PHARMACEUTICAL NANOTECHNOLOGY

Poly(ε-Caprolactone)/Eudragit Nanoparticles for Oral Delivery of Aspart-Insulin in the Treatment of Diabetes

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ABSTRACT: Nanoparticles prepared with a blend of a biodegradable polyester (poly(ε-caprolactone)) and a polycationic nonbiodegradable acrylic polymer (Eudragit® RS) have been used as a drug carrier for oral administration of a short-acting insulin analogue, aspart-insulin. Insulin-loaded nanoparticles, about 700 nm in diameter, encapsulated 97.5% of insulin and were able to release about 70% of their content *in vitro* in a neutral medium over 24 h. When administered orally to diabetic rats, insulin-loaded nanoparticles (50 IU/kg) decreased fasted glycemia for a prolonged period of time and improved the glycemic response to glucose in a time-dependent manner, with a maximal effect between 12 and 24 h after their administration. In parallel, plasma insulin levels increased. However, higher (100 IU/kg) and lower (25 IU/kg) doses of insulin did not exert any biological effect. It is concluded that polymeric nanoparticles composed of poly(ε-caprolactone)/Eudragit® RS are able to preserve the biological activity of the insulin analogue aspart-insulin; however, the postprandial peak suppression was prolonged more than 24 h by comparison with regular insulin working only 6–8 h. This effect may be explained by the monomeric configuration of aspart-insulin, which is probably better taken up by the intestinal mucosa than regular insulin. J Pharm Sci 99:879–889, 2010
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INTRODUCTION

Insulin is a 51 amino acid peptide discovered in 1921–1922 by Banting and Best, together with Macleod and Collip.[1] This hormone, produced by the pancreatic β cells, controls the level of sugar in blood by facilitating the uptake of glucose in the cells of the organism, especially liver, muscle, and adipose tissue. Thus, physiologically, insulin counteracts the increase in blood glucose levels after a meal. In type 1 diabetes (due to the autoimmune destruction of β cells) and in type 2 diabetes (characterized by a dysfunction of insulin, insulin resistance, and a reduction of insulin production due to a reduced number of β-cells), the parenteral administration of insulin normalizes blood glucose levels and prevents the complications of diabetes such as neuropathy, nephropathy, blindness, cardiac failure, stroke and amputation.[2]

Insulin is generally parenterally administered but alternative routes of administration (oral, nasal, rectal, pulmonary, and ocular) have been extensively investigated.[3] Among them, the oral route is the most physiological and comfortable. Indeed, insulin absorbed by the intestinal epithe-

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lium reaches the liver through the portal vein and can directly inhibit hepatic glucose output.[4] However, insulin is less absorbed by the gastrointestinal tract (<0.5% under physiological conditions) and in addition, it is strongly degraded by proteolytic enzymes, that is, the same enzymes that degrade dietary peptides and proteins. In order to protect insulin against degradation and to improve its absorption, insulin has been formulated with antiproteases,[5] hydrogels,[6] or absorption enhancers such as cyclodextrins,[7] bile salts, and surfactants.[8,9] Insulin was also modified chemically[10–13] or associated with oligoarginine, a cell-penetrating peptide.[14] Another strategy was to formulate insulin with drug delivery systems such as liposomes, lipid particles or polymeric nano- or microparticles.[4,15] These particles must be biocompatible, stable in suspension, and should be degraded under physiological conditions.

