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International Journal of Pharmaceutics 293 (2005) 271–280
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Pharmacological activity of peroral chitosan–insulin nanoparticles in diabetic rats

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Received 28 April 2004; received in revised form 11 November 2004; accepted 12 December 2004

Abstract

The objective of the present study was to evaluate the effects of formulation parameters on the in vivo pharmacological activity of the chitosan–insulin nanoparticles. Chitosan–insulin nanoparticles were prepared by ionotropic gelation at pH 5.3 and 6.1 and denoted as F5.3np and F6.1np, respectively. F5.3np and F6.1np administered orally at insulin doses of 50 U/kg and/or 100 U/kg were effective at lowering the serum glucose level of streptozotocin-induced diabetic rats. The 100 U/kg-dose F5.3np sustained the serum glucose at pre-diabetic levels for at least 11 h. In comparison, F6.1np had a faster onset of action (2 h versus 10 h) but lower efficiency. The effectiveness of peroral F5.3np and F6.1np in lowering the serum glucose level of streptozotocin-induced diabetic rats was ascribed to the local effect of insulin in intestine. Confocal micrographs showed strong interaction between rat intestinal epithelium and chitosan nanoparticles 3 h post-oral administration.

Keywords: Chitosan; Insulin; Nanoparticles; Oral delivery; Diabetes

1. Introduction

The oral route is the preferred route of drug administration for patients on chronic therapy. However, the oral delivery of many therapeutic peptides and proteins remain an unresolved challenge mainly because of the large size, hydrophilicity, and instability of these macromolecules (Lee and Yamamoto, 1990). In this study, we proposed to use chitosan nanoparticles as a carrier for the oral delivery of insulin.

Chitosan is a mucoadhesive, polycationic polymer that can facilitate drug absorption by localizing drug concentration around absorptive cells (Lehr et al., 1992) and prolonging drug residence in the gut (Leung et al., 1991). It is also an effective permeability enhancer because of its depolymerizing action on cellular F-actin and the tight junction protein ZO-1 (Schipper et al., 1997). Co-administered chitosan has been shown to enhance the transport of ^{14}C-mannitol (Schipper et al., 1996), buserelin (Kotéz et al., 1997), vasopressin

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doi:10.1016/j.ijpharm.2004.12.025

(Kotéz et al., 1997; Lueßen et al., 1997), and insulin (Kotéz et al., 1997) across the Caco-2 monolayers. Chitosan, in the form of glutamate solution and nanoparticles, is also reported to promote the transport of insulin through the nasal epithelium of sheep and rabbits, respectively (Illum et al., 1994; Fernandez-Urrusuno et al., 1999).

Oral delivery of insulin via polymer nanoparticles has also met with some success. The first report in 1988 suggested that insulin encapsulated in poly(isobutylcyanoacrylate) (PIBCA) nanocapsules had a long-term (up to 20 days) hypoglycemic effect in diabetic rats after oral administration (Démage et al., 1988). Peroral administration of insulin-loaded nanoparticles of poly(lactide-co-glycolide) (Barichello et al., 1999; Carino et al., 2000) and poly(fumaric-co-sebacic) anhydride (Mathiowitz et al., 1997) also controlled the plasma glucose levels of rats against an initial glucose load.

A recent study has shown that peroral chitosan–insulin nanoparticles could significantly lower the serum glucose levels of alloxan-induced diabetic rats (Pan et al., 2002). However, it is not known if the pharmacological activity was accompanied by increased plasma insulin levels. Neither did the authors control the pH at which the nanoparticles were prepared. In a previous study, we have found that the association and in vitro release of insulin were strongly influenced by the pH of the chitosan–insulin nanoparticle dispersion (Ma et al., 2002). Insulin association to chitosan nanoparticles at pH 5.3 was 50% less efficient than that at pH 6.1. However, the insulin association at pH 5.3 was stronger, where more than 75% of the associated insulin remained intact in vitro release studies. In contrast, insulin association at pH 6.1 was highly labile, the insulin rapidly and completely dissociating when the chitosan nanoparticles were diluted with aqueous media. These differences between the pH 5.3 and 6.1 formulations could have an impact on the in vivo pharmacological activity of the chitosan–insulin nanoparticles.

The objective of the present study was to evaluate the effects of formulation parameters on the in vivo pharmacological activity of the chitosan–insulin nanoparticles. In addition to formulation pH, we determined the effects of cross-flow filtration on the in vivo efficacy of chitosan–insulin nanoparticles prepared at pH 5.3 and 6.1. Both the serum glucose and serum insulin levels of the diabetic rats following the treatment were monitored in the study. Interaction between fluorescein isothiocyanate (FITC)-labeled chitosan nanoparticles and rat intestinal epithelium was also studied using confocal laser scanning microscopy.

