the fact that the ionic reaction of chitosan and PA results in stronger interaction than chitosan and TPP. The peak at 1602 cm^{-1} due to protonated amine group bending vibration also shifted to 1534 cm^{-1} , which is caused by the binding of phosphate on amine group of chitosan [29]. Based on these results, we may assume that the phosphoric groups of PA and TPP are linked with amino group of chitosan through intermolecular reactions.

Ionic cross-linked chitosan capsules exhibit remarkably pH responsive properties in gastrointestinal fluids [28]. Increased release of insulin was observed for both capsule types after transfer from SGF to SIF, probably due to the disintegration of chitosan capsules at near neutral pH values in SIF [30]. When these capsules were administered to mice, there was an interaction of chitosan with the cell membrane of intestine that resulted in structural reorganization of tight junction [31] as presented in Fig. 6. This is the evidence that chitosan is a hydrophilic bio-macromolecule, which has significant potential of reducing transepithelial electrical resistance and transiently opening tight conjunction between epithelial cells [32]. In the intestinal tract, insulin is released from the PA–chitosan capsules, which is caused by swelling, dissolution and subsequent diffusion of insulin through the hydrogel matrix [33]. The insulin encapsulated in PA–chitosan capsules was released gradually over a longer period by the distinguished adhesiveness of chitosan in epithelium, and then permeated through the paracellular pathway to blood stream [32].

We observed significant decreases in the glucose levels of diabetic mice due to enhancement of insulin absorption from PA–chitosan capsules in the lower intestinal tract, resulting in a much higher relative pharmacological bioavailability. It is supposed that the addition of phytic acid will improve the stability and lifetime of chitosan capsules to the extent that a PA–chitosan capsule can be used to delivery insulin orally. The sustained hypoglycemic effect with PA–chitosan capsules provides clear evidence of their ability to sustain the intestinal absorption and controlled release of insulin. Therefore, this study offers new insights to the potential use of phytic acid as a novel and effective cross-linker to prepare chitosan capsules for oral insulin delivery [34].

5. Conclusion

PA–chitosan capsules prepared using pH 6, 6% PA solutions were used to optimally entrap 3% insulin. *In vitro* studies showed that PA–chitosan capsules were more stable than TPP–chitosan capsules and more sustained release of insulin. PA–chitosan capsules also resulted in improved intestinal absorption of insulin and significantly decreased blood glucose levels in diabetic mice. The relative pharmacological

bioactivity of these capsules was approximately 6 times higher than that of TPP–chitosan capsules. PA appeared to be a promising cross-linking agent for encapsulation of insulin in chitosan matrix for oral delivery of this drug. Further research will be directed to investigate the effect of capsule size on insulin absorption and optimization of different parameters of PA–chitosan capsules.

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