As early as 1988[16] insulin was formulated with poly(alkylcyanoacrylate) nanocapsules and a prolonged reduction of hyperglycemia was observed after a single oral administration to diabetic rats. This reduction was dose dependent and lasted up to 20 days with a dose of 50 IU/kg of encapsulated insulin. These results were confirmed in dogs[17] and demonstrated the feasibility of oral delivery of insulin using polymeric biodegradable nanocapsules as a drug carrier. Later on, other types of nanoparticles were developed for oral delivery of insulin. The polymers were based on polymethacrylic acid,[18] poly(lactic acid),[19] poly(lactide-*co*-glycolide),[20–22] chitosan,[23–25] and alginate.[26–28] Nanoparticles composed of solid lipids,[29,30] calcium phosphate–PEG–insulin–casein (CAPIC),[31] or gold[32] were also developed. Generally, these formulations, orally administered mostly to diabetic animals, induced a sustained reduction in blood glucose.[4] In a recent work,[33] we used nanoparticles made of a biodegradable polymer (poly(ε-caprolactone)), and a nondegradable but biocompatible mucoadhesive polymer (Eudragit® RS) with polymers ratio 50/50. These insulin-loaded nanoparticles (100 IU/kg), administered orally in diabetic rats, reduced glycemia for a prolonged period of time (from 4 up to 24 h) with a maximal effect after 6–8 h (−40%). This effect was dose dependent. After labeling of insulin with fluorescein isothiocyanate, it was demonstrated that insulin was absorbed by the intestinal mucosa probably due to the mucoadhesive properties of the polycationic polymer, that is, Eudragit® RS.[33]

All insulin nanoparticles formulations developed for oral delivery of the peptide used regular insulin.[4] Regular insulin is a peptide of 5.8 kDa, composed of two peptide chains referred to as the A chain and B chain linked together by two disulfide bonds, and an intramolecular disulfide bond formed within the A chain. Insulin molecules have a tendency to form dimers in solution due to hydrogen bonding between the C-terminals of B chains. At high concentrations and even at low concentrations in presence of zinc ions, insulin dimers form hexamers. These interactions have important clinical implications. Monomers and dimers readily diffuse into blood while hexamers diffuse poorly. Thus, absorption of insulin preparations with a high proportion of hexamers is delayed. This property has been taken into account for the pharmaceutical development of long-acting insulin analogues.[34] In contrast, fast-acting insulin analogues based on the monomeric form of insulin have also been developed. In particular, aspart-insulin (marketed by Novo Nordisk, Copenhagen, Denmark, as NovoRapid®) was created through recombinant DNA technology so that the amino acid, B28, which is normally proline, is substituted with an aspartic acid residue. This analogue has increased charge repulsion which prevents the formation of dimers and hexamers; thus, monomeric insulin is absorbed three times faster than human insulin after subcutaneous injection, leading to a more rapid rise in plasma insulin concentration and an earlier hypoglycemic response.[35,36] We hypothesized that the aspart-insulin analogue, remaining monomeric, should be better absorbed by the intestinal mucosa, and in consequence should improve the biological efficacy of oral insulin-loaded nanoparticles. Thus, the present study was designed to develop a formulation of aspart-insulin-loaded nanoparticles made of poly(ε-caprolactone)/Eudragit® RS, to characterize them physico-chemically (diameter, zeta-potential, insulin loading, *in vitro* release of insulin) and to study the biological efficacy after oral administration in streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

Material

Aspart-insulin (Novorapid®) was kindly provided by Novo Nordisk (Paris, France). Poly(ε-caprolac-

tone) (MW = 42,000) was purchased from Sigma–Aldrich Chimie (L'Isle d'Abeau Chesnes, France) and Eudragit® RS (methacrylic acid esters with a small proportion of trimethylamonioethyl methacrylate chloride) (MW = 150,000) was a gift from Röhm Pharma (Darmstadt, Germany). Polyvinylalcohol (PVA, MW = 30,000, 88% hydrolyzed) and streptozotocin were obtained from Sigma–Aldrich Chimie. All other chemicals or solvents were of analytical grade.

Preparation of Nanoparticles

Nanoparticles were prepared by the multiple emulsion technique previously described by Hoffart et al.[37] Briefly, 1 mL of an aqueous solution of insulin (100 IU) was first emulsified, by sonification for 30 s, in methylene chloride (10 mL) containing 250 mg of polymers (PCL/Eudragit® RS 50/50). The resulting water-in-oil emulsion was thereafter poured into 40 mL of a polyvinyl alcohol aqueous solution (0.1%, m/v) and sonicated for 1 min, involving the formation of the second water-in-oil-in-water emulsion. After evaporation of methylene chloride under reduced pressure, the nanoparticles were isolated by centrifugation (45,000 g, 20 min). The nanoparticles were washed three times with deionized water then centrifuged again and kept in suspension in water until use. For control experiments, empty nanoparticles were formulated in the same way.