2. Materials and method

2.1. Materials

Chitosan, from Aldrich Chemical Co. (Milwaukee, WI), was determined by dilute solution viscometry (Wang et al., 1991) to have a molecular weight of 186 kDa, and by the first derivative method of UV spectrometry (Tan et al., 1998) to have a degree of deacetylation of 85%. Fluorescein isothiocyanate (FITC), streptozotocin (SZ) and insulin (from porcine pancreas, 1 mg was equivalent to 27.8 USP units) were from Sigma Chemical Co., St. Louis, MO, USA; pentasodium tripolyphosphate (TPP) was from Merck, Darmstadt, Germany; and pentobarbitone sodium was from Nembutal, Rhone Merieux, Australia. All organic solvents were of HPLC grade and all other chemicals were of analytical grade.

2.2. Preparation and characterization of chitosan–insulin nanoparticles

Chitosan–insulin nanoparticles were prepared by an ionotropic gelation method (Ma et al., 2002). Insulin (1 mL, 56 U/mL in 0.01 M HCl) was premixed with TPP (4 mL, 0.1% w/v in 0.05 or 0.075 M NaOH) before added dropwise to 8 mL of chitosan solution (0.2% w/v in 0.25% v/v CH_3COOH) and agitated at 1000 rpm (Corning Stirrer, PC-420, USA) at ambient temperature. The resultant chitosan–insulin nanoparticle dispersions had pH of 5.3 and 6.1, and were denoted as F5.3np and F6.1np, respectively.

Particle size and zeta potential of the nanoparticles were measured using a particle sizer (Zetasizer 3000 HAS, Malvern Instruments Ltd., Worcs, UK). To determine the insulin association efficiency (AE), the nanoparticle dispersions were centrifuged at 74,200 × g at 20 °C for 30 min (Avanti^{TM} J-25 centrifuge, Beckman, USA), lyophilized (Dynavac Freeze-drier, Auckland, New Zealand), and weighed, while the insulin concentration in the supernatant was quantified.

AE was calculated as follows:

$$\mathrm{AE} = \frac{\text{total amount of insulin used} - \text{insulin in supernatant}}{\text{total amount of insulin used}} \times 100\%$$

Insulin was assayed by a reversed-phase liquid chromatography (RP-HPLC) method (Ma et al., 2002) in a chromatograph (Shimadzu LC-10AT$_{VP}$, Kyoto, Japan) equipped with a UV detector and a Waters Spherisorb column (ODS1, S10, 4.6 mm × 250 mm, Waters Corporation, Milford, MA, USA). Gradient elution was used. The acetonitrile concentration in the mobile phase, which comprised acetonitrile and phosphate buffer (0.1 M monobasic sodium phosphate adjusted to pH 2.0 with phosphoric acid), was increased from 26% to 32% in 30 min. The injection volume was 20 μL, the flow rate was 1.5 mL/min, and the detection wavelength was 214 nm.

2.3. Simulated gastrointestinal transit

To monitor the changes in the characteristics of the F5.3np and F6.1np during gastrointestinal transit in the rats, the transit was simulated by incubating the nanoparticles in an acid medium (phase 1) and gradually raising the pH of the medium to that of the intestinal fluid (phase 2). The nanoparticle dispersions (1 mL) were incubated with 0.1 M HCl (without any enzyme) at volume ratios of 1:1, 1:2 and 1:4 for 30 min at 37 °C at 100 rpm (Orbit shaker bath, Lab-line, IL, USA). The above volume ratios were chosen based on the estimated volume of gastric fluid in sacrificed rats. The nanoparticles were incubated for a further 30 min after the pH of the medium was raised to pH 6.8–7.4 by the addition of 1 M NaOH. Particle size, zeta potential, and AE of the nanoparticles were measured before and after phases 1 and 2.

2.4. Cross-flow filtration

Cross-flow filtration was explored as a method to remove free ions and insulin molecules from the chitosan–insulin nanoparticle dispersion because of the instability of the dispersion to ultracentrifugation. Cross-flow filtration was performed with MicroKros^{TM} cross flow syringe filters (Spectrum, Laguna Hills, CA) fitted with hollow fiber filters (pore size of 0.1 μm and surface area of 6 cm^2). Five-milliliter aliquots of F5.3np or F6.1np were concentrated to 1 mL by repeated passage across the filter, and the concentrate was reconstituted to 5 mL by adding water. The filtration process was repeated twice. Particle size, polydispersity, and zeta potential of the nanoparticle dispersions were measured before and after each cycle of filtration. Insulin loss in pooled filtrates was determined by HPLC. Nanoparticle recovery was determined by lyophilizing the reconstituted dispersions and expressing the weight of the lyophilizate as a percent of that obtained from corresponding dispersions not subjected to cross-flow filtration.

