PHARMACEUTICAL NANOTECHNOLOGY

Nanoparticulate Biopolymers Deliver Insulin Orally Eliciting Pharmacological Response

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ABSTRACT: The aim of this study was to characterize and evaluate a novel oral insulin nanoparticulate system based on alginate-dextran sulfate core, complexed with a chitosan-polyethylene glycol-albumin shell. Insulin-loaded nanospheres (25, 50, 100 IU/kg) administered orally to diabetic rats reduced glycemia in a dose dependent manner. This effect lasted over 24 h with a maximal effect after 14 h. Nanospheres increased insulin plasma level and improved glycemic response to an oral glucose overload. After 4 days oral administration (50 IU/kg/day), the metabolic status of diabetic rats improved with a reduction in water intake, urine excretion and proteinuria. FITC-insulin-loaded nanospheres administered to an isolated intestinal loop were taken up by the intestinal mucosa. They strongly adhered to villus apical enterocytes and markedly labeled Peyer's patches. It is concluded that nanospheres preserve insulin and exert an antidiabetic effect after oral administration. This is explained by a protective effect against proteolytic enzymes by the albumin coating, by the mucoadhesive properties of chitosan-polyethylene glycol, and by the possibility of chitosan reversibly altering tight junctions leading to an improved absorption of insulin. This formulation demonstrates beneficial effects on diabetic symptoms and will be of interest in the treatment of diabetes with oral insulin. J Pharm Sci 97:5290–5305, 2008

Keywords: diabetes; insulin delivery; biomaterials; nanospheres; oral absorption

INTRODUCTION

Diabetes mellitus is a metabolic illness requiring strict glycemic control to reduce its progression and complications. Insulin replacement therapy provides the most effective means for glycemic control. Replicating physiological insulin secretion as a means of restoring normal metabolism, minimizes complications, and has thus becomes the essential goal of diabetes treatment.[1]

Amongst alternative routes for insulin administration, the oral route is potentially the most convenient and physiological. After gastrointestinal absorption, insulin undergoes a first hepatic bypass, thus triggering a primary effect by inhibiting hepatic glucose production.[1] However, peroral bioavailability of insulin is relatively low mainly due to high proteolytic activity in the gut and low permeability of the intestinal epithelium.[2] As with other peptides, insulin shows a poor physical and

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chemical stability and a relatively short plasma half-time.[3] Several strategies to overcome these barriers for perorally administered insulin include the addition of enzyme inhibitors[4] and/or permeation enhancers,[5] chemical modification,[6] cell penetration peptides,[7] vitamin B_{12}[8] or cyclodextrin conjugation,[9] polymeric carriers,[2,10–12] liposomes[13] or a colon targeting of the drug delivery system where the enzymatic activity is relatively low.[14] A promising strategy is the use of multifunctional polymers exhibiting permeation enhancing and mucoadhesive properties.[15] The interest of using natural materials such as alginate as part of drug development has increased in the past two decades.

Alginates are naturally occurring polymers and are known to form a reticulated structure when in contact with calcium ions. This particular characteristic has been used to produce sustained release particulate systems for a variety of drugs, proteins and even cells.[16,17] Previously, we developed an insulin formulation[18] contained alginate as matrix polymer and dextran sulfate as additive polymer. Dextran sulfate prevented insulin release at low pH, however, the entrapped insulin was rapidly released when nanospheres were transferred to intestinal fluid.[18] Moreover, the entrapped insulin showed a high sensitivity to pepsin attack, being rapidly degraded in simulated gastric fluid containing pepsin. *In vivo* studies also showed that alginate-dextran sulfate nanospheres were ineffective in reducing glycemia following oral administration to fasted diabetic rats. Thus, coating strategies were developed to improve enzymatic resistance and thus improve insulin oral absorption. The first strategy involved applying chitosan as coating material. Chitosan, a biocompatible polysaccharide was selected to several reasons. Polycationic polymers such as chitosan can be used to increase the stability of polyanionic alginate nanospheres and to minimize the loss of encapsulated material.[19] Secondly, chitosan exerts mucoadhesive properties and facilitates drug absorption by localizing drug around absorptive cells and by prolonging drug residence in the gut. Finally, chitosan is an effective permeability enhancer because it reversibly alters the tight junctions by its depolymerizing action on cellular F-actin and the tight junction protein ZO-1.[20] In our case, chitosan coating decreased encapsulation efficiency of insulin but *in vitro* release was now controlled in contrast with uncoated nanopsheres. As well, and as been observed in a similar system but when using microspheres,[21] chitosan-coated alginate-dextran sulfate core nanospheres were still highly sensitive to pepsin and did not reduce glycemia during *in vivo* assays. Albumin was then added as a second coating material. Albumin coating decreased encapsulation efficiency but for the first time, we observed insulin protection against pepsin. *In vivo* hypoglycaemic effect was even more pronounced when PEG was applied as a stabilizing polymer. Albumin was selected as a shell component for the possible protection of insulin against proteolytic enzymes in the gastrointestinal tract. As stabilizing polymer, PEG was selected for several reasons. PEG is nontoxic, is known to increase stability of polymeric nanoparticles[22] and it improves the transport of large proteins across nasal and intestinal barriers.[23,24] PEG can also improve peptide physicochemical and biopharmaceutical properties.[25]

The new nanosphere formulation described in the present report consists of an alginate-dextran sulfate core containing insulin, coated with chitosan-polyethylene glycol and finally albumin. Nanospheres were physicochemically characterized (diameter, zeta-potential, insulin loading) and insulin release investigated under simulated gastrointestinal conditions. Biological efficacy was analyzed in diabetic rats after a single and repeated oral administration. Finally, in order to examine the mechanism of intestinal uptake, insulin was labeled with fluorescein isothiocyanate (FITC) and the uptake of FITC-insulin-loaded nanospheres was visualized and quantified after intraluminal administration to an isolated intestinal segment. Water and electrolyte turnover during a 4 days treatment with oral insulin-loaded nanospheres was also evaluated.

MATERIALS AND METHODS

Materials

Low viscosity sodium alginate (viscosity of 2% solution at 25°C, 250 cps) was purchased from Sigma (St. Louis, MO). Chitosan (50 kDa), albumin, pepsin and streptozotocin were purchased from Sigma–Aldrich Chimie (L'Isle d'Abeau Chesnes, France). Setacarb calcium carbonate was obtained from Omya (Orgon, France). Paraffin oil was supplied by Vaz Pereira (Lisbon, Portugal). The emulsifier, Span 80, dextran sulfate (5 kDa) and PEG 4000 were purchased from Fluka, Chemie GmbH (Buchs,

Switzerland). Insulin was kindly donated by *Hospitais da Universidade de Coimbra* (Actrapid Insulin®, 100 IU/mL from Novo Nordisk, Bagsvaerd, Denmark).