Characterization of Nanoparticles

The mean diameter and zeta-potential of nanoparticles were determined by photon correlation spectroscopy (ZetaSizer II, Malvern Instruments, Orsay, France). The amount of insulin entrapped within polymeric nanoparticles was determined by high performance liquid chromatography (HPLC).[38]

In Vitro Experiments

Fifty milligrams of freeze-dried insulin-loaded nanoparticles were suspended in 20 mL of saline phosphate buffer (PBS, pH 7.4) containing 0.1% Tween® 80 and incubated at 37°C under gentle magnetic stirring (300 rpm). At determined intervals (5, 15, 30, 45 min, 1, 2, 3, 4, 6, 8, and 24 h), 1 mL samples were removed and replaced by 1 mL of fresh phosphate buffer added to the suspension of nanoparticles. Insulin was determined in each sample by HPLC as previously described.[38]

In Vivo Experiments in Diabetic Rats

Animals

Adult male Wistar rats (Depré, St Doulchard, France) were housed in air-conditioned quarters under a photoperiod schedule of 12 h light/12 h dark. They received standard laboratory chow diet (UAR, Villemoisson-sur-Orge, France) and tap water, available ad libitum. All experiments were carried out in accordance with the European Community Council Directive of November 24, 1986 (86/609/EEC).

Induction of Diabetes

Diabetes was induced in male Wistar rats (250 ± 30 g) by an injection of streptozotocin (65 mg/kg) in sodium chloride (0.9%) as previously described.[33] Rats were considered diabetic when glycemia was higher than 300 mg/dL about 2 weeks after streptozotocin treatment.

Subcutaneous (s.c.) Administration of Insulin-Loaded Nanoparticles and Free Insulin

Prior to oral administration and in order to verify that insulin encapsulated into nanoparticles was still active, loaded nanoparticles were injected s.c. to fasted diabetic rats (10 IU/kg body weight). Nonencapsulated insulin (10 IU/kg) and saline were administered in control animals. Glycemia, in blood samples withdrawn from the tail vein, was measured before injection and 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 h thereafter using a glucometer (Accu-check Go, Roche Diagnostics, Meylan, France). Rats were maintained fasted during the experiment up to 8 h and fed thereafter.

Intragastric Administration of Empty and Insulin-Loaded Nanoparticles

In order to investigate the biological efficacy of insulin-loaded nanoparticles administered orally, two sets of experiments were performed. In the first experiment, the effect of oral insulin on glycemia was analyzed as a function of insulin concentration. To this intent, unloaded (control) and insulin-loaded nanoparticles were given intragastrically as a single administration to overnight fasted (water at libitum) diabetic rats

at the dose of 25, 50, and 100 IU/kg body weight. The suspension of insulin-loaded nanoparticles contained 24 IU insulin per mL. On the other hand, there was also 1.5 g of polymer per mL making 24 IU per 1.5 g of polymer or 16 IU per gram of polymer (i.e., nanoparticles). Thus, the rats which received 25, 50, and 100 IU insulin-loaded nanoparticles/kg b.w. under a volume of 1 mL, received 1.56, 3.12, and 6.24 g of polymer, respectively. In the second experiment, the response of oral insulin to a glucose overload was investigated. Thus, unloaded (control) and insulin-loaded nanoparticles (50 IU/kg) were given intragastrically as a single administration to overnight fasted diabetic rats. Then 2, 5, 12, 24, 48, and 72 h later, these animals received orally glucose (2 g/kg). Glycemia was measured in blood sampled from the tail vein before oral glucose and subsequently at regular time intervals thereafter from 10 min up to 120 min.

