

Research Article

Preparation and Characterization of Water-Soluble Chitosan Microparticles Loaded with Insulin Using the Polyelectrolyte Complexation Method

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Polymeric delivery systems based on microparticles have emerged as a promising approach for peroral insulin delivery. The amount of insulin was quantified by the improved Bradford method. It was shown that water-soluble chitosan/insulin/tripolyphosphate (TPP) mass ratio played an important role in microparticles formation. Stable, uniform, and spherical water-soluble chitosan microparticles (WSC-MPs) with high insulin association efficiency were formed at or close to optimized WSC/insulin/TPP mass ratio. WSC-MPs had higher association efficiency in the pH 4.0 and pH 9.7 of TPP solution. The results showed that association efficiency and loading capacity of insulin-loaded WSC-MPs prepared in 0.01 mol/L HCl of insulin were $48.28 \pm 0.90\%$ and $9.52 \pm 1.34\%$. The average size of insulin-loaded WSC-MPs was 292 nm. The presented WSC microparticulate system has promising properties towards the development of an oral delivery system for insulin.

1. Introduction

In the present paper, insulin was chosen as the model protein. It is a well-known 51 amino acids protein, and the oral approach remains the most attractive due to convenience and high patient compliance [1]. However, administering drugs orally is by far the most widely used route of administration, although it is generally not feasible for protein drugs. The main reasons for the low oral bioavailability of biologicals are presystemic enzymatic degradation and poor penetration of the intestinal membrane [2, 3]. Much has been learned in the past few decades about macromolecular drug absorption from the gastrointestinal (GI) tract, including the barriers that restrict GI absorption [4]. One approach to improve the gastrointestinal uptake of low molecular weight proteins is to bind them to colloidal systems like microparticles (MPs), protecting them from degradation in the gastrointestinal tract and promoting the transport into systemic circulation [5–8].

MPs are defined as particulate dispersions or solid particles with a size in the range of 100–1000 nm. The drug is dissolved, entrapped, encapsulated, or attached to a micro-particle matrix. The major goals in designing microparticles as a delivery system are to control particle size, surface properties, and release of pharmacologically active agents in order to achieve the site-specific action of the drug at the therapeutically optimal rate and dose regimen [9]. More recently, researches have attempted to study chitosan microparticles (CS-MPs) as follows: preparation, modification, properties of loading various drugs, and their physiological characters, such as CS-MPs coated PLGA [10] and PEG [11], MPs loaded insulin [12], DNA [13], vaccine [14], peptide [15], enzyme [16], and anticancer drug 5-fluorouracil [17]. But some important factors affecting drug properties have not always been investigated, such as the type of water-soluble chitosan (WSC), and were seldom evaluated in insulin delivery system of nanoparticles [18]. Polyethylene glycol (PEG-) coated nanoparticles have been

found to be important potential therapeutic application for controlled release of drugs and site-specific drug delivery [19]. Therefore, we investigated a series of factors affecting delivery properties, concentration of WSC, and initial insulin which were all evaluated.

The objective of the present study was to evaluate the effects of formulation parameters on the insulin association efficiency of the insulin-loaded WSC-MPs and determine the effects of insulin association efficiency on different pH of TPP and resolution medium of insulin and release properties during incubation in phosphate buffer saline (PBS) of pH 7.4 and pH 1.2 and investigate the physicochemical structure of insulin-loaded WSC-MPs by TEM and particle size analyzer. A series of insulin-loaded WSC-MPs with various parameters were prepared. The physical and morphological properties of insulin-loaded WSC-MPs were investigated in accordance with formulation parameters, and the release profile of insulin was also determined regarding its potential for oral delivery.

2. Materials and Methods

2.1. Materials. WSC (degree of deacetylation 87%, molecular weight 21 kDa) was obtained from Shandong AK Biotech Ltd. Porcine insulin ($27.8 \text{ IU} \cdot \text{mg}^{-1}$) was purchased from Xuzhou Biochemical Plant, China. TPP was purchased from Tianjin Hongyan Chemical Reagent Factory. Coomassie Brilliant Blue G-250 was obtained from MBCHEM. All other chemicals and solvents were of analytical grade.

2.2. Equipment. UV-2201 UV-analyzer (Shimadzu, Japan), JB-3 Magnetic stirrer (Shanghai Instrument, China), SORVALL Biofuge Stratos low-temperature high-speed centrifuge (Thermo, Germany), THZ-051 high-precision CNC shaker (Thermo, Germany), ALPHA1-4 LSC Freeze Dryer (Martin Christ, Germany), Zetasizer 3000 HAS (Malvern, UK), TEM-100CXII (Electronics Company, Japan).