Preparation of Insulin-Loaded Nanospheres

Nanosphere preparation involved formulation of an alginate-dextran sulfate core, through nanoemulsion dispersion followed by triggered instantaneous particle gelation.[18] The following formulation was optimized in terms of insulin release profile and the ability of the particles to provide resistance against pepsin. A sodium alginate (2%, w/v), dextran sulfate (0.75%, w/v) solution was prepared by stirring (100 rpm) overnight. Following stationary deaeration for 1 h, insulin was added and dissolved (100 IU/mL, 10 mL). An aqueous suspension of ultrafine calcium carbonate (5%, w/v) was added at calcium-alginate ratio of 7% (w/w), and the dispersion was emulsified within paraffin oil facilitated by Span 80 emulsifier (1.5% v/v) at high speed (1600 rpm). After 15 min, gelation was induced by addition of 20 mL paraffin oil containing glacial acetic acid (acid-calcium molar ratio, 3) to solubilize calcium dispersed in the alginate-dextran nanodroplets. After 60 min, an acetate buffer solution (pH 4.5) (made according United States Pharmacopeia, USP XXVIII) with dehydrating solvents (acetone, isopropanol, and hexane)[26] was added to the oil-nanoparticle suspension and nanospheres were recovered by centrifugation (12500*g* during 10 min). The coatings were applied simply through electrostatic interaction. Chitosan-PEG solution at pH 4.5 (PEG-chitosan mass ratio of 5 with high calcium level at 1.5% (w/v)) was added to uncoated nanospheres under magnetic stirring during 30 min. Then, the supernatant containing unbound polymer was decanted and albumin-coating (1% albumin (w/v) in 100 mL at pH 5.1) applied to chitosan-PEG nanospheres under magnetic stirring during 30 min. Unbound coating protein contained in the supernatant was decanted. Insulin-free nanospheres (empty nanospheres) were also prepared.

Characterization of Insulin-Loaded Nanospheres

Size distribution analysis was performed by laser diffraction spectrometry using a Coulter LS130 granulometer (Beckman Coulter, Inc., Fullerton, CA). Mean diameters of aqueous suspensions were determined in triplicate and size distribution was represented by number. Surface charge was determined by laser doppler anemometry on a Malvern Zetasizer 3000HS (Malvern Instruments, Worcestershire, UK) at pH 4.5 and 25°C, the result of which is expressed as Zeta Potential. For scanning electron microscopy (SEM), insulin-loaded nanospheres were mounted on metal stubs using double-sided adhesive tape, drying in a vacuum chamber, sputter-coating with a gold layer and viewing under a scanning electron microscope (JSM-840, Jeol Instruments, Tokyo, Japan) to characterize shape and morphology and confirm particle size.

Determination of Encapsulation Efficiency of Insulin

Encapsulation efficiency was determined after chitosan-PEG-albumin-coating by analyzing filtrate, wash and dehydration solutions using insulin enzyme-linked-immuno-sorbent assay insulin kit (ELISA, Mercodia, Sweden) at 450 nm. Encapsulation efficiency (%) was determined by the following equation:

$$\text{Encapsulation efficiency}\ (\%) = \frac{\text{Initial insulin}^{*} - \text{lost Insulin}}{\text{Initial insulin}^{*}} \times 100$$

*Initial insulin added to formulate nanospheres.

In Vitro Release of Insulin Under Simulated Gastrointestinal Conditions

In vitro release behavior was assayed by incubating 5 mL of nanosphere suspension in 5 mL of HCl buffer at pH 1.2 (USP XXVIII), under magnetic stirring (100 rpm, 2 h) at 37°C, simulating stomach conditions without enzymes under sink conditions. The medium volume was calculated to ensure that sufficient volume be available to dissolve the insulin from the nanospheres. Samples at appropriate intervals were withdrawn and protein assayed using HPLC where the mobile phase was water (A): acetonitrile (B) with 0.04% trifluoroacetic acid with linear gradient B 30–40% over 5 min, flow rate 1.2 mL/min at 25°C. A reversed-phase X-Terra C-18 column, 5 μm, 4.6 mm × 250 mm (Waters Corporation, Milford, MA, USA), with a Purospher® STAR RP-18 precolumn, 5 μm, 4 mm × 4 mm (Merck, Darmstadt, Germany) was employed. The UV detector was set

at 210 nm and HPLC analysis carried out 27°C.[21] An insulin retention time around 5 min was obtained. To simulate the progress of nanospheres moving from the stomach into the upper small intestine, the buffer was changed to higher pH after 120 min. Nanospheres were centrifuged, then resuspended in 10 mL phosphate buffer at pH 6.8 (USP XXVIII), under magnetic stirring (100 rpm) at 37°C during 840 min. Samples at appropriate intervals were withdrawn and insulin assayed by HPLC. A calibration curve was prepared for each medium using fresh insulin. Experiments were performed in triplicate. Cumulative insulin release was then calculated as percentage of initial insulin content.

Insulin Molecular Integrity Under Simulated Gastrointestinal Conditions

Insulin molecular integrity was evaluated by HPLC after 1 mL nanosphere (8.5 IU of insulin/mL) matrix dissolution with 5 mL of sodium citrate (55 mM) in phosphate buffer pH 7.4 (USP XXVIII). Insulin was quantified initially, and after 2 h incubation in simulated gastric fluid containing pepsin at pH 1.2 (USP XXVIII) in a shaking water bath at 37°C and 100 rpm. Insulin-loaded nanospheres were recovered by centrifugation and transferred to citrate solution with stirring for 1 h, then aliquots collected, centrifuged and analyzed by HPLC (same conditions as previous experiment). HPLC chromatograms were collected and nonencapsulated insulin served as reference. Assays were conducted in triplicate. Intact insulin has a retention time around 5 min using same HPLC conditions as previous assay. Enzyme resistance was verified by insulin content after enzyme incubation as percentage of initial insulin content.

In Vivo Efficacy of Insulin-Loaded Nanospheres on Diabetic Rats

Male Wistar rats (250 g) were housed in a 12–12 h light–dark cycle, constant temperature environment of 22°C, relative humidity 55% and allowed free access to water and food during acclimatization. Animals received standard laboratory chow diet (UAR, Villemoisson-sur-Orge, France) and tap water, available *ad libitum*. All treatments began between 8.00 and 9.00 h. All experiments were carried out in accordance with the European Community Council Directive (86/609/EEC). To minimize the diurnal variance of blood glucose, all experiments were performed in the morning. Diabetes was induced with intravenous injection of 65 mg/kg streptozotocin in citrate buffer (pH 4.5) as previously described.[10] Ten days after the treatment, rats with frequent urination, loss of weight and blood glucose levels higher than 300 mg/dL were included in experiments. Blood glucose levels were determined by glucose oxidase/peroxidase method using a glucometer (Accucheck Go, Roche, Strasbourg, France).

In order to verify that the particles contained bioactive insulin, insulin-loaded nanospheres were injected subcutaneously to fasted diabetic rats (4 IU/kg body weight). Nonencapsulated insulin (4 IU/kg) was administered as control. Glycemia in blood samples withdrawn from the tail vein was measured before insulin injection and at intervals to 8 h. Rats were fasted 12 h prior to dosing, during the experiment and fed thereafter.

In order to investigate the effects of oral insulin-loaded nanospheres, 12 h fasted diabetic rats were gavaged with insulin-loaded nanospheres (25, 50, or 100 IU/kg) or empty nanospheres as control. Glucose was measured on a drop of blood from the tail vein before and at different intervals up to 24 h after oral nanosphere administration. Serum glucose time course was plotted, and the area below the 100% cut-off line determined using the trapezoidal method during 0–14 h. Pharmacological availability of peroral delivered insulin (AUC_{0-8h}) was determined based on 100% availability of the nanosphere suspension administered subcutaneously at a dose of 4 IU/kg. In the previous study,[18] nanospheres showed 100% pharmacological availability in comparison to insulin solution when administered subcutaneously. In order to eliminate potential interferences of the encapsulation process and the role of polymers unloaded nanospheres were then applied as controls. Finally, in order to confirm albumin and PEG effects, chitosan-coated alginate-dextran core nanospheres without albumin and PEG, were orally administered to fasted diabetic rats at dose level of 50 IU/kg. The same assay was performed with nonencapsulated insulin and water as negative controls. Serum glucose time course was plotted.

The effect of insulin-loaded nanospheres on postprandial rise in blood glucose was tested. Empty or insulin-loaded nanospheres (50 IU/kg) were given orally 10 h before the glucose gavage. Blood glucose level was measured as described

previously at 0, 20, 40, 60, 90, 120, and 180 min after glucose gavage (2 g/kg body weight).