2.5. In vivo pharmacological activity

Male Wistar rats (200–250 g, Laboratory Animal Centre, National University of Singapore) were rendered diabetic by daily intraperitoneal injections of SZ (40 mg/mL in pH 4.5 citrate) at 40 mg/kg for three days. After two weeks, rats with fasted blood glucose levels above 250 mg/dL were used for experiments. These rats were fasted for 22 h prior to and remained fasted for 24 h during the experiment, but were allowed water ad libitum. Test samples were administered intragastrically by gavage needle and blood samples (0.2 mL) were collected by orbital sinus puncture after lightly anesthesizing the rats with ether. Serum was separated from the blood samples by centrifugation (5000 rpm for 10 min) and stored at −80 °C (Forma Scientific, Marietta, OH, USA) until analysis.

F5.3np and F6.1np were administered to the rats at insulin doses of 50 and 100 U/kg, based on the total insulin content (4.28 U/mL) of the nanoparticle dispersions. The volume of dispersion administered ranged from 2.4 to 2.6 mL for the 50 U-dose and from 4.8 to 5.2 mL for the 100 U-dose. Control rats were similarly administered with equivalent volumes of an insulin solution (I), a solution of insulin and chitosan (IC), or a dispersion of blank chitosan nanoparticles (CS). These control samples were prepared using methods similar to that described for the preparation of the chitosan–insulin nanoparticles, except for the omission of the chitosan polymer and TPP (I), TPP (IC), or insulin (CS).

In vivo evaluation was conducted with the final nanoparticle dispersion at volumes equivalent to insulin doses of 50 and 100 U/kg. For the latter dose, the nanoparticle dispersion was evaluated before and after crossflow filtration. The dose for the filtered sample

Table 1
The effects of cross-flow filtration on the mean particle size, polydispersity, zeta potential, insulin content and yield of chitosan–insulin nanoparticles

	Filtration cycle	Mean size (nm)	Polydispersity	Zeta potential (mV)	Insulin loss (%)	Recovery (%)
F5.3np	0	269 ± 7	0.52 ± 0.05	34.9 ± 0.9	0	100
	1st	323 ± 15	0.68 ± 0.07	39.8 ± 2.0	49.5 ± 3.6	85.5 ± 6.6
	2nd	431 ± 34	0.91 ± 0.08	48.6 ± 1.3	56.1 ± 3.3	74.5 ± 4.8
	3rd	528 ± 53	1.00 ± 0.00	49.2 ± 0.4	60.8 ± 5.2	67.1 ± 2.4
F6.1np	0	339 ± 8	0.50 ± 0.06	21.8 ± 0.6	0	100
	1st	421 ± 9	0.67 ± 0.15	30.1 ± 1.0	54.6 ± 3.1	85.7 ± 5.4
	2nd	539 ± 51	0.95 ± 0.09	42.4 ± 2.1	79.1 ± 4.2	72.7 ± 3.0
	3rd	688 ± 50	1.00 ± 0.00	49.8 ± 0.5	88.5 ± 8.6	66.8 ± 8.3

F5.3np and F6.1np were nanoparticles prepared with insulin loading concentration of 4.28 U/mL at pH 5.3 and 6.1, respectively (mean ± S.D., $n = 3$).

was calculated based on the amount of free and associated insulin present in the sample prior to filtration. The objective for the comparative experiments was therefore to determine whether the removal of free insulin from a volume of nanoparticle dispersion by crossflow filtration would modify its pharmacological profile. The amount of insulin present in the filtered dispersion as compared with the original dispersion is given in Table 1.

Serum glucose level was determined using the Trinder® Diagnostic Glucose reagent (Sigma), and expressed as a percent of the baseline serum glucose level. Serum insulin concentration was measured by radioimmunoassay (RIA) (Porcine insulin RIA kit, Linco Research Inc., St. Charles, MO, USA). Both assays were performed according to the instructions of the manufacturers.

To evaluate whether the RIA was capable of detecting insulin associated with the chitosan nanoparticles, the total insulin content of the F5.3np and F6.1np dispersions, as well as the control I solution, were quantified by RIA. Standard insulin solutions supplied by the manufacturer were assayed with and without the addition of an equal volume of serum from untreated rats to evaluate the effect of serum on the RIA measurements.