Plasma Insulin After Oral Administration of Insulin-Loaded Nanoparticles

Insulin-loaded nanoparticles (50 IU/kg) were administered orally to overnight fasted diabetic rats. Blood samples from the tail vein were collected under a slight anesthesia prior and sequentially from 30 min to 24 h after the administrations. Plasma insulin concentrations were measured by radioimmunoassay (Insulin-CT kit from CIS Bio International, Gif-sur-Yvette, France).

Statistical Analysis

The means and standard errors for all values were calculated. For group comparisons a one-way analysis of variance (ANOVA) followed by a Dunnett or a Bonferroni multicomparison test were applied, using the Instat 2.00 Macintosh software (Graph Pad Software, San Diego, CA). The difference was considered as significant when $p < 0.05$.

RESULTS

Structure and Characterization of Nanoparticles

When analyzed by laser light scattering, insulin-loaded and empty nanoparticles showed a homogeneous size distribution with a mean diameter of 695 ± 56 and 340 ± 25 nm, respectively. Insulin-loaded and unloaded nanoparticles were positively charged (40.5 ± 0.7 and 36.7 ± 1.4 mV, respectively). These nanoparticles encapsulated a high amount of insulin ($97.5 \pm 0.4\%$) (Tab. 1) and contained 16 IU insulin/g polymer.

In Vitro Release of Insulin

Figure 1 illustrates the *in vitro* release profile of insulin from insulin-loaded nanoparticles in PBS at 37°C and pH 7.4. The release profile showed two phases: an initial burst release during which a significant amount of insulin was released within 30 min ($43.1 \pm 2.6\%$). This level increased up to $46.84 \pm 1.78\%$ after 1 h and slightly increased up to $68.6 \pm 4.6\%$ after 24 h.

Effect of Subcutaneous Administration of Insulin-Loaded Nanoparticles on Glycemia

Blood glucose profiles following subcutaneous injection of insulin-loaded nanoparticles and nonencapsulated insulin to diabetic rats at the dose of 10 IU/kg are illustrated in Figure 2. When compared to the glycemic profile of control animals receiving saline, encapsulated insulin as well as nonencapsulated insulin rapidly reduced insulin by 32% ($p < 0.001$) after half an hour and 55–59% ($p < 0.001$) after 1 h. The maximal reduction (−85 to −90%) was observed between 3 and 6 h. Then, glycemia increased slowly but faster after encapsulated insulin injection (−61%, $p < 0.05$ after 12 h) than after free insulin injection (−78%, $p < 0.001$ after 12 h). Finally, the rats were fed again after 12 h; the glycemia of the three groups increased and

Table 1. Characteristics of Insulin-Loaded Nanoparticles

	Unloaded NP	Insulin-Loaded NP
Mean diameter (nm)	340 ± 25	695.2 ± 55.8
Polydispersity index	0.20 ± 0.03	0.36 ± 0.05
Zeta potential (mV)	36.7 ± 1.4	40.5 ± 0.7
Encapsulation efficiency (%)	—	97.5 ± 0.4

Data are means ± SD ($n = 3$).

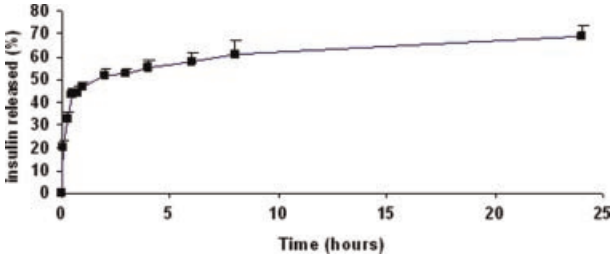

Figure 1. *In vitro* release profile of insulin from insulin-loaded nanoparticles in pH 7.4 saline phosphate buffer containing 0.1% Tween® 80. Results are means ± SD of three experiments.

after 24 h, the values of both groups treated with encapsulated and nonencapsulated insulin reached the control group treated with saline. None significant difference was observed between the three groups.