Plasma insulin levels were also determined after oral dosage of insulin-loaded nanospheres. Twelve hours fasted diabetic rats were anaesthetized with ketamine and xylazine. Blood samples were collected before oral administration of insulin-loaded nanospheres (50 IU/kg) and 4, 8, and 12 h thereafter, then centrifuged, and the plasma was stored at -20°C. Plasma immunoreactive insulin was measured by radioimmunometric assay (Insulin-CT kit from CIS Bio International, Gif-sur-Yvette Cedex, France) for up to 12 h.

Intestinal Uptake of Insulin-Loaded Nanospheres

Fluorescein isothiocyanate (FITC)-labeled insulin-loaded nanospheres (50 IU/kg) were administered in an *in situ* isolated intestinal loop. Rats were fasted overnight and anesthetized with ketamine (100 mg/kg). After laparotomy, a 30 cm ileal loop, situated above the ileocaecal valve, was isolated from the digestive tract by a ligature at each side. A 2 mL suspension of FITC-insulin-loaded nanospheres (50 IU/kg) was injected in the intestinal lumen of this ileal segment. The volume of the formulations is an important factor on the gastrointestinal tract particle distribution.[27]

The animals (6 per group) were sacrificed at time intervals up to 8 h after the intra-luminal injections. Blood from the mesenteric vein, the intestinal content and the intestinal mucosa were removed and frozen until analysis. Biopsies of the follicular (Peyer's patches) and the nonfollicular mucosa were performed at the same time intervals. Tissue samples were embedded in a cryostat medium (Tissue Tek, Miles Diagnostics, Elkhart, Indiana, USA), cryofixed and 10 μm thick frozen sections were prepared using a cryostat (Leica, CM3050 S, France), and sections observed under fluorescence microscope (Olympus DP70, Rungis, France). As well, micrographs were taken with a BioRad MRC-1024 confocal laser scanning microscope equipped with an argon/krypton laser (Bio-Rad Laboratories GmbH, Munich, Germany). The excitation was performed using $l = 488$ nm for green fluorescence. Images were processed using Volocity® software (Improvision Germany, Tuebingen, Germany).

Fluorescent activity in mesenteric blood, gut contents and mucosa homogenates was measured by spectrofluorometry using a fluorescence spectrophotometer (BioRad, VersaFluor™, Marnes-la-Coquette, France). The fluorescence measurement was performed at an excitation wavelength of 480 nm and an emission wavelength of 520 nm.

Water and Electrolyte Turnover During 4-Day Treatment With Oral Insulin-Loaded Nanospheres

Twelve animals were maintained in individual metabolic cages under standard laboratory conditions. The individual metabolic cages permitted the quantification of the exact amounts of water and food consumed, as well as volume of urine which was collected in a bottle. Following a 24 h familiarization period, rats were divided into two groups of six rats, and treated orally either with insulin-loaded nanospheres (50 IU/kg) or empty nanospheres for 4 successive days. On the last day of treatment, water and food intake were measured and urine was collected. Insulin-loaded nanospheres or empty nanospheres were administered each day after a 6 h fast.

Statistical Analysis of *In Vivo* Data

Results were expressed as means $\pm$ standard deviation (SD) or means $\pm$ standard errors of means (SEM). For group comparison, an analysis of variance with a one-way layout was applied. Significant differences in mean values were evaluated by a Student's *t*-test. For multiple comparison group tests, a Bonferroni or a Dunnett multicomparison test was applied, using Instat 2.00 Macintosh software (Graph Pad Software, San Diego, CA). The differences were considered significant when $p < 0.05$.

RESULTS

Characterization of Insulin-Loaded Nanospheres

Insulin-loaded nanospheres showed a unimodal size distribution with 90% of the particles having a diameter less than 1842 nm, and 50% less than 812 nm as seen in Figure 1. Small agglomerates were observed under SEM as seen in Figure 2 however agglomerates readily dispersed after manual mixing. SEM images showed that individual particles were mainly less than 1000 nm. The charge of uncoated nanospheres was negative (-16 ± 2 mV) as shown in Table 1 but addition of polycationic chitosan coating reversed the charge to positive ($+15 \pm 1$ mV). Subsequent application of albumin-coating reversed the charge once again

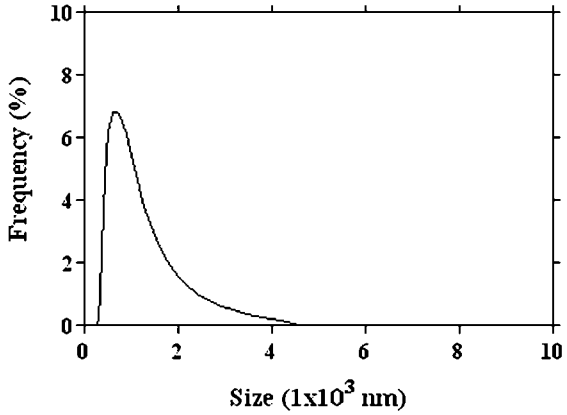

Figure 1. Size distribution of albumin-chitosan-PEG coated insulin-loaded nanospheres.

to negative (-12 ± 5 mV), but less so, compared to uncoated nanospheres. Application of PEG reduced the negative charge of albumin-chitosan coated nanospheres to -7 ± 4 mV. Changes in the zeta potential of the nanospheres indicate effective coating by the respective materials. Finally, when insulin-loaded nanospheres were incubated for 2 h at 37°C with pepsin, nanospheres reverted to positive ($+21 \pm 3$ mV) due to hydrolysis of the albumin coat. Thus, coating changes the physicochemical properties of the nanospheres.

Determination of Encapsulation Efficiency and *In Vitro* Release of Insulin Under Simulated Gastrointestinal Conditions

Encapsulation efficiency of insulin within the nanospheres was high at $85 \pm 4\%$. *In vitro* release of insulin under simulated gastric and intestinal conditions is illustrated in Figure 3. At low pH, only 25% of the encapsulated insulin (2.1 IU/mL) was released while an additional 45% of the insulin was released when pH was changed to 6.8. Thus, 70% of the overall insulin was released after 960 min assay and under simulated experimental conditions.

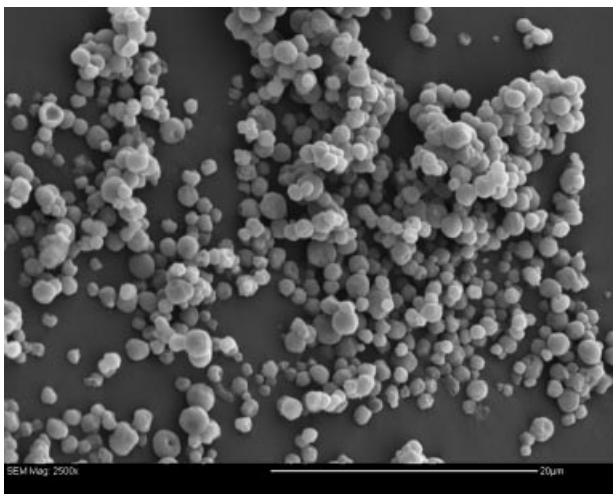

Figure 2. Scanning electron micrograph of chitosan-PEG coated insulin-loaded nanospheres before BSA coating. The bar represents 20 μm.