Pharmacological availability (PA) of peroral chitosan–insulin nanoparticles was determined based on a 100% availability of the control I solution administered peritoneally to the diabetic rats at a dose of 5 U of insulin/kg. Serum glucose levels were plotted against time, and the area under the curve (AUC) below the 100% cut-off line was determined using the trapezoidal method. PA for the chitosan–insulin nanoparticles was calculated according to the following equation:

$$\mathrm{PA} = \frac{\mathrm{AUC}_{0\rightarrow 24\mathrm{oral}}/\mathrm{Dose}_{\mathrm{oral}}}{\mathrm{AUC}_{0\rightarrow 24\mathrm{ip.}}/\mathrm{Dose}_{\mathrm{ip.}}} \times 100\%$$

2.6. Synthesis of FITC-labeled chitosan (fCS) and chitosan nanoparticles (fNP)

FITC was covalently conjugated to the chitosan molecule to enable the visualization of the chitosan nanoparticles under a confocal microscope. The synthesis of FITC-labeled chitosan (fCS) was based on a previously reported method (Huang et al., 2002). Briefly, 100 mL of dehydrated methanol was mixed with 100 mL of chitosan solution (1% in 0.1 M CH_3COOH) with continuous stirring. FITC (2 mg/mL in methanol) was added to give a final FITC to D-glucosamine residue ratio of 1:50. After 3 h of reaction in the dark at ambient temperature, fCS was precipitated by raising the pH to 8–9 with 0.5 M NaOH, and collected by centrifuging at 40,000 × g for 10 min. The fCS was purified by repeated wash cycles with water using centrifugation until no fluorescence was detected in the supernatant (Perkin-Elmer LS-5B luminescence spectrometer, Beaconsfield, England, $\lambda_{ex} = 490$ nm, $\lambda_{em} = 520$ nm). Purified fCS was then dissolved in 80 mL of 0.1 M acetic acid and dialyzed in the dark against 5 L of water for three days, the water being changed every 24 h. The purified fCS was freeze-dried and stored at −80 °C. The content of fluorescein thiocarbamyl moiety (FTC) was determined by measuring the fluorescence (Perkin-Elmer LS-5B luminescence spectrometer at $\lambda_{ex} = 490$ nm and $\lambda_{em} = 520$ nm) of a 26.3 μg/mL solution of fCS in 0.01 M acetic acid with reference to

Table 2
Changes in the characteristics of F5.3np and F6.1np dispersions after 30 min incubation with 0.1 M HCl at different volume ratios (1:1, 1:2 and 1:4) at 37 °C (phase 1), followed by another 30-min incubation after the pH of the medium was raised to pH 6.8–7.4 (phase 2)

Volume ratio of mixing	F5.3np			F6.1np		
	Particle size (nm)	Zeta potential (mV)	AE (%)	Particle size (nm)	Zeta potential (mV)	AE (%)
Control	269 ± 7	34.9 ± 0.9	38.5 ± 1.5	339 ± 8	21.8 ± 0.6	78.5 ± 2.3
Characteristics after phase 1						
1:1	294 ± 13	44.7 ± 5.3	33.6 ± 3.5	371 ± 41	42.5 ± 5.8	48.7 ± 5.6
1:2	303 ± 44	48.9 ± 12.2	31.8 ± 4.8	383 ± 18	49.6 ± 11.5	40.3 ± 6.1
1:4	145 ± 61	58.0 ± 15.6	31.7 ± 4.2	172 ± 63	58.8 ± 14.7	35.9 ± 5.7
Characteristics after phase 2						
1:1	188 ± 10	15.7 ± 9.9	33.4 ± 4.1	218 ± 11	17.0 ± 4.8	39.3 ± 5.3
1:2	140 ± 9	13.7 ± 13.3	30.6 ± 4.3	169 ± 10	13.4 ± 12.6	35.8 ± 3.7
1:4	129 ± 1	17.0 ± 6.5	31.9 ± 3.2	153 ± 14	16.9 ± 13.7	30.3 ± 6.5

Data presented as mean ± S.D., $n = 3$.

FITC standard solutions (2.31–36.92 ng/mL of FITC in 0.01 M acetic acid). The FITC:chitosan stoichiometry has been shown to be stable to dilution by a variety of aqueous media including culture media (Huang et al., 2002).

Blank fluorescent chitosan nanoparticles (fNP) at pH 5.5 were prepared with 0.2% fCS solution (0.25% acetic acid as solvent) and 0.1% TPP solution (0.05 N NaOH as solvent) according to the procedures described for unlabelled chitosan nanoparticles. The fNP were characterized by size and zeta potential measurements.

2.7. Association of fNP in the intestinal epithelium of rats

Freshly prepared fNP dispersion (2.5 mL, containing fCS 1.33 mg/mL) was administered by oral gavage to Wistar rats ($n = 8$) fasted overnight. The rats were sacrificed 3 h later with a peritoneal injection of 80 mg/kg of pentobarbitone sodium. Intestinal segments were localized after laparotomy and the ileum was isolated and cut into 1–2 cm lengths. After washing thoroughly with isotonic saline, the tissues were solubilized by incubation overnight at 60 °C with 10% NaOH (1 mL of solution to 0.1 g of tissue). The fluorescence of the resultant solutions was quantified and translated to the amount of fCS internalized per gram of tissue. Ileal tissues of untreated animals dissolved at 0.1 g/mL in 10% NaOH served as the blank solution against which the fluorescence of treated samples was measured. The blank solution was also used to dilute the fNP dispersions to produce standard samples for constructing the calibration curve.