2.3. Preparation of Chitosan Microparticles. WSC-MPs were prepared by the ionic gelation of WSC and tripolyphosphate (TPP) as described by Fernández-Urrusuno [20]. Briefly, WSC was dissolved in aqueous solution in various concentrations. Then, tripolyphosphate (TPP) was dissolved in distilled water. Microparticles were spontaneously obtained upon the addition of 1 mL of a TPP aqueous solution to 5 mL of the WSC aqueous solution under magnetic stirring at room temperature. The resultant mixtures were broadly characterized as either a clear solution, an opalescent suspension (MPs), or aggregates. The optimal solution concentrations were identified from these experiments as 0.8–3.0 mg/mL WSC in aqueous solution and 0.5–2.0 mg/mL TPP in distilled water. These concentrations were subsequently used for the preparation of micro-particle suspensions.

2.4. Preparation of Insulin-Loaded WSC-MPs. Insulin-loaded WSC-MPs were prepared by the polyelectrolyte complexation of WSC with insulin and TPP as a counteranion,

in which the positively charged amino groups of WSC interact with the negatively charged TPP [21]. Water-soluble chitosan (1.0–2.0 mg/mL) as well as TPP (0.7–1.5 mg/mL) were dissolved in distilled water. Insulin was dissolved in 0.01 M HCl at various concentrations. Insulin solution (2 mL) was premixed with 2 mL of TPP solution or 4 mL of WSC solution before the addition of the TPP solution dropwise into the WSC solution under magnetic stirring at ambient temperature. Insulin-loaded WSC-MPs were isolated by centrifugation ($15,000 \times g$, 30 minutes, 4°C) and resuspended in 10 mL of purified water after discarding the supernatants. Insulin-loaded WSC-MPs were also prepared on a large scale by adding 20 mL of the TPP solution and 20 mL of the insulin solution to 40 mL of WSC, maintaining the stirring conditions (at room temperature). Insulin-loaded WSC-MPs were centrifuged ($15,000 \times g$, 30 minutes, 4°C), and the resuspension volumes were proportionally adapted to. Freeze-drying was performed using the freeze dryer. Particles were dried for 24 h corresponding to a temperature of -45°C .

2.5. Micro-Particle Characterization. The particle size and size distributions of the insulin-loaded WSC-MPs were determined by particle sizer (Zetasizer 3000 HAS, Malvern Instruments Ltd., Worcs, UK). Insulin-loaded WSC-MPs separated from suspension were dried by a freeze dryer. The morphology of the MPs was observed by TEM-100CXII.

2.6. Insulin Association Efficiency. The association efficiency (AE) and loading capacity (LC) of the process were determined upon separation of the insulin-loaded WSC-MPs by ultracentrifugation at $15,000 \times g$, 4°C for 30 minutes from the aqueous medium containing nonassociated insulin. The amount of free insulin in the supernatant was measured using the improved Bradford method [22]. The pH of supernatant of insulin-loaded WSC-MPs were adjusted to 7–9 using saturated NaOH, and the precipitations (free chitosan) were separated from solution by centrifuging for about 10 minutes (4000 r/min). The supernatant in a volume of 0.1 mL was pipetted into 5 mL test tubes, and 3 mL of Coomassie Brilliant Blue G-250 dye reagent was added to the test tube, then the contents were mixed by vortexing. The absorbance at 595 nm was measured after 10–15 minutes. The standard curve was plotted between insulin content and corresponding absorbance. The AE and LC of the insulin-loaded WSC-MPs were calculated according to (1). The amounts of insulin were determined in triplicate:

$$\begin{aligned} \text{AE (\%)} &= \frac{\text{Total amount of insulin} - \text{Free insulin in supernatant}}{\text{Total amount of insulin}} \\ &\times 100, \\ \text{LC (\%)} &= \frac{\text{Total amount of insulin} - \text{Free insulin in supernatant}}{\text{Total weight of nanoparticles}} \\ &\times 100. \end{aligned} \quad (1)$$

