Preparation and Characterization of Nanoparticles Shelled with Chitosan for Oral Insulin Delivery

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Received August 9, 2006; Revised Manuscript Received October 12, 2006

Nanoparticles (NPs) composed of chitosan (CS) and poly(γ-glutamic acid) (γ-PGA) were prepared by a simple ionic-gelation method for oral insulin delivery. Fourier transform infrared (FT-IR) spectra indicated that CS and γ-PGA were ionized at pH 2.5−6.6, while X-ray diffractograms demonstrated that the crystal structure of CS was disrupted after it was combined with γ-PGA. The diameters of the prepared NPs were in the range of 110−150 nm with a negative or positive surface charge, depending on the relative concentrations of CS to γ-PGA used. The NPs with a positive surface charge (or shelled with CS) could transiently open the tight junctions between Caco-2 cells and thus increased the paracellular permeability. After loading of insulin, the NPs remained spherical and the insulin release profiles were significantly affected by their stability in distinct pH environments. The in vivo results clearly indicated that the insulin-loaded NPs could effectively reduce the blood glucose level in a diabetic rat model.

Introduction

An oral route is the most convenient and comfortable means of administering protein drugs and eliminates pain caused by an injection, the stress associated with multiple daily injections, and possible infections.[1] However, oral administration of protein drugs also has certain obstacles, as drugs must overcome various significant barriers in the gastrointestinal (GI) tract prior to delivery to the bloodstream.[2,3] First of all, protein drugs are readily degraded by the low pH of gastric medium in the stomach. Second, different digestive enzymes in the stomach and small intestine may lead to degradation of protein drugs.[3−5] Therefore, a carrier system is required to protect protein drugs from the GI environment.

However, the intestinal epithelium is another major barrier to the absorption of hydrophilic macromolecules, as they cannot diffuse across the cells through the lipid-bilayer cell membranes.[4−8] Attention has been given to improving the paracellular transport of hydrophilic macromolecules.[9] The transport of hydrophilic macromolecules via the paracellular pathways is, however, severely restricted by the presence of tight junctions, which are located at the luminal aspect of adjacent epithelial cells.[10] A number of intestinal permeation enhancers have been explored to improve the oral bioavailability of hydrophilic macromolecules.[9] It was suggested that an ideal intestinal permeation enhancer should be nontoxic and act in a reversible way on the tight junctions between adjacent epithelial cells.[11]

Therefore, this study was to develop a novel nanoparticle (NP) delivery system that is able to provide protection from the GI environment and enhance absorption of protein drugs in the intestinal epithelium via the paracellular pathways. The NP delivery system is composed of low molecular weight (MW) chitosan (CS) and poly(γ-glutamic acid) (γ-PGA) prepared by a simple and mild ionic-gelation method. This technique is promising as the NPs can be prepared under aqueous-based conditions at room temperature.

CS is a nontoxic, biocompatible, and mucoadhesive polymer that has been proven to be a safe and efficient intestinal permeation enhancer for the absorption of protein drugs.[9,11,12] Given a low MW, the polycationic characteristic of CS can be used together with good solubility at a pH value close to physiological ranges.[13] Loading of protein drugs at physiological pH ranges may prevent their bioactivity from decreasing. γ-PGA, an anionic peptide, is a natural compound produced as capsular substance or as slime by members of genus *Bacillus*.[14] It is known that γ-PGA is water-soluble, biodegradable, nontoxic, and relatively nonimmunogenic.[15,16]

In this study, preparation of the NPs composed of low MW CS and γ-PGA at various weight ratios was reported and their physicochemical characteristics were examined by Fourier transform infrared spectroscopy (FT-IR), X-ray diffraction (XRD), and dynamic light scattering. Evaluation of the prepared NPs in enhancing intestinal paracellular transport was investigated in vitro in Caco-2 cell monolayers. The change of transepithelial electrical resistance (TEER) for the tightness of cell monolayers was measured, and the paracellular transport of NPs was visualized by use of confocal laser scanning microscopy (CLSM). Additionally, the release profiles of insulin from test NPs were studied in simulated GI and bloodstream media. The efficacy of oral administration of the insulin-loaded NPs was also investigated in a diabetic rat model.

Experimental Section

Preparation of Low MW CS and γ-PGA. CS (MW ~280 000, Challenge Bioproducts Co., Taichung, Taiwan) with a degree of

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10.1021/bm0607776 CCC: $37.00
Published on Web 12/08/2006

deacetylation of approximately 85% was treated with cellulase (1.92 units/mg, Sigma, St. Louis, MO) to produce low MW CS according to a method described by Qin et al.[17] The depolymerized CS was precipitated with aqueous NaOH at pH 7.0−7.2. The obtained CS had an average MW of 80 000, determined by a gel-permeation chromatography system. γ-PGA was produced by *Bacillus licheniformis* (ATCC 9945, Bioresources Collection and Research Center, Hsinchu, Taiwan) and purified as per the method reported by Yoon et al.[18] with slight modifications. The purified γ-PGA was then hydrolyzed in a tightly sealed steel container at 150 °C for 1.5 h and the average MW of the obtained γ-PGA was about 60 000. Details of the methodology used in the preparation of low MW CS and γ-PGA were reported in our previous publication.[6] The obtained low MW CS and γ-PGA were then used for the preparation of NPs.