Isolated intestinal tissues were also frozen in liquid nitrogen in the presence of Jung® tissue freezing medium (Leica Instruments, Germany), sectioned into 10-μm thick specimens, mounted on slides and stored at 4 °C. They were examined under a confocal laser scanning microscope (Fluoview FV300, Olympus, Japan) within 6 h of isolation. Auto-fluorescence was removed by adjusting the conditions for the confocal microscopy experiments such that untreated tissues showed negligible fluorescence.

2.8. Statistics

Data are expressed as the mean ± S.D. and analyzed by one-way ANOVA (SPSS 10, SPSS Inc. Chicago, USA) with the student's unpaired *t*-test applied for paired comparisons of means. *p* values of 0.05 or less were considered significant, while values of 0.01 or less were considered very significant.

3. Results

3.1. Characterization of chitosan–insulin nanoparticles

The F6.1np had a mean size about 70 nm larger than the F5.3np (Table 1), but this size difference might not be important in view of the relatively large polydispersity of the dispersions (∼0.5). The larger mean size

of the F6.1np was related to its lower zeta potential, which resulted in the formation of fewer crosslinks via the TPP ions. The removal of free TPP and insulin by cross-flow filtration caused significant increases in the mean size, polydispersity and zeta potential of the F5.3np and F6.1np (Table 1). In addition, 60.8% of the insulin load of F5.3np, which had AE of 38.5% (Table 2), was removed after three cycles of filtration (Table 1). This loss comprised mainly of the free insulin because 97% of the residual insulin in the filtered dispersion could not be separated from the nanoparticles by centrifugation at 74,200 × *g*. In contrast, although the F6.1np had a higher AE of 78.5% (Table 2), 88.5% of its total insulin load was removed after three cycles of filtration (Table 1), confirming our previous finding (Ma et al., 2002) that the insulin-nanoparticle association of this dispersion was very labile.

The characteristics of the F5.3np and F6.1np were changed under simulated gastrointestinal transit conditions, the changes being dependent on the volume of 0.1 M HCl used in phase 1 (Table 2). Incubation with increasing volume of 0.1 M HCl raised the zeta potential but decreased the AE of the nanoparticle dispersions. More significantly, the differences in particle size, zeta potential and AE between the F5.3np and F6.1np were abolished after 30 min of incubation with four times their volume of 0.1 M HCl. Under these conditions, the particle size of both dispersions decreased by about 50% to 145–172 nm while the zeta potential increased to 58–59 mV. More than 50% of the associated insulin in F6.1np was released after 30 min incubation with 1:4 v/v of 0.1 M HCl, the AE decreasing to 35.9%. AE for F5.3np incubated under similar conditions was reduced by about 7% to 31.7%. Subsequent increase in the pH of the medium to the intestinal pH range did not produce significant changes to the AE of the F5.3np and F6.1np despite falling mean particle size and zeta potential values. Comparable AE (ranging from 30% to 40%) and zeta potential were obtained for the F5.3np and F6.1np after incubation in the simulated intestinal fluid, but the F5.3np had a smaller mean particle size compared to the F6.1np at every volume ratio of mixing.

3.2. Pharmacological response of chitosan–insulin nanoparticles

A marked and sustained lowering of the serum glucose levels was observed in the diabetic rats 10 h after the oral administration of the F5.3np dispersion equivalent to an insulin dose of 50 U/kg body weight (Fig. 1). In contrast, rats fed with the control I, IC and CS samples did not register significant changes in serum glucose levels over 24 h post-administration (Fig. 2). Maximum pharmacological response (serum glucose at 59.1% of baseline level) of the F5.3np was seen at 16 h (Fig. 1). Compared to rats administered with the control I solution, the F5.3np produced significant pharmacological responses at 16 h ($p = 0.032$) and 19 h ($p = 0.026$). The hypoglycemic activity of the F5.3np dispersion was dose-dependent, the serum glucose level falling to 48.6% of baseline level (19 h) in

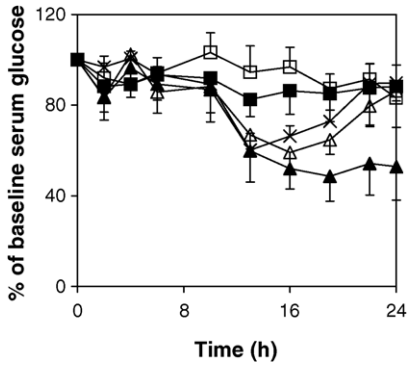

Fig. 1. Serum glucose levels of streptozotocin-induced diabetic rats (mean ± S.D., $n = 8$) after the oral administration of (a) F5.3np at insulin doses of 50 U/kg (△) and 100 U/kg (▲); (b) insulin solution at 50 U/kg (□) and 100 U/kg (■); (c) F5.3np after cross-flow filtration, whose insulin dose was 100 U/kg before cross-flow (×).