2.7. In Vitro Release Studies. To establish the insulin release profile from insulin-loaded WSC-MPs at simulated gastric and intestinal pH, insulin-loaded WSC-MPs were placed into test tubes containing 1.5 mL of pH 1.2 and pH 7.4 phosphate buffer at 37°C, respectively. The concentrations of insulin-loaded WSC-MPs in the release medium were adjusted in order to achieve sink conditions for insulin release. At appropriate time intervals of 0.5, 2, 4, 6, 8, 10, 12, and 24 h, 1 mL of samples removed and ultracentrifuged at $15,000 \times g$ for 30 minutes, and 1 mL of the supernatants was replaced by fresh medium. The individual sample was centrifuged, and the amount of insulin in the supernatant was measured by the improved Bradford method. All release experiments were done triplicately, and the mean values were recorded. The insulin cumulative release was calculated according to

$$CR(\%) = \frac{C_t \times V + V_i \sum_{n=0}^{t-1} C_i}{W \times X} \times 100, \quad (2)$$

where C_t is the sample concentration at T , V was the total volume of release medium, V_i was the sampling volume at T_i , C_i was the sample concentration at T_i (both V_0 and C_0 were equal to zero), W was the weight of lyophilized particles used for drug content determination, and X was the drug content.

3. Results and Discussion

3.1. Preparation of WSC-MPs. Experiments were performed by using various WSC aqueous solutions and TPP concentration, and the optimal condition was selected to achieve microparticles by polyelectrolyte complexation. The results are presented in Table 1.

The zone of the opalescence corresponding to a suspension of WSC-MPs was associated with a formulation containing a final WSC aqueous solution concentration in the range 1.0–2.0 mg/mL and a final TPP concentration in the range 0.5–1.0 mg/mL, respectively. These conditions were selected for preparation of the WSC-MPs to reach final theoretical WSC/TPP ratios of 6 : 1–8 : 1 (w/w).

3.2. Preparation of Insulin-Loaded WSC-MPs

3.2.1. Effect of Resolution Medium of Insulin. Table 2 represents the insulin association efficiency of the insulin-loaded WSC-MPs prepared by the ionotropic gelation in different resolution media of insulin. Insulin was premixed with WSC aqueous solution prior to MPs' formation.

The results showed higher AE for insulin-loaded WSC-MPs in 0.01 mol/L HCl than other resolution medium of insulin. This finding can probably be explained by the competition between insulin and TPP in the interaction with WSC. Insulin in 0.01 mol/L HCl with higher negative charge density resulted in prior formation of insulin-loaded WSC-MPs; the particles were formed spontaneously by the electrostatic interactions of positively charged WSC and negatively charged insulin. Compared to insulin, TPP is a much smaller molecule with higher negative charge density. It can dominate the interaction of insulin with positively

charged polymers causing a reduction in the positive charge density of WSC.

3.2.2. Optimization of Insulin-Loaded WSC-MPs. Many studies have reported that the quantity of TPP in a given formulation has a significant effect on the insulin encapsulation and characteristic of CS-MPs [5, 6, 8, 20]. Therefore, the optimal amount of TPP in formulation was investigated in detail.

Results of the investigations on the experimental conditions for the formation of insulin-loaded WSC-MPs could be obtained by varying the concentrations of TPP, WSC, and insulin. As concluded from the results given in Table 3, association efficiency of the insulin-loaded WSC-MPs was affected by the insulin concentration in 0.01 mol/L HCl solutions and the amount of insulin incorporated, with increasing amount of insulin leading to a slight decrease of association efficiency. The mechanism of insulin association to insulin-loaded WSC-MPs was mediated by an ionic interaction between both macromolecules. The electrostatic interactions between the acidic insulin groups and the amino groups of WSC might have played a role in association of insulin to the insulin-loaded WSC-MPs.

Formulation of insulin-loaded WSC-MPs contained a final WSC aqueous solution concentration of 1.5 mg/mL and a final TPP concentration of 1.0 mg/mL, respectively. This condition was selected for preparation of the insulin-loaded WSC-MPs to reach final theoretical WSC/TPP ratios of 3 : 1 (w/w). Insulin concentration of 0.5 mg/mL (0.01 mol/L HCl) could be loaded efficiently, and the relatively higher association efficiency and loading capacity of insulin-loaded WSC-MPs were $48.28 \pm 0.90\%$ and $9.52 \pm 1.34\%$ ($n = 3$), respectively. However, the preparation of chitosan-insulin microparticles with about 30% of AE by a polyelectrolyte complexation method using insulin in 0.01 mol/L HCl was reported [23].