Insulin Molecular Integrity Under Simulated Gastrointestinal Conditions

The resistance of insulin to pepsin was analyzed by HPLC after exposure for 2 h at 37°C. As represented in Figure 4a, insulin shows one peak at 5 min. Another peak at 8.3 min is related to an insulin additive, metacresol, which is not present in nanospheres because it is easily discarded during the nanosphere recovery process. Insulin released from nanospheres before pepsin incubation showed an insulin peak at the same retention time (Fig. 4b). When nonencapsulated insulin was exposed to pepsin, the insulin peak disappeared (Fig. 4c), indicating proteolytic degradation. In contrast, when insulin-loaded nanospheres were exposed to pepsin, the insulin peak was maintained showing that albumin coated nanospheres prevent enzymatic degradation of insulin (Fig. 4d).

Glycemic Response of Diabetic Rats in Response to a Subcutaneous Injection of Insulin-Loaded Nanospheres

Blood glucose profiles following subcutaneous injection of insulin-loaded nanospheres and nonencapsulated insulin to diabetic rats at dose level of 4 IU/kg are illustrated in Figure 5. Both promoted a rapid and intense decrease of glycemia with glucose levels approaching 13% of initial values after 4 h. Then, blood glucose levels increased reaching 40–50% of basal values at 8 h. In the untreated control group, blood glucose level decreased slowly from 2 to 8 h, due to fasting. There were no statistical differences between groups treated with the same dose level of nonencapsulated insulin and insulin-loaded nanospheres showing that the nanoparticulate insulin was as effective as the same dose of free insulin, demonstrating full retention of insulin activity.

Glycemic Response of Diabetic Rats in Response to Oral Administration of Insulin-Loaded Nanospheres

The glycemic response of insulin-loaded nanospheres was examined by orally dosing 12 h fasted

Table 1. Zeta Potential of Insulin-Loaded Nanospheres and its Variation After Processing, Coating and Pepsin Exposure

Nanosphere Production	Zeta Potential (mV)
Uncoated nanospheres	-16 ± 2
Coating process	
Chitosan-coated nanospheres	$+15 \pm 1$
Albumin-chitosan-coated nanospheres	-12 ± 5
Albumin-chitosan-PEG-coated nanospheres	-7 ± 4
Enzyme assay	
Albumin-chitosan-PEG-coated nanospheres before pepsin incubation	-7 ± 4
Albumin-chitosan-PEG-coated nanospheres after pepsin incubation	$+21 \pm 3$

Data are means ± SD of three experiments.

diabetic rats with 25, 50, and 100 IU/kg or empty nanospheres as control. As illustrated in Figure 6A, insulin-loaded nanospheres decreased glycemia in a dose-dependent manner in contrast to rats treated with empty nanospheres. The lowest insulin dose (25 IU/kg) was effective (-18%, $p < 0.05$) from 6 h while higher doses (50 and 100 IU/kg) significantly reduced glycemia from 4 h (respectively by 17% and 21%, $p < 0.05$). The glycemic reductions attained were -44 ($p < 0.05$), -55 ($p < 0.05$), and -76% ($p < 0.01$) with 25, 50, and 100 IU/kg insulin-loaded nanospheres respectively. Rats were subsequently refed after 14 h fast, then 24 h after the administration of insulin-loaded nanospheres, glycemia was still reduced by -21% (not significant), -33 and -38% ($p < 0.05$) with 25, 50, and 100 IU/kg insulin-loaded nanospheres respectively. Reduction of glycemia was not observed with insulin solution, demonstrating that insulin alone cannot be absorbed sufficient to elicit a biological response by the oral route in the absence of a suitable carrier. As well, a hypoglycaemic effect was not observed with empty nanospheres, the decrease between 10 and 14 h being attributed to the fast, over the experiment.

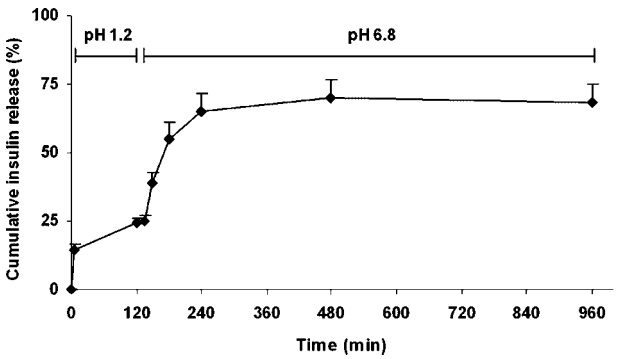

Figure 3. *In vitro* release profile of albumin-chitosan-PEG coated insulin-loaded nanospheres in simulated gastric medium at pH 1.2 and intestinal medium at pH 6.8. Results are means ± SD of three experiments.

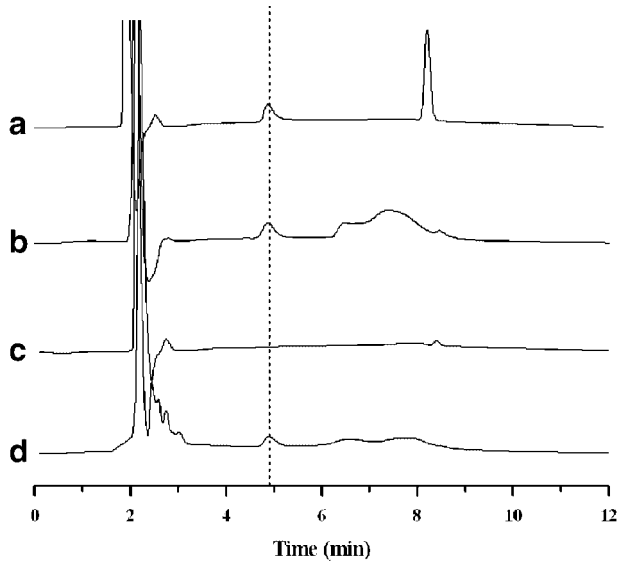

Figure 4. Insulin HPLC chromatograms: (a) insulin nonencapsulated as control (insulin retention time around 5 min represented as dotted line); (b) insulin extracted from nanospheres before pepsin incubation; (c) insulin nonencapsulated as control after pepsin incubation; (d) insulin extracted from nanospheres after pepsin incubation. Peak around 2 min corresponds to mobile phase peak for all situations.Peak around 8 min in nonencapsulated insulin chromatogram corresponds to an additive, metacresol, which is not present in nanospheres. This additive is generally discarded after nanospheres recovery process. Large band between 6 and 9 min corresponds to coating material of insulin-loaded nanospheres.

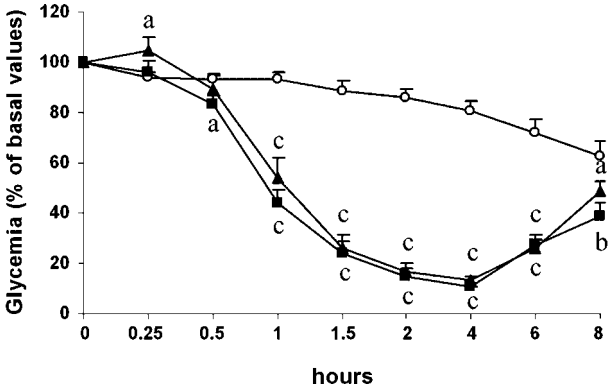

Figure 5. Glycemia after a subcutaneous administration of insulin nonencapsulated (squares, $n = 6$) and albumin-chitosan-PEG coated insulin-loaded nanospheres (triangles, $n = 7$) or saline (empty circles, $n = 9$) in fasted diabetic rats. Insulin was administered at the concentration of 4 IU/kg. Before the injections, glycemia was 327 ± 21 mg/dL. Results are expressed as means $\pm$ SEM. Statistically different from saline control: (a) $p < 0.05$; (b) $p < 0.01$; (c) $p < 0.001$.