Effect of Oral Administration of Insulin-Loaded Nanoparticles on Glycemia

As illustrated in Figure 3A, insulin-loaded nanoparticles intragastrically administered at the dose of 50 IU/kg in diabetic fasted rats, significantly reduced glycemia from half an hour (−29%, $p < 0.05$) when compared to controls receiving empty nanoparticles. The glycemia continued to decrease and the maximum decrease was observed after 6 to 8 h (−53%, $p < 0.01$). In contrast, a lower (25 IU/kg) and a higher dose (100 IU/kg) of encapsulated insulin slightly reduced glycemia by comparison with the profile observed after oral administration of

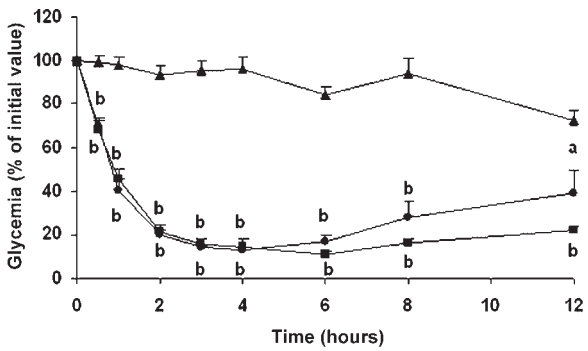

Figure 2. Profiles of glycemia after a subcutaneous administration of free aspart-insulin (squares), insulin-loaded nanoparticles (circles), or saline (triangles) in fasted diabetic rats. The administrated dose of insulin was 10 IU/kg. Before the injections, glycemia was 324 ± 26 mg/dL. Results are expressed as means ± SEM SEM ($n = 6$–8 animals per group). Statistically different from saline (a) $p < 0.05$; (b) $p < 0.001$.

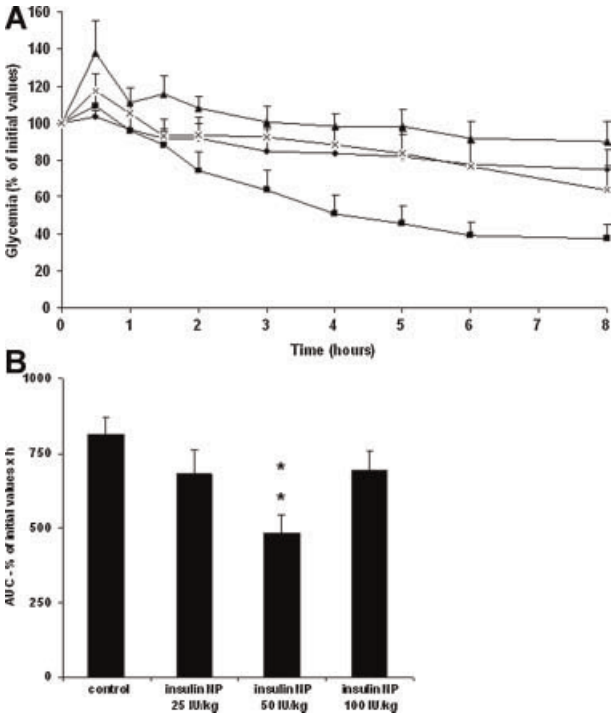

Figure 3. (A) Profiles of glycemia after an oral administration of aspart-insulin-loaded nanoparticles at the dose of 25 IU/kg (diamonds), 50 IU/kg (squares), and 100 IU/kg (crosses) or empty nanoparticles as controls (triangles) in fasted diabetic rats. Before the oral administrations, glycemia was 206 ± 16 mg/dL. Results are expressed as means ± SEM of 8 animals per group. (B) Areas under the curves (0–8 h) of each glycemia profile. Statistically different from control $^{**}p < 0.01$.

empty nanoparticles but this decrease was not significant. This was confirmed by the calculation of the areas under the curve (trapezoidal method) over 8 h (Fig. 3B). Indeed, 50 IU/kg insulin-loaded nanoparticles reduced this area by 40% ($p < 0.01$) whereas the two other doses (25 and 100 IU/kg) reduced it only by 16 and 14% (NS), respectively compared to the area obtained with unloaded nanoparticles.