Preparation of NPs. NPs were prepared by a simple ionic-gelation method under magnetic stirring at room temperature. In brief, aqueous γ-PGA (1.0 mg/mL, 2 mL, pH 7.4) was added by flush mixing with a pipet tip into aqueous CS at various known concentrations (0.15, 0.30, 0.60, 0.90 or 1.20 mg/mL, 10 mL, pH 6.0). The obtained NPs were collected by ultracentrifugation at 32 000 rpm for 50 min. Supernatants were discarded and NPs were resuspended in deionized (DI) water for further studies.

Characterization of NPs. The crystalline forms of CS and the CS/γ-PGA NPs were determined by XRD (MXP18, MAC Science, Japan). The diffraction patterns were determined over a range of diffraction angle $2\theta = 5-40°$.[19] The size distribution and ζ potential of the prepared NPs at pH 1.2 (0.1 N HCl), pH 2.5 (0.01 N HCl), and pH 6.6 or 7.4 (10 mM phosphate-buffered saline, PBS), simulating the environments of the GI tract or bloodstream, were measured on a Zetasizer (3000HS, Malvern Instruments Ltd., Worcestershire, U.K.).[20,21] FT-IR (Perkin-Elmer Spectrum RX1 System, Buckinghamshire, England) was used to examine the peak variation of amino groups and carboxylic groups on the prepared NPs at distinct pH values.

TEER Measurements. Caco-2 cell monolayers were cultured on the tissue-culture-treated polycarbonate filters (diameter 24.5 mm, growth area 4.7 cm^2) in Costar Transwell 6 wells/plates (Corning Costar Corp., Corning, NY) and were used for the transport experiments 24−30 days after seeding (TEER values in the range of 600−800 $\Omega \cdot cm^2$).[22] Subsequently, evaluation of the prepared NPs in enhancing intestinal paracellular transport at pH 6.6 or pH 7.4 was investigated in vitro in Caco-2 cell monolayers. The change of TEER for the tightness of cell monolayers was measured with a Millicell electrical resistance system (Millipore Corp., Bedford, MA) connected to a pair of chopstick electrodes.

Fluorescein Isothiocyanate-Labeled NPs and CLSM Visualization. Synthesis of the FITC-labeled CS was based on the reaction between the isothiocyanate group of FITC and the primary amino groups of CS as reported in the literature.[23] To remove the unconjugated FITC, the precipitate was subjected to repeated cycles of washing and centrifugation (40000*g* for 10 min) until no fluorescence was detected in the supernatant. The obtained FITC-labeled CS was used to prepare the NPs for the CLSM study as per the procedure described above.

The transport medium (pH 6.6 or 7.4) containing the FITC-labeled NPs (0.2 mg/mL, 2 mL) was introduced into the donor compartment of Caco-2 cells, which were precultured on the Transwell for 24−30 days. After incubation for 60 min at 37 °C, test samples were aspirated. The cells were then washed twice with prewarmed PBS solution before they were fixed in 3.7% paraformaldehyde.[23] Cells were examined under an inverted CLSM (TCS SL, Leica, Germany). Fluorescence images were observed by use of an argon laser (excitation at 488 nm, emission collected at a range of 510−540 nm) at 0.2-μm intervals, and a maximum intensity projection and 3D images were created with LCS Lite software (version 2.0).

Visualization of Opening Tight Junctions. Caco-2 cell monolayers were incubated with the FITC-labeled NPs (0.2 mg/mL, 2 mL). After 60 min, the incubated NPs were removed and the cells were fixed in 3.7% paraformaldehyde. The cells were washed three times with PBS and permeabilized with 0.2% Triton X-100 and RNase (100 μg/mL) for 15 min at 37 °C. The wash was repeated and the cells were blocked with 5% normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS for 60 min at 37 °C. Subsequently, cells were treated with rabbit anti-ZO-1 mAb (Zymed Laboraties, San Francisco) at 1:50 dilution for 60 min at 37 °C. After being washed three times with PBS, the cells were incubated in Cy-5-conjugated goat anti-rabbit IgG at 1:100 dilution (Jackson ImmunoResearch Laboratories). Before the samples were mounted for CLSM examination, the cells were again washed with PBS and DI water. The stained cells were evenly mounted on the slides and examined with excitation at 488 and 633 nm, respectively, by CLSM. Superimposed images were performed with the LCS Lite software as mentioned above.

Insulin Loaded Capacity of NPs. A sample of 100 mg of insulin (from bovine pancreas, 27.4 IU/mg) was dissolved in 10 mL of 0.01 N HCl and this solution was neutralized with 0.1 N NaOH.[24] The insulin solution was then diluted with DI water to make a 1 mg/mL insulin stock solution. The insulin stock solution (1 mL) was premixed with aqueous γ-PGA (2 mg/mL, 1 mL) and added into aqueous CS (0.9 mg/mL, 10 mL) under magnetic stirring at room temperature as described before. To determine the loading content and loading efficiency, the insulin-loaded NPs were collected by ultracentrifugation at 32 000 rpm, 4 °C for 50 min, and the amount of free insulin concentration was assayed in the supernatant by high-performance liquid chromatography (HPLC). The drug loading content and loading efficiency of the NPs were determined as described in the literature and calculated from the following equations:[25]

$$\text{loading content (\%)} = \frac{\text{total amount of insulin} - \text{amount of free insulin}}{\text{weight of NPs}} \times 100$$

$$\text{loading efficiency (\%)} = \frac{\text{total amount of insulin} - \text{amount of free insulin}}{\text{total amount of insulin}} \times 100$$