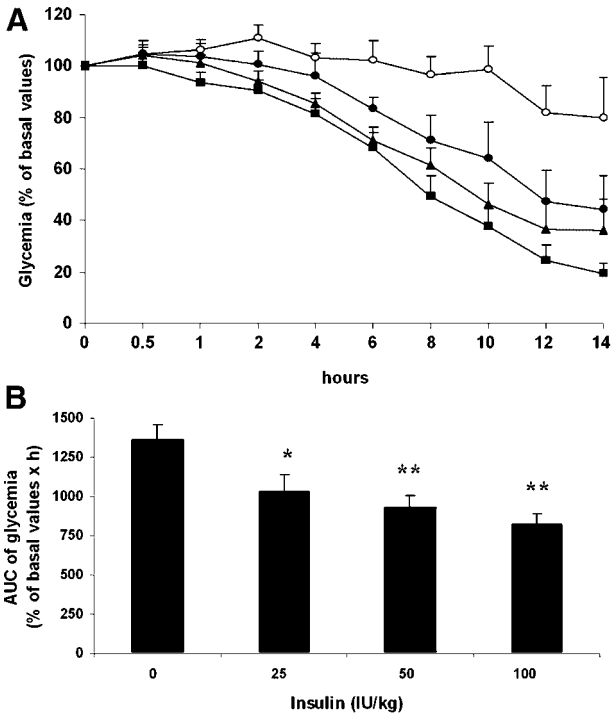

Figure 6. (A) Glycemia after a single oral administration of albumin-chitosan-PEG coated insulin-loaded nanospheres: 25 IU/kg (full circles, $n = 8$), 50 IU/kg (triangles, $n = 9$), and 100 IU/kg (squares, $n = 8$) or empty nanospheres as control (empty circles, $n = 9$) in fasted diabetic rats. Results are expressed as means $\pm$ SEM. Mean basal values at T_0 were 390 ± 21 mg/dL. (B) Areas under the curves (0–14 h) of each glycemia profile. Statistically different from empty nanospheres: $^{*}p < 0.05$; $^{**}p < 0.01$.

Calculation of the areas under the curves over a 14 h period (Fig. 6B) showed that 25, 50, and 100 IU/kg insulin-loaded nanospheres reduced the areas by 24 ($p < 0.05$), 32 ($p < 0.01$), and 40% ($p < 0.01$), respectively.

Pharmacological availability was calculated based on AUC_{0-8h} and summarized in Table 2. Values ranged from 10% to 42% of subcutaneous administration for doses of 100 to 25 IU/kg, respectively.

To confirm albumin and PEG effects, chitosan-coated alginate-dextran sulfate core nanospheres, without albumin and PEG, were orally administered to fasted diabetic rats. Figure 7 depicts blood glucose profiles following oral administration. Glycemia was not reduced for both nanospheres and negative controls except at 6–8 h where the fasting effect was observed for all tested groups. Even after chitosan coating, alginate-dextran sulfate core nanospheres were ineffective in producing a pharmacological response, which is consistent with previous results.[21]

Glycemic Response of Diabetic Rats Treated by Oral Insulin-Loaded Nanospheres to a Glucose Tolerance Test (OGTT)

When diabetic rats were dosed orally with glucose (2 g/kg body weight), glycemia increased reaching a maximal level (347%, $p < 0.001$) after 40 min (Fig. 8). Then glycemia decreased again to 180 min. When insulin-loaded nanospheres (50 IU/kg) were administered 10 h before glucose challenge, glycemia also increased but to a lesser extent, attaining 246% of initial values 40 min after glucose administration ($p < 0.001$), and decreased slowly thereafter. Differences in response between empty and insulin-loaded nanosphere treatments were significant statistically ($p < 0.05$). Consequently, insulin-loaded nanospheres improved the response to a glucose challenge.

Insulinemia in Diabetic Rats Treated Orally With Insulin-Loaded Nanospheres

After oral administration of insulin-loaded nanospheres (50 IU/kg), insulinemia increased with a maximal effect 4 h after gavage by factor seven of basal value as illustrated in Figure 9. The detection kit used is known to have some cross-reaction with rat insulin, which may explain the low basal concentration due to weak residual

Table 2. Pharmacological Availability of Insulin-Loaded Nanospheres Administered to Diabetic Rats, Calculated from Glycemia Obtained After a Subcutaneous Administration of Free Insulin (4 IU/kg) and an Oral Administration of Insulin-Loaded Nanospheres (25, 50, and 100 IU/kg)

Route	Dose of Insulin (IU/kg)	$AUC_{0\text{-}8h}$	Pharmacological Availability (%)	t_{max} (h)
Subcutaneous	4	265 ± 18	100	4
Oral	25	696 ± 43	42	14
Oral	50	670 ± 31	21	14
Oral	100	630 ± 36	10	14

Data are means ± SEM of 6–9 animals per group.

insulin production which can occur in streptozotocin induced diabetic rats. Basal insulin concentration was 35.0 ± 8.2 μU/mL ($t = 0$) but insulin levels increased to 250.8 ± 19.4 μU/mL ($p < 0.001$) at 4 h following oral administration of human insulin. At 8 h, plasma insulin level was still high at 149.4 ± 12.7 μU/mL ($p < 0.001$) and increased again at 12 h, being 225.1 ± 20.2 μU/mL ($p < 0.001$). These results showed that insulin absorption was markedly enhanced by the action of insulin-loaded nanospheres.

Intestinal Uptake of Insulin-Loaded Nanospheres

When FITC-labeled insulin-loaded nanospheres (50 IU/kg) were administered to an *in situ* isolated intestinal loop, a strong fluorescence appeared after 5 min in the lumen (Fig. 10a). Fluorescent label was strongly adherent to the apical part of the *villi* and appeared as a film in close contact with enterocytes (Fig. 10b). No labeling appeared in the lumen of the crypt nor in the crypt cells. In addition, an intense fluorescent labeling was present in the connective axis of the *villi* where it probably reflects the presence of labeled insulin inside vascular structures. Indeed, focused fluorescent points had deep intestinal location as observed by confocal microscopy analysis, 30 min after *in situ* administration (Fig. 11a and b). There was a strong labelling of Peyer's patches (Fig. 10c) indicating a preferential uptake of insulin-loaded nanospheres by these follicles.

In contrast, when FITC-labeled insulin in solution was administered in the intestinal lumen, lumen and *villi* remained unlabeled. Occasionally, a very smooth material appeared in the dome of Peyer's patches.

Fluorescent activity in rat gut contents and mucosa homogenates from isolated intestinal loop

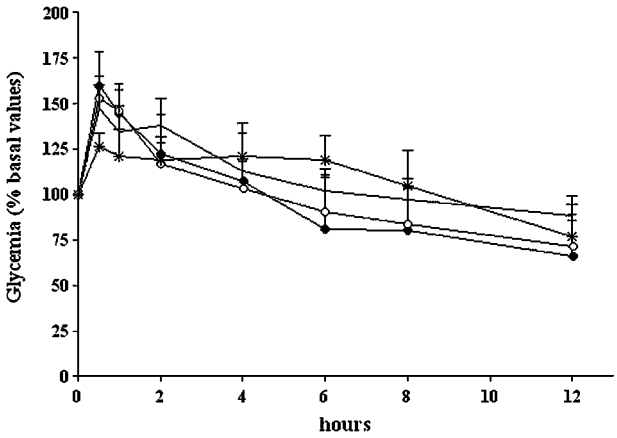

Figure 7. Glycemia after a single oral administration of chitosan-coated alginate-dextran core nanospheres without albumin coating and PEG stabilizing effect: 50 IU/kg (full circles, $n = 6$), empty nanospheres (empty circles, $n = 6$), nonencapsulated insulin (stars, $n = 6$) and water (line, $n = 4$) as control in fasted diabetic rats. Results are expressed as means ± SEM.