Thus, aspart-insulin formulated with nanoparticles remains biologically active after oral administration to fasted diabetic rats at a dose of 50 IU/kg but lower and higher doses were less efficient.

Effect of Oral Administration of Insulin-Loaded Nanoparticles on an Oral Glucose Tolerance Test (OGTT)

Because insulin-loaded nanoparticles exert a long-term effect on fasted glycemia after oral

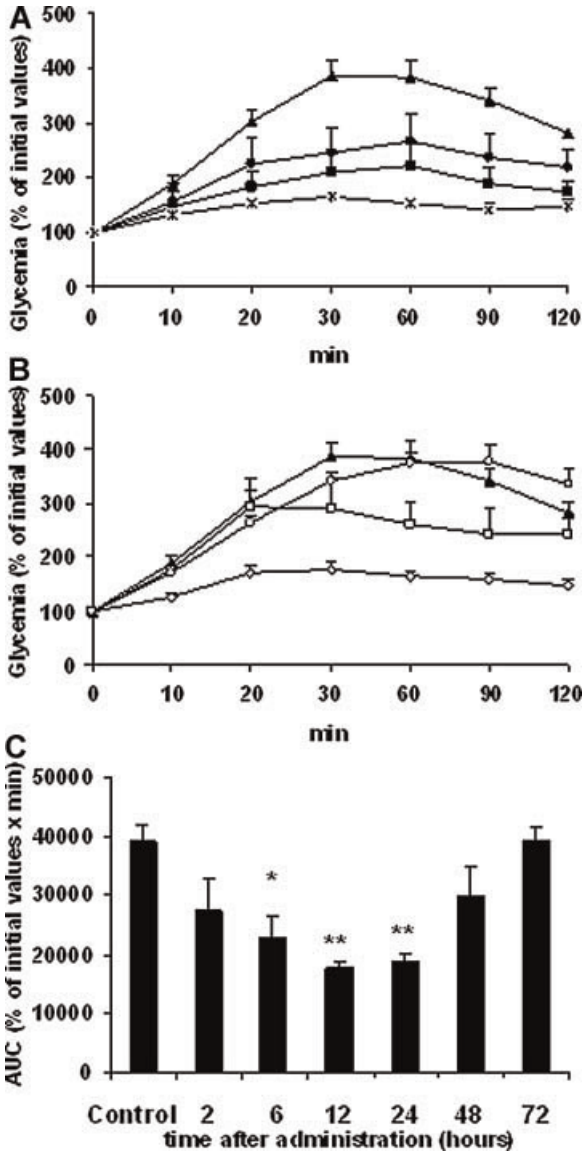

Figure 4. Glycemia after an oral glucose challenge (2 g/kg) performed 2 h (A, full circles), 6 h (A, full squares), 12 h (A, crosses), 24 h (B, empty diamonds), 48 h (B, empty squares), and 72 h (B, empty circles) after a single oral administration of aspart-insulin-loaded nanoparticles (50 IU/kg) or empty nanoparticles for controls (A, B, full triangles) in fasted diabetic rats. (C) Corresponding areas under the glycemia curves (0–120 min). Results are expressed as means ± SEM of 6–8 animals per group except for controls where $n = 23$. Comparisons between control and aspart-insulin-treated animals $^{*}p < 0.05$; $^{**}p < 0.01$.

administration, we investigated the glycemic response to an oral glucose overload (2 g/kg body weight) several hours (from 2 to 72 h) after the single administration of insulin-loaded nanoparticles. As shown in Figure 4 A and B, in control animals receiving empty nanoparticles, oral glucose increased glycemia immediately, reaching a maximal increase (385%, $p < 0.001$) after 30–60 min. Then glycemia decreased slightly but never came back to control values after 120 min.