3.2.3. Effect of pH of TPP. Table 4 represents the insulin association efficiency of the microparticles prepared by the polyelectrolyte complexation at different pH of TPP. WSC aqueous solution presented $-\text{NH}_3^+$ sites. Triphosphoric acid ($\text{H}_5\text{P}_3\text{O}_{10}$) is a weak polyprotic acid-like phosphoric acid, the K_α decrease from $K_{\alpha 1}$ to $K_{\alpha 2}$, and the decreased quantity is one to several orders. Due to this reason, sodium triphosphate ($\text{Na}_5\text{P}_3\text{O}_{10}$) dissolved in water to dissociate both OH^- and triphosphoric ions in the TPP solution. In original TPP solution (basic, pH was not adjusted), the concentration of $\text{P}_3\text{O}_{10}^{5-}$ and $\text{HP}_3\text{O}_{10}^{4-}$ was high, but the concentration of OH^- was also present. The OH^- or triphosphoric ions ($\text{P}_3\text{O}_{10}^{5-}$ and $\text{HP}_3\text{O}_{10}^{4-}$) in this medium could competitively react ionically with the bind site $-\text{NH}_3^+$ in chitosan by deprotonation and covalent bonds, respectively. The pH value of prepared chitosan (0.01 M) or TPP aqueous solution (0.01 M) was 4.0 and 9.7, respectively. The TPP ions ($\text{P}_3\text{O}_{10}^{5-}$) competed with OH^- ions to bind with $-\text{NH}_3^+$ in chitosan, resulting in the decrease of binding sites for OH^- ions and increased pH. When titrating acetic acid (pH 4.0) with TPP (pH 9.7), it was found that the pH of

TABLE 1: Effect of water-soluble chitosan (WSC) and TPP concentrations on the formation of microparticles.

WSC (mg/mL)	TPP (mg/mL)				
	0.5	0.7	1.0	1.5	2.0
0.8	↓	↓	↓	↓	↓
1.0	◦	↓	↓	↓	↓
1.5	×	◦	↓	↓	↓
2.0	×	◦	◦	↓	↓
3.0	×	×	×	↓	↓

×: clear solution; ◦: opalescent suspension (nanoparticles); ↓: aggregation.

TABLE 2: Effect of resolution medium of insulin on association efficiency (AE) ($n = 3$).

Resolution medium	% AE (mean \pm SD)
0.01 mol/LHCl	48.28 \pm 1.2
0.01 mol/LNaOH	18.80 \pm 2.3
pH 7.4 phosphate buffer	40.07 \pm 4.3
distilled water	34.82 \pm 3.6

mixing increased quickly. This is due to the buffer ability of TPP ions in weak acid. However, the pH increase of the TPP (pH 9.7)/chitosan (pH 4.0) mixing solution was significantly slower than the pH increase of TPP (pH 9.7)/acid (pH 4.0) mixture. This suggests that the TPP ion lost its buffer ability due to binding to the protonated chitosan. This hypothesis shows that the precipitations of complexes in this state were formed both by deprotonation and covalent bonds. By adjusting the pH value of TPP solution from 9.7 (initial) to 4.0, only $P_3O_{10}^{5-}$ anions existed. The pH value of the TPP (pH 4.0)/chitosan (pH 4.0) mixture was kept almost constant with the increase of TPP/chitosan mixing ratio due to no OH^- ions being released, but chitosan-TPP precipitation was also formed. This result shows that the precipitations of complexes were formed only by ionic-crosslinking between $-NH_3^+$ and TPP ions [24]. So insulin-loaded WSC-MPs had higher association efficiency in the pH 4.0 and pH 9.7 of TPP solution.

3.3. Morphology of the Microparticles. According to the size distribution, a photograph and a TEM photograph are shown in Figures 1 and 2, respectively. Morphologically, the insulin-loaded WSC-MPs look round to oval in shape and have a relatively smooth surface. The average size of Insulin-loaded WSC-MPs was 292 nm.

3.4. Insulin Release Studies. The representative insulin release profile from insulin-loaded WSC-MPs in conditions, simulating the gastric and intestinal pH, is illustrated in Figure 3, as a suitable application of this method. The profile of drug release from nanoparticles can be affected by method of preparation and also by ionic interaction between the drug and addition of auxiliary ingredients. If the drug was loaded by incorporation method, the system would have a relatively small burst effect and then sustained release characteristics. When the drug was involved in interaction

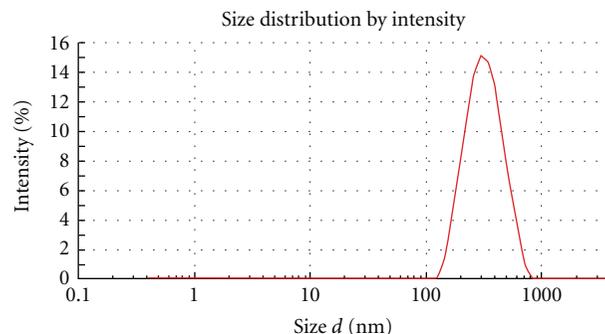


FIGURE 1: Size distribution of the insulin-loaded WSC-MPs made by the polyelectrolyte complexation.