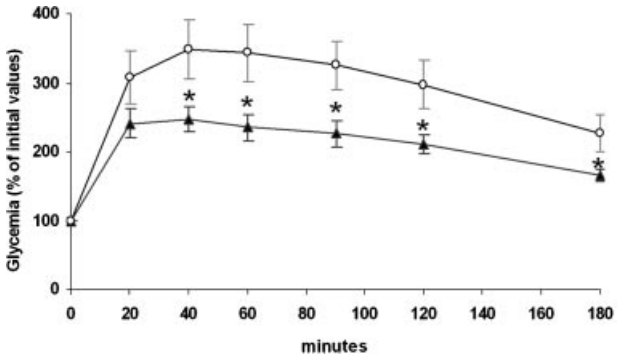

Figure 8. Blood glucose levels after an oral glucose challenge (2 g/kg) performed 10 h after a single oral administration of albumin-chitosan-PEG coated insulin-loaded nanospheres (50 IU/kg) (triangles, $n = 12$) or empty nanospheres (empty circles, $n = 10$) in fasted diabetic rats. Results are expressed as means ± SEM. The value of glycemia before the administration of nanospheres for all animals was 492 ± 23 mg/dL. This value decreased 54% and 1% after 10 h and before glucose administration for animals dosed with albumin-chitosan-PEG coated insulin-loaded nanospheres and empty nanospheres, respectively. Statistically different from empty nanospheres: $^{*}p < 0.05$.

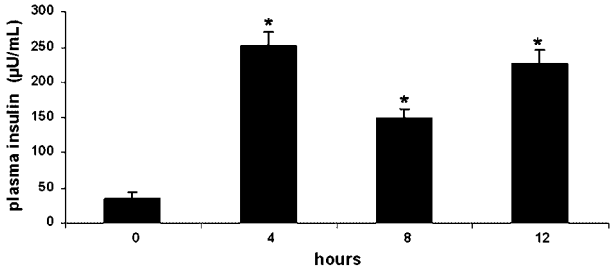

Figure 9. Insulinemia bar plot following oral administration of albumin-chitosan-PEG coated insulin-loaded nanospheres (50 IU/kg) in diabetic rats fasted for 12 h. Each value represents mean ± SEM ($n = 14$). Mean basal values at time zero were: 35.0 ± 8.2 µU/mL ($t = 0$). Comparisons versus time zero: $^{*}p < 0.05$.

and mesenteric blood was measured by fluorescence spectrophotometry and is represented in Figure 12. Strong FITC-fluorescence spreading through gut content decreased over time. In contrast, fluorescence of mucosal contents progressively increased and achieved a maximum at 8 h. Concerning FITC-insulin levels in mesenteric blood, a strong increase of fluorescence activity was observed over the experiment demonstrating insulin passage through intestinal mucosa.

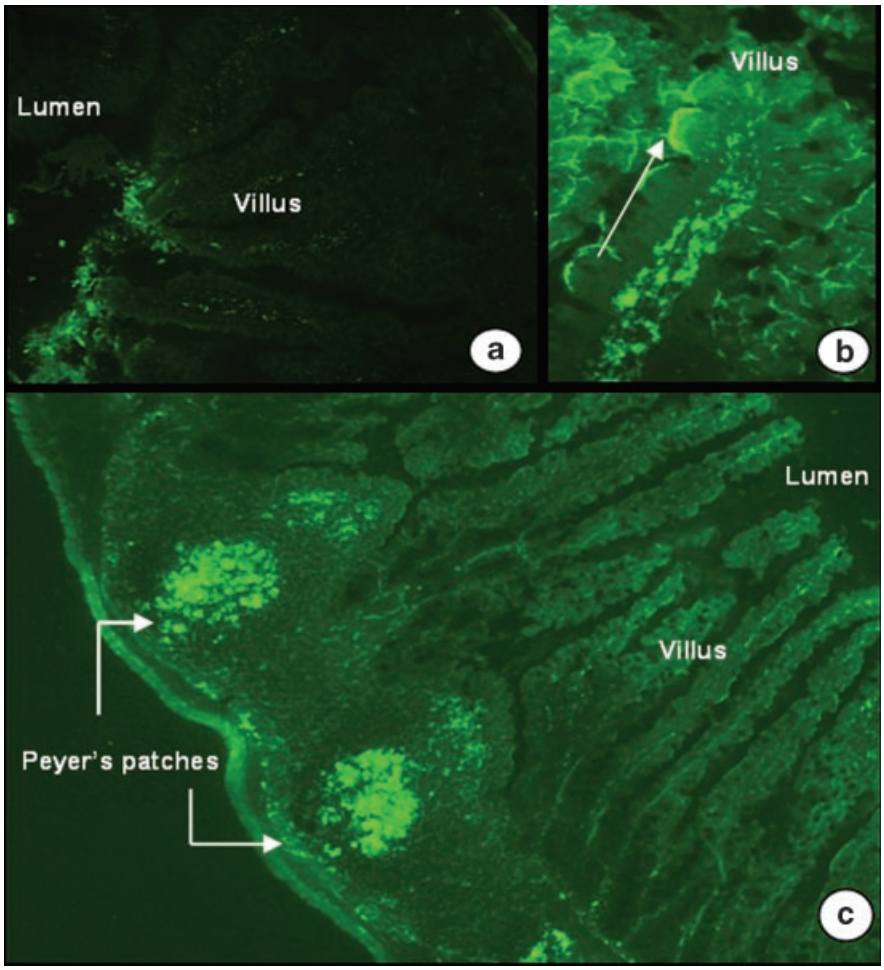

Figure 10. Fluorescence microscopy of tissue slices through the intestinal epithelium 5 min (a) and 30 min (b and c) after the intraluminal administration of albumin-chitosan-PEG coated FITC-labeled insulin-loaded nanospheres (50 IU/kg) in an *in situ* isolated intestinal segment. Note in (a) the presence of fluorescent material inside the intestinal lumen. Part (b), fluorescent material is adherent to the apical pole of enterocytes (arrow) and is present inside the core of the *villus*. Part (c), a strong fluorescent material in Peyer's patches. Magnifications: (a) 100×; (b) 200×; (c) 100×.