In Vitro Release Study. The release profiles of insulin from the prepared NPs were investigated in simulated dissolution media (pH 2.5, 6.6, and 7.4, simulating GI tract and bloodstream conditions) at 37 °C under agitation (100 rpm, Distek-2230A, North Brunswick, NJ). At particular time intervals, the samples were taken out and centrifuged and the supernatants were used for the HPLC analysis. The amount of insulin released was expressed as a percentage of the total insulin associated with the NPs as calculated from the loading efficiency.[26] The stability of the released insulin was determined by analyzing the conformation of the released insulin at pH 7.4 on an Aviv 202 spectropolarimeter and comparing the spectrum with that of standard insulin. The ellipticity (q, millidegrees) was measured from 300 to 200 nm.[27]

In Vivo Study. Animal care and use was performed in compliance with the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996. Male Wistar rats (180−200 g) were rendered diabetic by daily intraperitoneal injection of streptozotocin (STZ, 80 mg/mL in pH 4.5 citrate) at 75 mg/kg body weight for 3 days.[2,28] The diabetic rats were fasted for 12 h prior to and remained fasted for 10 h during the experiment but were allowed water ad libitum. Formulations (in DI water, 2 mL, pH 6.0) following were administered to the diabetic rats by use of an oral feeding needle: oral empty NPs (without insulin), oral insulin solution (15.0 or 30.0 IU/kg), oral insulin-loaded NPs (15.0 or 30.0 IU/kg), and subcutaneous (SC) injection of insulin solution (2.5 IU/kg).

Blood samples were collected from the tails of the rats prior to oral administration to establish the baseline glucose levels, and at different times after dosing blood samples were collected in the same way.[29] The blood glucose levels were determined by use of a glucose meter (LifeScan Inc., CA).

Table 1. Effects of Various Weight Ratios (or Charge Ratios) of Chitosan to Poly(γ-glutamic acid) on Mean Particle Size and ζ Potential of Nanoparticles[a]

CS:γ-PGA ratio (w/w)	CS:γ-PGA charge ratio[b]	mean particle size (nm)	ζ potential (mV)
0.8:1.0	0.5:1.0	114.8 ± 9.7	−23.7 ± 2.5
1.5:1.0	1.0:1.0	[c]	[c]
3.0:1.0	2.0:1.0	131.9 ± 1.9	26.4 ± 1.4
4.5:1.0	3.0:1.0	145.6 ± 1.9	32.1 ± 1.6
6.0:1.0	4.0:1.0	147.3 ± 2.0	33.4 ± 1.5

[a] Prepared in deionized water (pH 6.0, $n = 5$). [b] Ratio of positively charged -NH_3^+ groups on CS to negatively charged -COO^- groups on γ-PGA. [c] Precipitation of aggregates was observed.

Statistical Analysis. Statistical analysis for the determination of differences in the measured properties between groups was accomplished by one-way analysis of variance and determination of confidence intervals, performed with a computer statistical program (Statistical Analysis System, Version 6.08, SAS Institute Inc., Cary, NC). All data are presented as mean values with standard deviations indicated (mean ± SD). Differences were considered to be statistically significant when the p values were less than 0.05.

Results and Discussion

Preparation of NPs. NPs were produced by ionic gelation of the positively charged CS with the negatively charged γ-PGA. As shown in Table 1, CS:γ-PGA at distinct weight ratios formed complexes on the nanometer scale, with the exception of the weight ratio at 1.5:1.0. At this weight ratio, theoretical calculations demonstrated that the overall charge ratio (of the positively charged -NH_3^+ groups on CS to the negatively charged -COO^- groups on γ-PGA) of the NPs was approximately 1.0:1.0, thus resulting in aggregation and precipitation of this formulation.

The diameters of the prepared NPs were in the range of 110−150 nm with a negatively or positively charged ζ potential, depending on the relative concentrations of CS and γ-PGA used. When the amount of negatively charged γ-PGA sufficiently exceeded that of positively charged CS (CS:γ-PGA = 0.8:1.0), the NPs formed tended to have γ-PGA exposed on their surfaces and thus had a negative surface charge. In contrast, when the amount of the positively charged CS significantly exceeded that of the negatively charged γ-PGA (CS:γ-PGA = 3.0:1.0, 4.5:1.0, or 6.0:1.0), some of the excessive CS molecules were entangled onto the surfaces of the obtained NPs. Thus, the resulting NPs displayed a positive surface charge (Table 1).

The X-ray diffractograms of CS and CS/γ-PGA NPs are shown in Figure 1. There were two strong crystalline peaks in the diffractogram of CS at 2θ at 10.4° and 21.8°, while the crystalline peaks observed in the CS/γ-PGA NPs decreased significantly. This observation indicated that the crystal structure of CS was disrupted after being combined with γ-PGA via electrostatic interactions.

CS has special features of adhering to the mucosal surface and opening the tight junctions between epithelial cells.[11] It was suggested that interactions of positively charged amino groups of CS with negatively charged sites on cell surfaces and tight junctions induce a redistribution of F-actin and the tight junction's protein ZO-1, which accompanies the increased paracellular permeability.[10] Therefore, only those NPs with a positive surface charge (or CS dominant or shelled on the surface) were selected for the rest of the study.