When the glucose tolerance test was performed after the administration of insulin-loaded nanoparticles, the glycemic profiles were different. When it was performed 2, 6, 12, 24, and 48 h later, glycemia also increased but less than after unloaded nanoparticles intake. Finally, when insulin-loaded nanoparticles were administered 72 h before the glucose tolerance test, the profile of glycemia was identical to that of controls (Fig. 3B). The calculation of the areas under the curves during the 120 min glucose challenge, confirmed these observations and showed that insulin-loaded nanoparticles improved the glycemic response performed 2, 6, 12, 24, and 48 h later by 30% (NS), 41% ($p < 0.05$), 55% ($p < 0.01$), 52% ($p < 0.01$), and 24% (NS), respectively.

When the doses of insulin-loaded nanoparticles were lower (25 IU/kg) or higher (100 IU/kg), the profile of glycemia during an oral glucose challenge did not differ from that observed after empty nanoparticles as control, regardless the time after nanoparticles administration.

Consequently, oral insulin-loaded nanoparticles improved the oral glucose challenge for a prolonged time, with a maximal effect 12–24 h after intake of insulin-loaded nanoparticles at the dose of 50 IU/kg.

Plasma Insulin in Rats Treated Orally With Aspart-Insulin-Loaded Nanoparticles

Plasma insulin responses are illustrated in Figure 5. When insulin-loaded nanoparticles

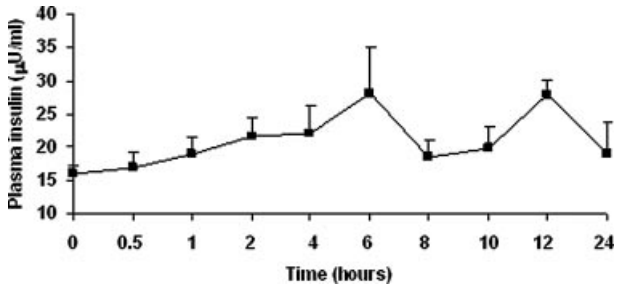

Figure 5. Plasma insulin levels over time after a single administration of aspart-insulin-loaded nanoparticles (50 IU/kg) in fasted diabetic rats. Results are expressed as means ± SEM of five animals.

were administered orally at the dose of 50 IU/kg, plasma insulin level increased, with a first peak value (+75%, $p < 0.05$) 6 h after the administration. Then, insulinemia decreased again slightly and a second peak appeared after 12 h. Finally, insulinemia decreased again but remained slightly over basal values after 24 h.

DISCUSSION

In a previous work,[33] we have shown that nanoparticles prepared with a blend of a biodegradable polyester (poly(ε-caprolactone)) and a polycationic nonbiodegradable acrylic polymer (Eudragit® RS) reduced glycemia in a dose-dependent manner after a single oral administration in diabetic rats and improved the glycemic response to oral glucose ingestion. In this previous study, regular insulin (Actrapid®, Novo Nordisk) was used. In the present study regular insulin was replaced by the insulin analogue, aspart-insulin, in which proline, normally in position B28, was substituted by an aspartic acid residue. This molecular change of human insulin leads to a more rapid absorption and achieves higher plasma insulin concentrations than human soluble insulin following subcutaneous injection in human.[35,36] Thus, it has a faster and more effective glucose-lowering effect, with superior control of postprandial hyperglycemia, compared with human soluble insulin.[35,36]