FIGURE 2: TEM photograph of the insulin-loaded WSC-MPs made by the polyelectrolyte complexation (7 KV, $\times 1,600$).

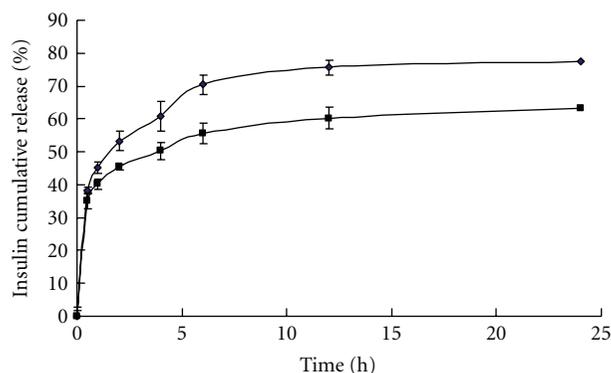
with auxiliary ingredients to form a less water-soluble complex, the drug release can be very slow with almost no burst effect. The release results showed good incorporation of insulin in polymers by ionic interaction between insulin and the polymers. Insulin release was characterized by no burst effect, in both media at 0.5 h ($<40\%$). The preliminary insulin release test from insulin-loaded WSC-MPs in vitro proved that they had a sustained release form as shown in Figure 3. Ionic strength in release medium may affect significantly the release properties of insulin-loaded WSC-MPs based on ionic gelation. The in vitro insulin release profiles obtained for each formulation showed three phases [25]: (1) a first initial burst release of 50% (in 2 h), due to the drug desorption from the particles surface; (2) a plateau for the following 12 h, resulting only from the diffusion of the drug dispersed in the polymer matrix; (3) a constant

TABLE 3: The optimal conditions for the formulation of insulin-loaded WSC-MPs by orthogonal design ($L_9(3^4)$) ($n = 3$).

NO.	pH of WSC	C_{WSC} (mg/mL)	C_{INS} (mg/mL)	C_{TPP} (mg/mL)	AE(%)
1	3.8	1.0	0.5	0.7	32.01
2	3.8	1.5	0.7	1.0	27.27
3	3.8	2.0	1.0	1.5	26.32
4	4.5	1.0	0.7	1.5	21.79
5	4.5	1.5	1.0	0.7	31.24
6	4.5	2.0	0.5	1.0	44.56
7	5.0	1.0	1.0	1.0	34.91
8	5.0	1.5	0.5	1.5	27.65
9	5.0	2.0	0.7	0.7	15.95

TABLE 4: Effect of pH of TPP on association efficiency (AE) ($n = 3$).

pH of TPP	% AE (mean \pm SD)
0.01 mol/LHCl	18.44 \pm 5.4
3.0	33.35 \pm 4.1
4.0	46.26 \pm 2.6
5.0	29.75 \pm 3.2
7.0	32.39 \pm 2.8
9.7 (distilled water)	48.31 \pm 1.6

FIGURE 3: Insulin release from WSC-MPs at gastric pH 1.2 (◆) and phosphate buffer, simulating intestinal pH 7.4 (■) ($n = 3$).

sustained release of the remaining drug, resulting from the diffusion of the insulin through the polymer wall as well as its erosion.

4. Conclusion

In summary, the results suggested that, while the polyelectrolyte complexation is easy to be used for the production of insulin-loaded WSC-MPs, the insulin in 0.01 mol/L HCl seems to be more suitable for future studies. The insulin-loaded WSC-MPs obtained by the polyelectrolyte complexation method had higher insulin association efficiency and had higher association efficiency in pH 4.0 and pH 9.7 of TPP solution. These insulin-loaded WSC-MPs have narrow size distributions look round to oval in shape, and have a relatively smooth surface. The in vitro release

profiles of insulin from insulin-loaded WSC-MPs showed relatively small initial burst release followed by a slowly sustained release phase. Quantification of insulin released from insulin-loaded WSC-MPs by the improved Bradford method supported the application of these insulin-loaded WSC-MPs as oral delivery systems for insulin. Present investigations are being performed regarding the assessment of insulin in vivo oral bioactivity.

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