It was reported that the natural pH environment of the GI tract varies from acidic in the stomach to slightly alkaline in the small intestine.[30] The fasting pH of the stomach is about

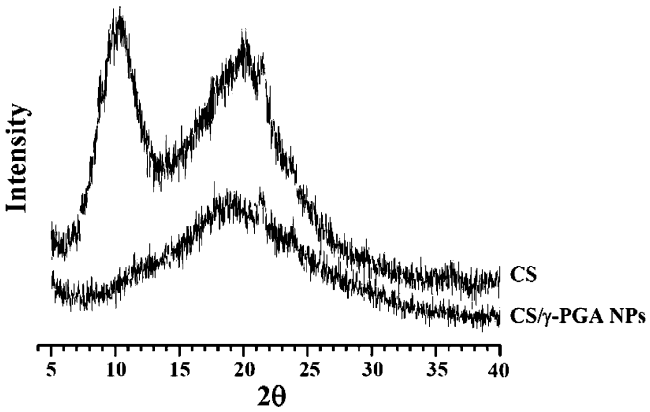

Figure 1. X-ray diffractograms of CS (top trace) and CS/γ-PGA NPs (bottom trace).

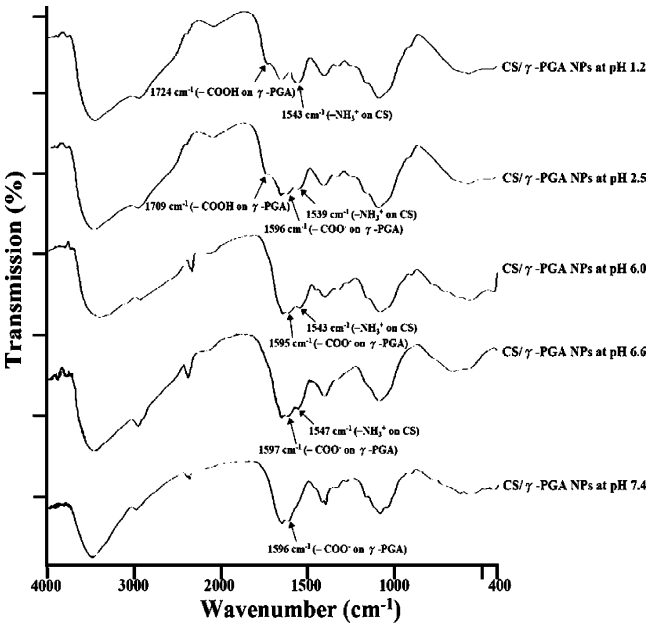

Figure 2. FT-IR spectra of CS/γ-PGA NPs in distinct pH environments.

2.5−3.7.[31,32] In the presence of food, the stomach pH is about 1.0−2.0, due to hydrochloric acid secreted by parietal cells.[33] The proximal part of the small intestine (duodenum) has a pH of about 6.0−6.6 due to neutralization of the acid by bicarbonates secreted by the duodenal mucosa and pancreas.[30,33] The jejunum and ileum are the middle portion and terminal part of the small intestine, respectively, and their pH values are about 7.4.[30,32] Therefore, characterization of the prepared NPs at distinct pH values, simulating the environments of the GI tract or bloodstream, must be investigated.

Characterization of NPs at Distinct pH Values. The peak variation of the NPs (CS:γ-PGA = 4.5:1.0) at distinct pH values was examined by FT-IR (Figure 2). As shown, at pH 6.0 (the condition used to prepare NPs in DI water) and pH 6.6 (simulating the pH environment at the duodenum), the characteristic peaks observed at 1543 (1547) and 1595 (1597) cm^{-1} were the protonated amino groups (-NH_3^+) on CS and the carboxylic ions (-COO^-) on γ-PGA, respectively.[6] The ionized CS and γ-PGA were therefore able to form polyelectrolyte complexes via electrostatic interactions, resulting in a matrix structure with a spherical shape (~140 nm in diameter, Figure 3 and Table 2). At pH 2.5 (simulating the fasting pH of the stomach), some of the -COO^- groups on γ-PGA became protonated (-COOH, 1709 cm^{-1}). The electrostatic interaction

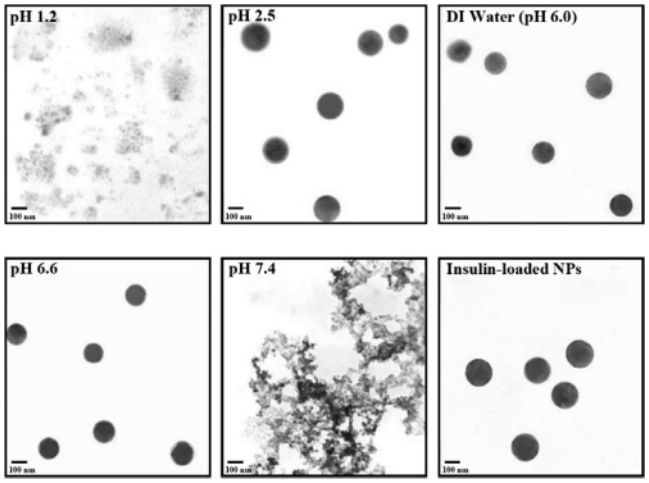

Figure 3. TEM micrographs of the NPs (CS:γ-PGA = 4.5:1.0 w/w) in distinct pH environments and the NPs loaded with insulin.