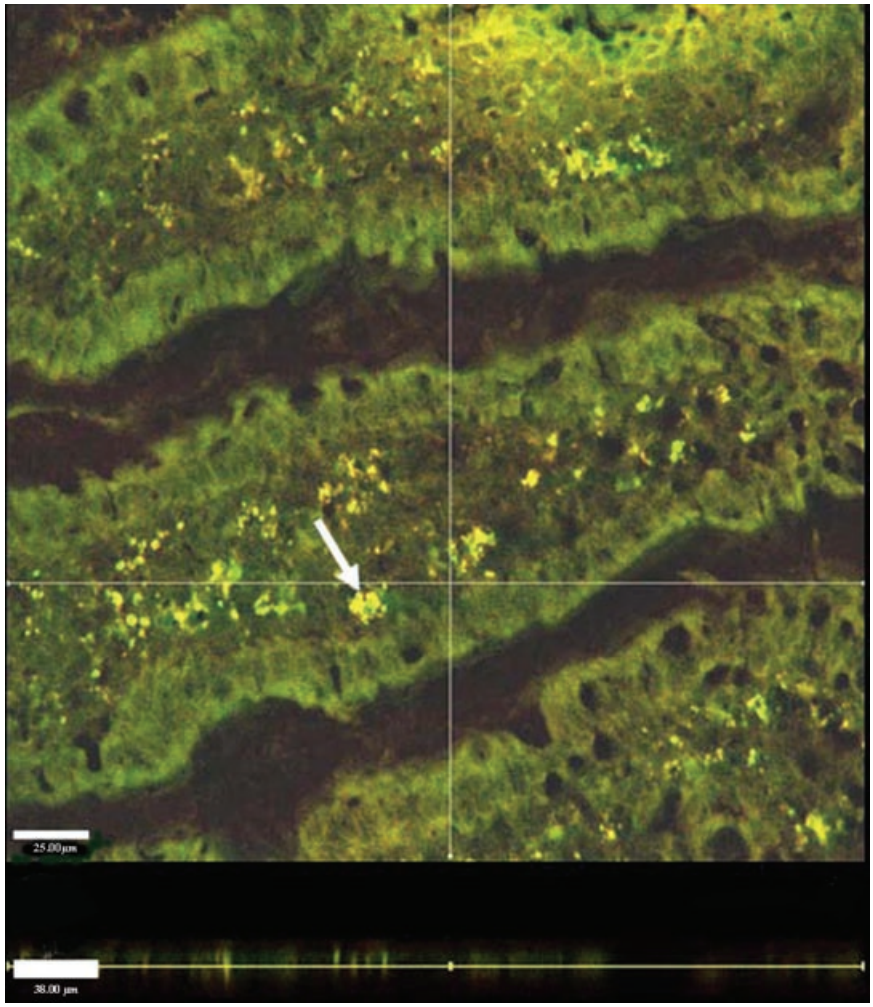

Figure 11. Confocal microscopy of intestinal *villi* after 30 min of intraluminal administration of albumin-chitosan-PEG coated FITC-insulin-loaded nanospheres in an *in situ* isolated intestinal segment. The fluorescent labeling (arrow) represents insulin-loaded nanospheres in the core of the *villus* (a). Lines indicate where the pictures were taken and analyzed (b). The bar represents 25 μm in (a) and 38 μm in (b).

Metabolic Status of Diabetic Rats After 4-Day Treatment With Insulin-Loaded Nanospheres

Diabetes is generally characterized by several metabolic parameters such as hyperphagia, polydipsia and polyuria together with albuminuria and proteinuria. As illustrated in Table 3, insulin-loaded nanospheres administered orally to diabetic rats for 4 successive days at the concentration of 50 IU/kg, improved these metabolic parameters when compared to rats treated with empty nanospheres. Indeed, water intake, volume of collected urine and proteinuria over 24 h were significantly reduced by 42 ($p < 0.001$), 47 ($p < 0.01$), and 67% ($p < 0.05$), respectively. However, food intake was only slightly reduced (−15%, not significant). Thus, a daily oral administration of insulin-loaded nanospheres for four successive days improves the metabolic status of diabetes.

DISCUSSION

The oral route of free insulin delivery takes advantage of the portal-hepatic route of absorption. However, insulin is degraded by proteolytic enzymes in the GIT and, being a small peptide, is less absorbed by the intestinal mucosa. Alginate-dextran sulfate nanospheres coated with chitosan-PEG-albumin preserve the biological activity of entrapped insulin, and strongly elicit an

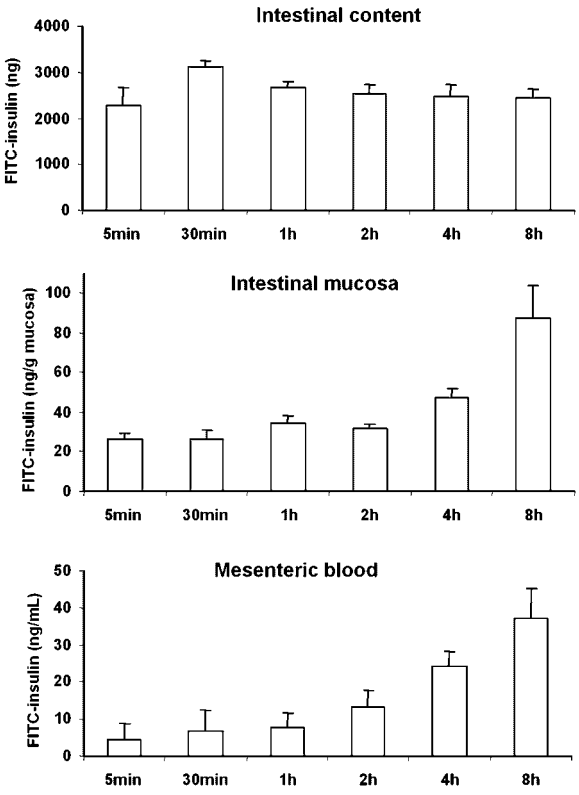

Figure 12. Fluorescent activity from FITC-insulin in the intestinal content, intestinal mucosa and mesenteric blood after injection of albumin-chitosan-PEG coated FITC-insulin-loaded nanospheres (50 IU/kg) in an *in situ* isolated intestinal segment. Results are expressed as means ± SEM of six experiments at each time.

hypoglycaemic response when administered subcutaneously or orally. Albumin-coating and PEG stabilizing effect seem to be crucial in producing a pharmacological response, as nanospheres coated with only chitosan did not elicit hypoglycemia following oral administration. With the newly developed chitosan-PEG-albumin coated nanospheres glucose reduction was higher than 75% of basal values after an oral administration of 100 IU/kg insulin while empty nanospheres or nonencapsulated insulin did not affect glycemia. Glycemic response was dose-dependent and lasted for a prolonged time, at least for 24 h, with a maximal effect 14 h postadministration. In addition, the improvement of the glycemic response to an oral glucose tolerance test confirms the efficacy of orally administered particulate insulin. Finally, plasma insulin levels increased by a factor seven indicating that insulin was protected and well absorbed by the intestinal mucosa.

Insulin can be incorporated into nanospheres with high efficiency. At pH 4.5, positive amino groups of insulin (p$I = 5.3$) strongly interact with carboxylic groups of alginate (pK_a 3.38 and 3.65) and with sulfate groups of dextran (first pK_a negative and second pK_a 2).[18] The nanoencapsulation process is essential to protect insulin from the acidic environment and proteolytic attack during gastrointestinal tract passage. By itself, insulin was not bioactive after oral administration. This data is not in agreement with a very similar nanosystem which involved alginate-chitosan nanoparticles where chitosan coating produced an hypoglycemic response around 45% of basal values after 14 h of oral administration.[28] Albumin coating and PEG stabilizing effect appear to play an important role on insulin protection and particle stabilization. Protection of nanoencapsulated insulin was demonstrated by three experiments: firstly, insulin-loaded nanospheres had equivalent pharmacological activity to nonencapsulated insulin when administered subcutaneously; secondly, nanoparticulate insulin was detected after incubation of insulin-loaded nanospheres in presence of pepsin and thirdly, only 25% of initial insulin content was released from nanospheres incubated in an acidic environment simulating gastric medium, with an additional 45% being released in a neutral simulated

Table 3. Metabolic Status of Rats After Four Successive Days Oral Administration of Insulin-Loaded Nanospheres (50 IU/kg)

	Control	Insulin-Loaded Nanospheres
Water intake (mL/24 h)	133.0 ± 3.6	76.8 ± 10.0***
Food intake (g/24 h)	28.8 ± 4.1	24.3 ± 1.0
Urine volume (mL/24 h)	118.0 ± 11.3	62.3 ± 12.5**
Proteinuria (mg/24 h)	46.0 ± 15.2	15.1 ± 2.2*

Control animals received empty nanospheres. Water and food intake, and urine volume were measured for 24 h. Proteinuria was measured on urines collected for 24 h. Results are means ± SEM of six animals per group. Statistically different from control.
*$p < 0.05$.
**$p < 0.01$.
***$p < 0.001$.