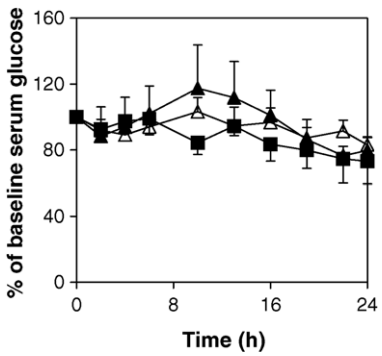

Fig. 2. Serum glucose levels of streptozotocin-induced diabetic rats after the oral administration of control samples: blank chitosan nanoparticle dispersion (■); insulin solution at 50 U/kg (△), and a solution of insulin and chitosan at insulin dose of 50 U/kg (▲). Data represent mean ± S.D., $n = 8$.

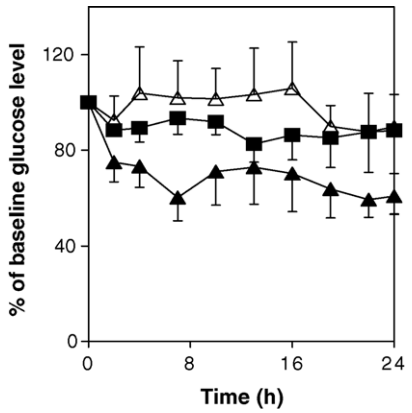

Fig. 3. Serum glucose levels of streptozotocin-induced diabetic rats after the oral administration of (a) F6.1np equivalent to a dose of 100 U insulin/kg (▲); (b) F6.1np after cross-flow filtration, whose insulin dose was 100 U/kg before cross-flow (△). Control rats were administered with insulin solution at 100 U/kg (■). Data represent mean ± S.D., $n = 8$.

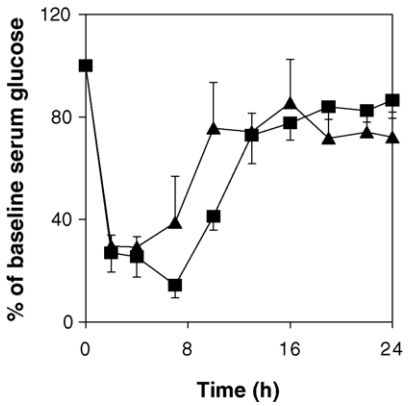

Fig. 4. Serum glucose levels of streptozotocin-induced diabetic rats after intraperitoneal injection with control insulin solution (■) and F5.3np (▲) at insulin dose of 5 U/kg (mean ± S.D., $n = 4$).

rats fed with double the dose of the dispersion (Fig. 1). The serum glucose level of the rats was maintained at close to pre-diabetic level for at least the next 11 h. Cross-flow filtration did not affect the onset of action of the F5.3np but reduced the duration of action and maximum activity (to 60.2% of baseline level) of the dispersion (Fig. 1).

Peroral F6.1np produced a different pharmacological profile (Fig. 3). At an insulin dose of 100 U/kg, the F6.1np elicited a faster onset of action than F5.3np, producing a significant pharmacological response as early as 2 h after oral administration. However, the serum glucose levels observed with F6.1np were higher, in the range of 60–75% of baseline level over the time period of 6–24 h after administration. Unlike the F5.3np, cross-flow filtration abolished the in vivo activity of the F6.1np (Fig. 3).

Similar serum glucose-time profiles were obtained for diabetic rats injected peritoneally with control insulin solution or F5.3np dispersion at the insulin dose of 5 U/kg (Fig. 4). The serum glucose level of the rats fell sharply to 28% of the baseline value 2 h after the injections, and was maintained at low levels for the next few hours before climbing to near baseline level. Rats treated with the nanoparticles showed a faster recovery of the serum glucose levels to baseline levels.

The pharmacological availability (PA) was calculated by comparing the AUC of specific serum glucose-time graphs and that for the insulin solution injected intraperitoneally. PA for peroral F5.3np administered at insulin doses of 50 and 100 U/kg were 4.4% and 3.2%, respectively, while that for the F6.1np administered to give 100 U of insulin/kg was 3.5%. PA for the peroral filtered F5.3np dispersion was 5.1% while that for intraperitoneal F5.3np was 83.3%.