Table 2. Particle Sizes and ζ Potential Values of Nanoparticles Prepared at Distinct Weight Ratios of Chitosan to Poly(γ-glutamic acid) in Five Distinct pH Environments

pH	mean particle size (nm)	ζ potential (mV)
	CS:γ-PGA Ratio 3.0:1.0 (w/w)	
1.2	[a]	[a]
2.5	183.2 ± 7.3	30.7 ± 0.8
6.0	131.9 ± 1.9	26.4 ± 1.4
6.6	302.6 ± 1.4	5.1 ± 0.3
7.4	[a]	[a]
	CS:γ-PGA Ratio 4.5:1.0 (w/w)	
1.2	[a]	[a]
2.5	180.7 ± 2.5	35.6 ± 1.8
6.0	145.6 ± 1.9	32.1 ± 1.6
6.6	140.2 ± 4.8	12.2 ± 0.6
7.4	[a]	[a]
	CS:γ-PGA Ratio 6.0:1.0 (w/w)	
1.2	[a]	[a]
2.5	184.1 ± 2.6	36.8 ± 4.4
6.0	147.3 ± 2.0	33.4 ± 1.5
6.6	145.1 ± 3.7	13.2 ± 0.4
7.4	[a]	[a]

[a] Precipitation of aggregates was observed.

between CS and γ-PGA was therefore relatively weaker as compared to that at pH 6.0 or 6.6, thus leading to a relatively larger size of the NPs (~180.7 nm, Table 2, pH 2.5).

At pH 1.2 (simulating the pH in the stomach after meal), most carboxylic groups of γ-PGA were in the form of -COOH (1724 cm^{-1}). There was little electrostatic interaction between CS and γ-PGA, and the NPs became unstable and subsequently broke apart (Figure 3). Therefore, the NPs prepared in the study may be orally administered only before meals (pH 2.5−3.7), which was the case used in our in vivo study. Similarly, at pH 7.4 (simulating the pH at the jejunum and ileum or bloodstream), CS was deprotonated (indicated by the disappearance of the characteristic peak of -NH_3^+ on CS, Figure 2) and thus led to the collapse of NPs (Figure 3).

TEER Measurements. Caco-2 cell monolayers have been widely used as an in vitro model to evaluate the intestinal transport of hydrophilic macromolecules.[34] It was reported that measurements of TEER of Caco-2 cell monolyers can be used to predict their paracellular permeability.[35,36] As the tight junctions open, the TEER of Caco-2 cell monolayers would be significantly reduced, due to ion passages through the paracellular route. As shown in Figure 4, the TEER values of Caco-2

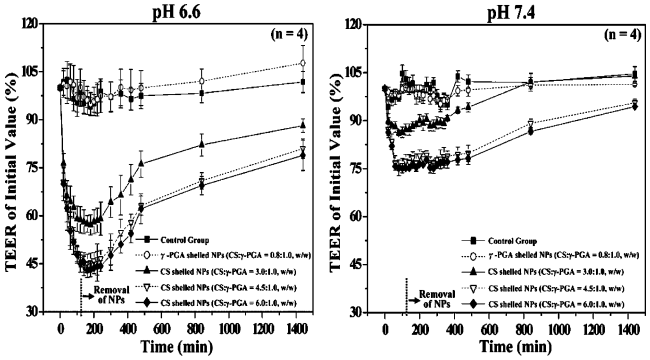

Figure 4. Effects of the NPs shelled with CS or γ-PGA on the TEER values of Caco-2 cell monolayers at pH 6.6 or 7.4. Control group: no incubation with NPs.

cell monolayers incubated with the NPs shelled with γ-PGA (with a negative surface charge) showed insignificant differences as compared to the control group without incubation with NPs. This indicated that γ-PGA does not have any effects on the opening of intercellular tight junctions.

In contrast, incubation of the NPs shelled with CS (with a positive surface charge) on the apical side of Caco-2 cell monolayers led to an immediate and pronounced reduction in TEER values as compared to the control group. After removal of the incubated NPs, a gradual increase in TEER values was observed. These observations indicated that the NPs shelled with CS could transiently open the tight junctions between Caco-2 cells.

The phenomenon described above was significantly less remarkable at pH 7.4 than at pH 6.6 (Figure 4). As mentioned above, the electrostatic interaction between positively charged CS and negatively charged sites of tight junction proteins leads to opening of the tight junctions between cells. At pH 7.4, CS became less positively charged and therefore had less effect on the intercellular permeability of Caco-2 cell monolayers. It was reported that the pK_a value of CS is about 6.5.[37]

As shown in Table 2, the NPs with a CS:γ-PGA weight ratio of 3.0:1.0 had a significantly larger size and a lower ζ potential (pH 6.6) than those with a weight ratio of 4.5:1.0 or 6.0:1.0, thus exhibiting less effect on reducing the TEER values of Caco-2 cell monolayers (Figure 4). In contrast, the latter two groups of NPs were comparable in particle size, ζ potential, and ability in opening intercellular tight junctions. Therefore, the NPs prepared at a CS:γ-PGA weight ratio of 4.5:1.0 were selected for the rest of the study.

CLSM Visualization. CLSM was used to visualize the transport of the FITC-labeled NPs across Caco-2 cell monolayers. This noninvasive method allows for optical sectioning and imaging of the transport pathways across Caco-2 cell monolayers, without disrupting their structures.[3] Figure 5 shows the fluorescence images of four optical sections (and those after 3D reconstruction) of Caco-2 cell monolayers that had been incubated with the FITC-labeled NPs at pH 6.6 (panel a) or pH 7.4 (panel b) for 60 min. At pH 6.6, intense fluorescence signals at intercellular spaces of Caco-2 cell monolayers were observed. This observation confirmed with our TEER results that the NPs shelled with CS were able to open the tight junctions between Caco-2 cells and thus increase the paracellular permeability (Figure 4). In contrast, at pH 7.4, the FITC-labeled NPs appeared to be broken apart and their ability to increase the permeability through Caco-2 cell monolayers via the paracellular pathways was inferior to that observed at pH 6.6.