intestinal medium. Acid pH causes the alginate polymer in the core of the particle to precipitate and thus collapse, forming a tight impermeable matrix. At subsequent neutral pH, the polymer begins to swell, releasing the insulin.[18] Therefore, it can be considered that insulin may be partly protected by nanospheres during passage through the stomach. It is likely that the albumin shell plays an important and crucial protective role against proteolytic enzymes in the stomach. Albumin may act as degradative target of pepsin leaving encapsulated insulin free of proteolytic attack. The dramatic change in zeta potential from negative to positive, demonstrates degradation of the albumin coating, exposing the underlying layer of mucoadhesive chitosan and therefore nanospheres may exert mucoadhesive properties. As well, chitosan with PEG chains may also influence the mesh size of the hydrogel networks,[12] forming an additional capsule shell, made up of an interpenetrating network near the surface of the core particle. Even if 25% of encapsulated insulin is released in the stomach, the main part of encapsulated insulin is active and resistant to pepsin and will be delivered to the intestine in its bioactive form. Since chitosan is a polysaccharide with positive charge, it promotes protein adsorption to the nanospheres since most proteins have isoelectric points lower than 7.4 and are negatively charged. In addition to particle charge, other properties may also influence mucoadhesion of nanospheres such as particle size and hydrophobicity. The introduction of PEG may not influence this property, but high concentrations of PEG may lead to the compact nanosphere structure as described above. PEG was also used to improve the stability of the polymers,[25,29] to improve the half-live of insulin and to increase residence time along the intestine.[30]

To enhance the intestinal absorption of peptides and especially of insulin associated with nanoparticles, researchers generally consider several factors: (i) mucoadhesion, (ii) particle endocytosis, and (iii) permeation—enhancing factors.[31] Mucoadhesive properties of chitosan and PEG may have promoted nanosphere adhesion and subsequent absorption by enterocytes. Measurement of zeta potentials showed that pepsin which is abundant in the stomach, reduced the negative charge of insulin-loaded nanospheres, so that nanospheres become more mucoadhesive once in the small intestine. In fact, it was reported that PEG chains enhanced adhesion due to the interdiffusion and entanglements with mucus layers.[32] Adhesion of insulin-loaded nanospheres to the intestinal mucosa may improve retention time, leading to higher concentrations of insulin at the site of absorption.[33] Previous results[15] in which positively charged insulin nanoparticles, but of a different composition (poly(ε-polycaprolactone)/Eudragit) are in agreement with this assumption. We also observed a sustained dose-dependent hypoglycemic effect in the present study after oral administration. However, previous work [15] showed less hypoglycaemic effect (about −40% of control values vs. −76% in the present study) and duration of the effect was lower (8 h vs. 14 h in the present study). Thus, it appears that insulin-loaded nanospheres composed of alginate-dextran sulfate coated with chitosan-PEG-albumin exert a better antidiabetic effect than poly(ε)caprolactone-Eudragit nanoparticles after oral administration in diabetic rats. This difference may be explained by the polymeric properties of the drug carriers. Indeed, the presence of chitosan coating which confers positive charges to the nanospheres contributes to the mucoadhesive properties of the particles and in addition transiently opens the tight junctions[20,34] allowing a better absorption of liberated or encapsulated insulin. Moreover, a recent work[28] which used negatively charged insulin nanoparticles made by complexation of alginate with chitosan led to a less pronounced hypoglycemic response after 14 h of oral administration to diabetic rats at dose 100 IU/kg (about −45% of control values vs. higher than −75% in the present study). This difference may be explained by the different methodology and by the presence of albumin coating and PEG stabilizing effect. Our data suggests that albumin coating is critical to preventing pepsin attack and PEG plays an important role as stabilizing agent.

Different mechanisms have been proposed to explain the translocation of particulate material across the intestinal barrier: uptake via Peyer's patches, isolated lymphoid follicles[35–38] abundant in the ileum, intracellular uptake via insulin surface receptor binding[39,40] and, intercellular/paracellular passage.[38,41] In order to better approach these mechanisms of insulin absorption, we labeled insulin with FITC and followed the uptake by spectrofluorometry and by the observation of cryosections of the intestinal mucosa after injection of labeled insulin-loaded nanospheres in an *in situ* isolated intestinal loop. FITC-insulin was taken up by the intestinal mucosa. Indeed, FITC-insulin concentration decreased as a

function of time in the intestinal content and in parallel increased in the intestinal mucosa and mesenteric blood indicating that it passed from the intestinal lumen to the blood compartment. Fluorescence microscopic observations confirm these data and showed at first an intense labeling of FITC-insulin in the intestinal lumen and then in the axis of the *villi* and Peyer's patches. These results taken together showed that insulin, either liberated from nanospheres in the intestinal lumen or associated with nanospheres, was able to be taken up by the intestinal mucosa. It is possible that a part of the insulin is liberated in close contact with the apical part of enterocytes then taken up by a receptor-mediated process as described by Bendayan et al.[39,40] Insulin-loaded nanospheres may also be taken up by a paracellular pathway.[41] Chitosan coating has been shown to transiently open tight junctions between cells,[42] allowing the passage of small particles. This process mainly occurs at the tip of the *villi* where there is a physiological desquamation of mature enterocytes and the presence of a thin fluorescent film was observed overlaying the enterocytes (Fig. 10b). However, the most intense labeling was found in Peyer's patches suggesting that nanospheres may be taken up by endocytosis through M cells. This labeling was more pronounced than with poly(ε)caprolactone-Eudragit loaded insulin nanoparticles[15] or with alginate-chitosan nanoparticles[28] and may be attributed to the combination of the advantageous properties of chitosan with those of PEG as observed by Prego et al.[29] who grafted PEG to chitosan.

In addition, this formulation had beneficial effects on diabetic symptoms, including thirst, frequent urination and proteinuria. Thirst develops because of osmotic effects as sufficiently high glucose above the renal threshold in the blood is excreted by the kidneys. This requires water causing increased fluid loss, which must be replaced. The lost blood volume will be replaced from water held inside body cells, causing dehydration. Water intake (polydipsia) was significantly reduced in a 4-day period due to lower glycemia levels following insulin-nanosphere administration. As well, polyuria was significantly reduced after 4 days of daily administration. Additional metabolic studies will be performed in order to characterize the metabolic and toxicological effects during continuous and prolonged oral administration. The potential extension to other therapeutic proteins will also be studied in the next future.

CONCLUSIONS

Alginate-dextran sulfate core, chitosan-polyethylene glycol-albumin coated nanospheres protect insulin during nanosphere formulation and from proteolytic degradation during gastrointestinal transit. Insulin-loaded nanospheres with small size (50% less than 812 nm) and encapsulation efficiency of 85% were produced by emulsion dispersion/triggered gelation, followed by polyelectrolyte coating. Blood glucose reduction following oral administration was higher than 70% of the basal value while empty nanospheres or nonencapsulated insulin were ineffective in producing oral hypoglycemic response. Nanospheres lacking albumin and PEG in the coating material were also ineffective, as it appears that albumin prevents protease attack on the insulin, and PEG serves as nanosphere stablizer. In contrast, chitosan-PEG-albumin coated nanospheres increased insulinemia by a factor of seven and significantly improved the response to the glucose oral tolerance test. Insulin-loaded nanospheres exert an antidiabetogenic effect when administered perorally in diabetic rats and show high intestinal uptake by Peyer's Patches and *villi*. The excipients used are well accepted in the pharmaceutical field, thus this formulation is able to markedly improve the intestinal absorption of insulin and will be of interest in the treatment of diabetes with oral insulin. The benefits are considerable in terms of diabetes, but also potentially toward the oral delivery of many other therapeutic peptides and proteins.

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