The in vivo pharmacological activity of peroral F5.3np and F6.1np was not accompanied by higher serum immunoreactive insulin concentrations. Rats treated with either dispersion had serum insulin levels ranging from 3.5 to 4.0 μU/mL at all the sampling time points, similar to the control rats. These values were also similar to the baseline serum insulin levels of the fasting streptozotocin-induced diabetic rats. The RIA had high detection sensitivity; the threshold insulin concentration detectable was 2 μU/mL, and exhibited a linear relationship ($y = -0.194 \ln x + 1.11$, $R^2 = 0.98$) between the measured radioactivity (y) and insulin concentration (x) in the range of 2–200 μU/mL. The addition of serum or chitosan molecules did not affect the insulin quantification.

To determine whether the RIA was capable of measuring insulin associated with the chitosan nanoparticles, the total insulin contents of the F5.3np and F6.1np were determined after the dispersions were diluted using blank serum to an insulin concentration of 50 μU/mL as recommended by the manufacturer. Based on the insulin release profiles of the two formulations (Ma et al., 2002), this dilution was likely to result in the dissociation of insulin to give final AE of 39%

and 32% for the F5.3np and F6.1np, respectively. The control solution for this experiment was a 50 μU/mL solution of insulin. Insulin content measured by the RIA was calculated as a percent of the insulin loading in the samples, and amounted to 99.42 ± 4.16%, 74.0 ± 1.94% and 78.52 ± 2.18% for the control solution, F5.3np and F6.1np, respectively. These results confirm the reliability of the RIA in the measurement of free insulin, but cast doubts on its ability to quantify insulin associated with the chitosan nanoparticles.

3.3. Association of fluorescent chitosan nanoparticles in rat intestinal epithelium

FITC was successfully conjugated to the chitosan molecule to give fluorescent chitosan (fCS) with a FTC content of 7.5 ± 0.53 μg/mg. Ionotropic gelation of fCS with TPP produced fluorescent chitosan nanoparticles (fNP) whose characteristics were similar to those of unlabelled chitosan nanoparticles prepared under the same conditions. A linear calibration curve obeying the equation of $y = 37.13x - 22.28$ ($R^2 = 0.993$) was obtained when the fluorescence of fNP dispersions (1.22–9.74 μg/mL) were plotted against concentration of fNP.

fNP associated to the rat intestinal epithelium was quantified after tissue homogenization. The amount of fNP associated with the rat ileal tissues 3 h after oral administration was 70.45 ± 12.23 μg/g of tissue. The confocal laser scanning electron microscope showed strong fluorescence in the intestinal villi of rats dosed with fNP (Fig. 5a and b). Sharp loci of fluorescence equivalent to particles of 3 μm or larger were observed throughout the ileal tissue. The location of the fluorescent particles suggests that the fNP were at various stages of internalization in the ileum (Fig. 5b). Particles in A were located in the space between the intestinal villi; particles in B were on the surface of the intestinal enterocytes; those in C were within the outer most layers of the intestinal enterocytes, while particles in D appeared to have been transported into the tissues underlying the absorptive cells. Under the same experimental conditions, the ilea of control rats given peroral PBS did not exhibit fluorescence under the confocal microscope (results not shown).

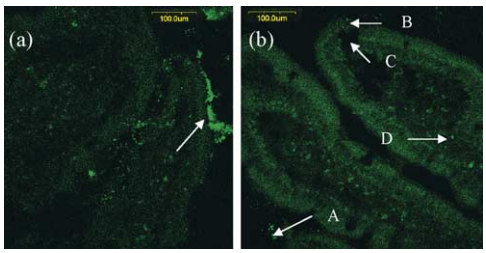

Fig. 5. Confocal micrographs of rat ilea isolated 3 h after the oral administration of FITC-chitosan nanoparticle dispersion (2.5 mL, chitosan concentration of 1.33 mg/mL).

4. Discussion

The F5.3np and F6.1np were effective in lowering the serum glucose levels of streptozotocin-induced diabetic rats when administered orally at insulin doses of 50 and/or 100 U/kg to the rats. Of particular interest was the activity of the higher dose F5.3np, which maintained the serum glucose level of the treated rats at pre-diabetic levels (60% of baseline) for at least 11 h post-administration. Results generated from the control dosing samples suggest that the pharmacological response of the chitosan–insulin nanoparticles was due primarily to the insulin associated with the nanoparticles, rather than to the chitosan nanoparticle per se, or to the dissolved insulin in the nanoparticle dispersion.