The NP-treated Caco-2 cell monolayers were also immunofluorescently stained for ZO-1 proteins (at pH 6.6, Figure 6).

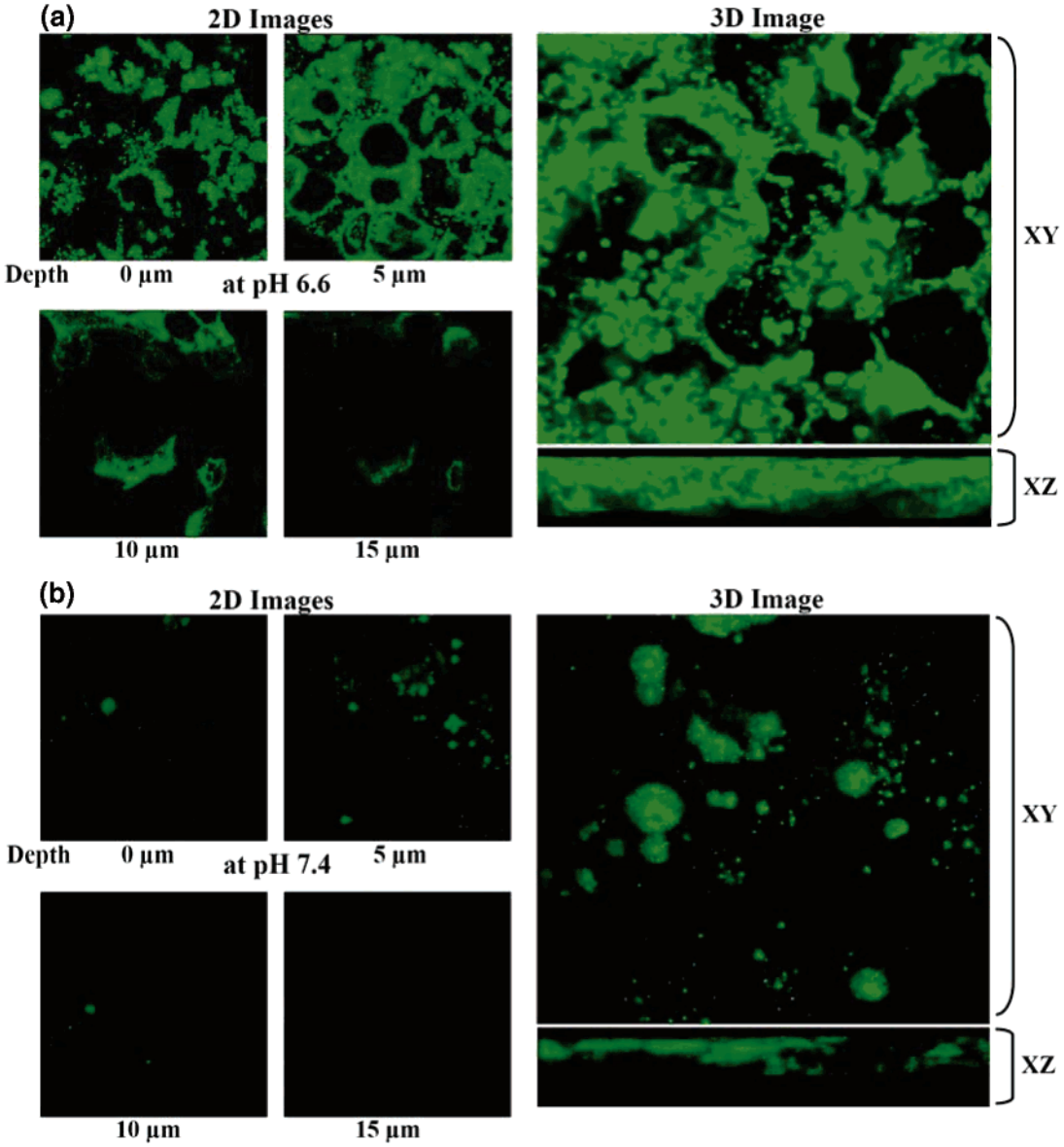

Figure 5. Fluorescence images (taken by an inverted confocal laser scanning microscope) of four optical sections (and those after 3D reconstruction) of Caco-2 cell monolayers that had been incubated with the FITC-labeled NPs for 60 min at (a) pH 6.6 or (b) pH 7.4. XZ shows a 3D confocal reconstruction of diffusion of the FITC-labeled NPs added at the apical sections of Caco-2 cultures grown on Transwell plates.

As shown, the staining for ZO-1 proteins showed a continuous ring appearance between adjacent cells before incubation with the FITC-labeled NPs (control). After incubation with the FITC-labeled NPs, the staining for ZO-1 proteins appeared discontinuous, indicating opening of tight junctions. After removal of NPs, the discontinuous staining for ZO-1 proteins appeared again, indicating restoration of cell tight junctions. Similar observations were also reported by Smith et al.[38] for treatment of Caco-2 cells with CS.