The hypoglycemic activity of the F5.3np and F6.1np was not accompanied by significant increase in the serum immunoreactive insulin levels. Similar phenomenon has been observed when insulin-loaded polyisobutylcyanoacrylate (PIBCA) nanoparticles (Lowe and Temple, 1994) were administered perorally to diabetic rats at an insulin dose of 50 U/kg. Although a single dose of the PIBCA nanoparticles produced a significant hypoglycemic activity that persisted for up to 3 weeks, there was no concomitant rise in the level of immunoreactive insulin in the plasma over the same time frame. The authors have ascribed this phenomenon to a local effect of the insulin in the intestine. Insulin receptors are present on the brush border membranes and insulin is an important regulatory peptide in the intestinal epithelium (Gallo-Payet and Hugon, 1984). Hyperglycemia in streptozotocin-induced diabetic rats has been attributed to two factors: (i) enhanced transport of glucose from the intestine to the general circulation through an increased number of glucose carriers in the basolateral membrane of the intestine (Csáky and Fisher, 1981); (ii) inhibition of glucose metabolism by

a decrease in the mucosal phosphofructokinase concentration and an increase in the susceptibility of the phosphofructokinase to inhibition by ATP (Jamal and Kellett, 1983). A local action of insulin in the brush border membrane could reverse these two mechanisms. In this study, the glucose carriers had a limited role in regulating the serum glucose level because the experimental rats were fasted for 24 h and would have low intestinal glucose concentrations. Reversal of the second factor requires the phosphofructokinase concentration to be replenished, and a lag time of 17–25 h is required to correct the streptozotocin-induced diabetes in the rats (Jamal and Kellett, 1983). This lag time could account for the delayed onset of response observed with the insulin-loaded chitosan nanoparticles.

The sustained hypoglycemic activity of peroral F5.3np and F6.1np is not unique because other types of polymer-insulin nanoparticles have been reported to exhibit sustained hypoglycemic activity when administered orally (Aboubakar et al., 2000; Démage et al., 1988). Aboubakar et al. (2000) suggested that this was due to the progressive arrival of the nanoparticles from the stomach to the absorptive site as well as the regeneration of free insulin from the nanoparticles in situ.

Of the total insulin content in the F5.3np formulation, about 38.5% of it was associated with the nanoparticles when this formulation was administered to the diabetic rats. The slow onset of action implied that the free insulin in the formulation did not contribute to its pharmacological response. The onset of action of the F5.3np was also not influenced by the dose administered or by cross-flow filtration, both of which regulated the amount of free insulin in F5.3np administered to the rats. In contrast, the AUC and maximum response of the F5.3np was modified by the volume of dispersion administered and by cross-flow filtration. Therefore, while the free insulin in the F5.3np did not affect the onset of action, it had an impact on the AUC and maximum efficacy of the formulation.

The different in vivo pharmacodynamic profiles of the F5.3np and F6.1np could not be attributed to their characteristics at the time of formulation. Changes in the characteristics of the nanoparticles under the simulated gastric transit conditions suggest that the differences in particle size, zeta potential and associated insulin content between the two formulations could be offset by their interaction with the gastric fluid. Extrapolating from the results of the simulated experiments, the faster onset of action and lower maximum response of the F6.1np might be related to the rapid release of associated insulin from the F6.1np in the rat stomach. However, it is not clear how a rapid regeneration of free insulin could expedite the onset of action, particularly when the released insulin was likely to be degraded by proteolysis in the GIT.

Similar AUC were obtained for equivalent doses of peroral F5.3np and F6.1np, attesting again to the dependence of the AUC on the total insulin content (free + associated) of the nanoparticle dispersion. A threshold content of insulin was important, because the loss of 89% of the insulin load through cross-flow filtration rendered the F6.1np ineffective in vivo, while the loss of 61% of the insulin load reduced but did not abolish the activity of the F5.3np.

Intraperitoneal injection with F5.3np at a dose of 5 U/kg produced a hypoglycemic response in the diabetic rats that was largely similar to that produced by an equivalent dose of intraperitoneal insulin solution. This is not surprising in view of the high proportion (61%) of free insulin in the F5.3np. Compared to the insulin solution, however, the free insulin content in the F5.3np was lower, which might account for the faster recovery of the rat serum glucose to near baseline level. The PA for intraperitoneal F5.3np was 83.3%, suggesting that the pharmacological response of this formulation was partially contributed by insulin associated with the nanoparticles.

5. Conclusions

Chitosan–insulin nanoparticles formulated with an insulin loading concentration of 4.28 U/mL at pH 5.3 and 6.1 were effective in lowering the serum glucose level of streptozotocin-induced diabetic rats when given at insulin doses of 50 and/or 100 U/kg to the rats. In particular, F5.3np administered at the insulin dose of 100 U/kg was able to maintain the rat serum glucose level at pre-diabetic levels for at least 11 h. The pharmacological activity of the nanoparticles was not accompanied by increases in the serum insulin concentration, and might be ascribed to the local action of the insulin in the intestinal epithelium. The formation of fCS into fNP significantly enhanced its interaction with the intestinal epithelium.

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