Insulin-Loaded NPs. Zinc is usually added in the biosynthesis and storage of insulin.[34] It is known that the histidine and glutamic acid residues in insulin may complex with Zn to increase its stability.[39] γ-PGA is unique in that it is composed of naturally occurring L-glutamic acids linked together through amide bonds. Therefore, insulin may conjugate with γ-PGA via Zn. The NPs remained spherical with a rather smooth surface after loading of insulin (Figure 3). The insulin-loaded NPs had a particle size of 196.7 $\pm$ 7.1 nm and a ζ potential of 29.7 $\pm$ 2.1 mV, while their insulin loading efficiency and loading content were 56.8% $\pm$ 3.2% and 14.9% $\pm$ 0.9%, respectively. It was found that the stability of the insulin-loaded NPs at distinct pH environments and their ability in opening tight junctions in Caco-2 cell monolayers were similar to those of unloaded NPs.

Figure 7a shows the release profiles of insulin from the prepared NPs at pH 2.5, 6.6, and 7.4, simulating the pH environments of the fasting stomach, the duodenum, and the bloodstream, respectively. The condition at pH 1.2 (simulating the pH environment in the stomach after meal) was not conducted in the study. This is because the NPs prepared in the study were unstable as described before. At pH 2.5, the amount of insulin released from the NPs was about 20% within the first hour. As discussed above, the size of the NPs at pH 2.5 appeared to be relatively larger than at pH 6.0 and, consequently, led to a higher permeability of insulin. This was followed by little release of insulin thereafter, possibly due to the conjugation of insulin with γ-PGA via Zn.

At pH 6.6, there was only a minimal amount of insulin released from the NPs. In contrast, at pH 7.4, the NPs became unstable and broken apart, resulting in a fairly fast release of insulin. These results suggested that the stability of the NPs in distinct pH environments significantly affected their insulin

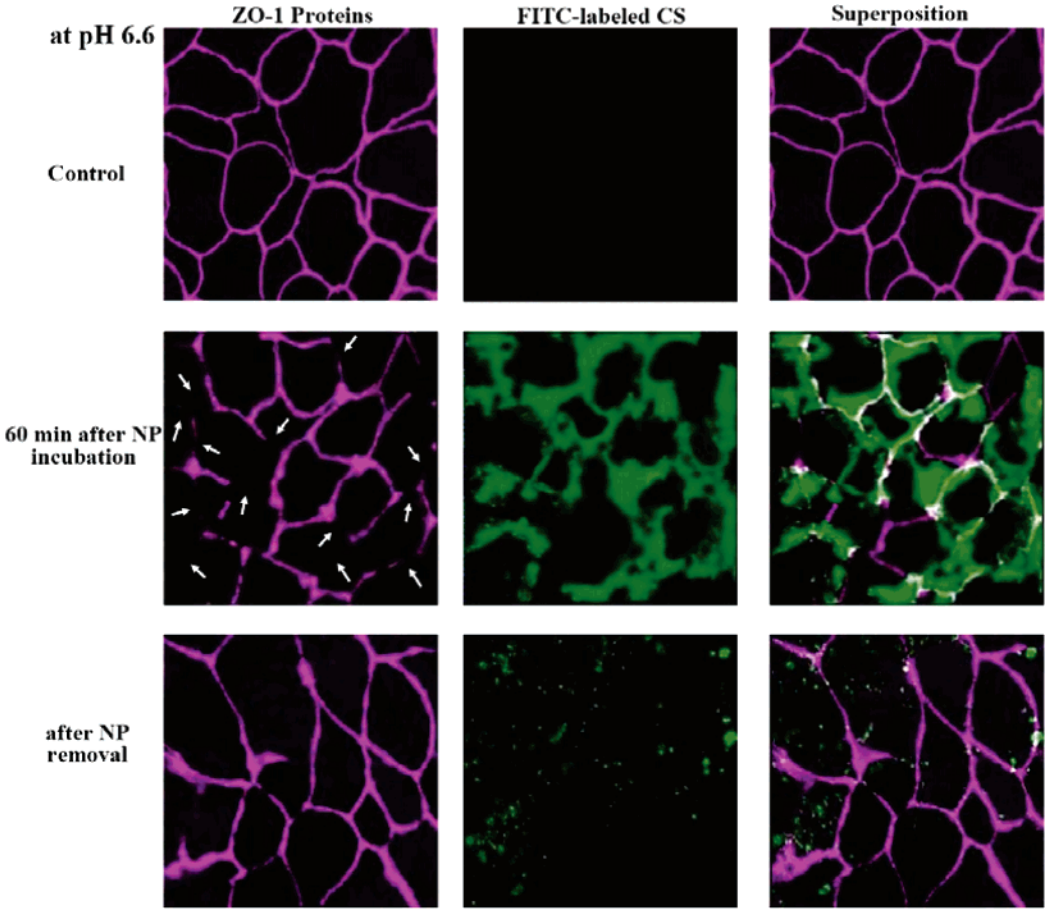

Figure 6. Fluorescence images of Caco-2 cell monolayers immunofluorescently stained for ZO-1 proteins after incubation with the FITC-labeled NPs for 60 min and their counterparts after removal of NPs.

release profiles. As indicated by the circular dichroic spectra (Figure 7b), no significant conformation change was noted for the insulin released from the NPs at pH 7.4 as compared to the standard insulin.

In Vivo Study. STZ has been used to induce diabetes in experimental animals since it can cause selective destruction of pancreatic β cells in several species.[40] At the time of the experiment, the blood glucose levels of the created diabetic rats (469.7 ± 34.2 mg/dL) were significantly higher than the normal ones (107.2 ± 8.1 mg/dL). As might be expected, for the group of SC injection of insulin, the blood glucose level was reduced sharply by 60% within 2 h and then increased gradually (Figure 8). Multiple daily injections of insulin are currently the standard treatment for insulin-dependent diabetic patients.[41,42] However, poor patient compliance is associated with this method. Therefore, better ways of insulin administration are being sought.

Ideally, the oral route is by far the most convenient and comfortable way of delivering insulin. However, oral administration of pure insulin solution (15 or 30 IU/kg) was totally ineffective in decreasing the blood glucose level (Figure 8). It was reported that the development of an effective oral insulin formulation has been hampered by problems of delivering a sufficient supply of insulin to the body at the time when it is needed.[2] For orally administered drugs to be effective, the drugs must be absorbed into the bloodstream. Protein drugs, like insulin, are readily degraded by the low pH in the stomach. Even those protein drugs that survive the hostile environment in the stomach cannot easily partition into the hydrophobic lipid membrane and travel through the epithelial barrier to the blood.[3]

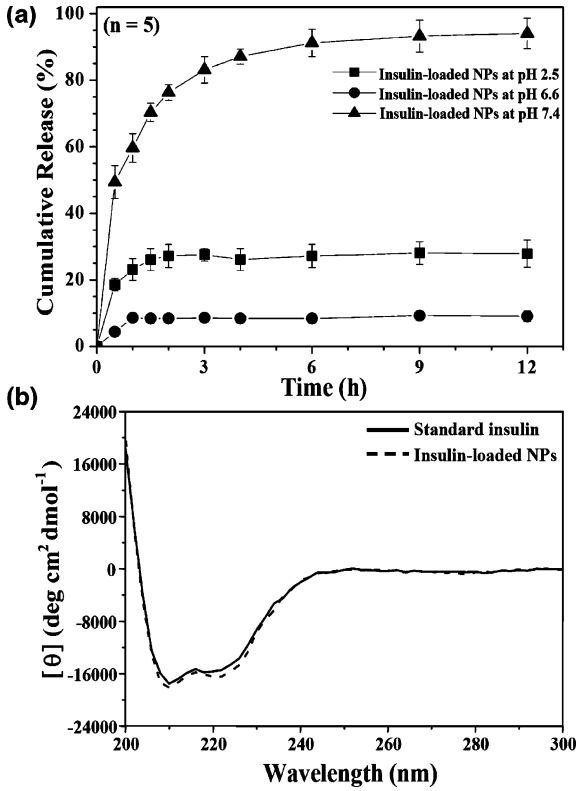

Figure 7. (a) Release profiles of insulin from test NPs in distinct pH environments at 37 °C. (b) Circular dichroic (CD) spectra of insulin released from test NPs at pH 7.4 and standard insulin.

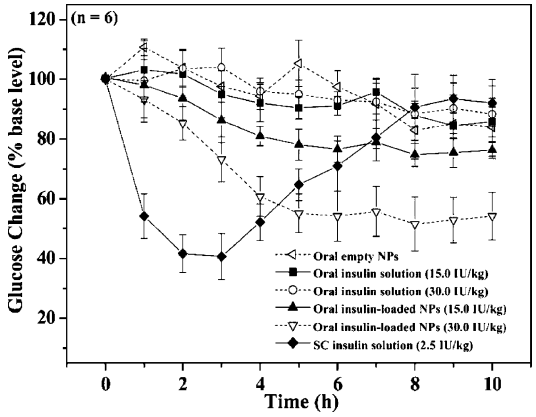

Figure 8. Changes in the blood glucose levels after formulations were administered to a diabetic rat model: oral empty NPs (without insulin); oral insulin solution (15.0 or 30.0 IU/kg); oral insulin-loaded NPs (15.0 or 30.0 IU/kg); and subcutaneous (SC) injection of insulin solution (2.5 IU/kg).

The developed NP delivery system, which may transiently open the tight junctions between the intestinal epithelial cells, provided an approach to overcome these obstacles. The NPs shelled with mucoadhesive CS may prolong their residence in the small intestine, infiltrate into the mucus layer, and subsequently mediate transiently opening the tight junctions between epithelial cells while becoming unstable and broken apart. The insulin released from the broken-apart NPs could then permeate through the paracellular pathway to the bloodstream. As shown in Figure 8, for the group receiving orally administered insulin-loaded NPs at a dose of 15 IU/kg, a significant decrease of the blood glucose level was found as compared to the group receiving oral intake of insulin solution. The reduction in blood glucose was greater in the animals receiving a higher dose of insulin (30 IU/kg). The hypoglycemic effects were present for up to 10 h in these animals. These results indicated that the reduction of the blood glucose levels depended on the insulin dose encapsulated in the NPs.

Conclusions

In conclusion, a novel NP system composed of low MW CS and γ-PGA was successfully prepared in the study by a simple ionic-gelation method under aqueous-based conditions. The results indicated that the NPs shelled with CS could transiently open the tight junctions between Caco-2 cell monolayers. Additionally, the in vivo results clearly indicated that the insulin-loaded NPs could effectively reduce the blood glucose level in a diabetic rat model.

Acknowledgment. This work was supported by a grant from the National Science Council (NSC 95-2120-M-007-010), Taiwan, Republic of China.

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BM0607776