Physico-Chemical Properties of Nanoparticles

Aspart-insulin-loaded poly(ε-caprolactone)/Eudragit® RS nanoparticles were larger than regular insulin loaded nanoparticles (695 ± 56 nm vs. 331 ± 11 nm) as reported previously.[33] The substitution of proline in position B28 by an aspartic acid could explain this difference. Indeed, the pH_i of aspartic acid is 2.8 whereas that of proline is 6.3. Thus, at a physiological pH, aspart-insulin is more negatively charged than regular insulin. Indeed, in these conditions, the carboxylic acid function of aspartic acid is almost fully ionized and aspartate residue has therefore a negative charge and forms a very polar and hydrophilic radical. It could consequently create electrostatic interactions with Eudragit® RS and modify the arrangement of insulin with polymers. This could explain the larger size of aspart-insulin-loaded nanoparticles compared to regular insulin-loaded nanoparticles.[33] However, the charge surface represented by the zeta potential was quite similar (40.5 ± 0.7 mV vs. 41.8 ± 3.4 mV for regular insulin-loaded nanoparticles).[33] This clearly suggests that insulin is probably located not only at the surface of the nanoparticle but also in its matrix. This hypothesis is supported by the comparison with heparin nanoparticles also composed of poly(ε-caprolactone)/Eudragit® RS.[37] Heparin, a negatively charged glycosaminoglycan, was found by confocal microscopy at the outer surface of negatively charged nanoparticles while empty nanoparticles were positive.[37] Thus, insulin (pI 5.4), which is mainly negatively charged in the external aqueous 0.1% PVA solution (pH 6.5) probably exerts electrostatic interactions with the positively charged polycationic polymer, leading to an overall positive zeta potential of nanoparticles.

The *in vitro* release study of insulin displays a behavior which is also similar to that previously observed with heparin nano- and microparticles.[37] The initial burst seems to be of value regarding the mechanism of absorption of insulin. Indeed, once it reaches the site of absorption, that is, the intestine, the encapsulated drug can be released very rapidly allowing a high drug gradient concentration followed by a good absorption. Since nanoparticles are an aqueous suspension, they will leave the stomach of fasted animals very rapidly and arrive in the duodenum where they will be able to release their cargo.

Subcutaneous Administration of Nanoparticles

In the first set of experiments, both aspart-insulin-loaded nanoparticles and the commercial aspart-insulin solution were subcutaneously administered at the dose of 10 IU/kg. The major objective of this initial study was to make sure that the incorporated aspart-insulin was still biologically efficient after the encapsulation process. Based on the results observed in Figure 2, it can be concluded that, despite many shearing and/or organic solvent stress during manufacturing, the encapsulated insulin is bioactive since there is no *in vivo* difference for the six first hours. The initial sharp fall in glycemia may be related with the observed *in vitro* burst. Later on, from 8 to 12 h, the two profiles of glycemia are different: while that of free aspart-insulin remains stable, that of encapsulated aspart-insulin increases again and tends to return to control values. Although the difference is nonstatistical, this may reflect the slower and incomplete release of insulin from nanoparticles as it was again observed *in vitro* during the release test. Nevertheless, the profile of glycemia

observed after subcutaneous administration of encapsulated aspart-insulin was quite similar to that of encapsulated regular insulin.[33] So it can be concluded that there might not be any advantage in encapsulating aspart-insulin for subcutaneous administration.

Oral Administration of Nanoparticles

Once it was known that the manufacturing process did not alter the encapsulated insulin, aspart-insulin-loaded nanoparticles were administered orally to fasted diabetic rats. They also reduced hyperglycemia for a sustained period, from half an hour postadministration up to more than 8 h (Fig. 3). However, the maximal effect observed after 8 h was more pronounced with aspart-insulin-loaded nanoparticles than with regular insulin-loaded nanoparticles (−52% vs. −24%) at the dose of 50 IU/kg.[33] Thus, encapsulated aspart-insulin was more efficient than regular insulin with respect to oral administration. This might be attributed to a better absorption of the monomeric insulin analogue compared to the hexameric human insulin.

Nevertheless, the most important difference between regular insulin and its analogue was that encapsulated aspart-insulin did not elicit a dose-dependent hypoglycemic effect. Indeed, the maximal effect was observed with 50 IU/kg insulin while lower (25 IU/kg) and higher doses (100 IU/kg) did not show any significant reduction in glycemia. These discrepancies might be explained regarding the mechanisms by which insulin-loaded nanoparticles administered orally elicit their biological response. At first insulin could be protected by the polymeric nanoparticles against proteolytic enzymes in the stomach and gastrointestinal tract.[4,33] Secondly, arriving in the intestine, several possibilities may occur. Insulin-loaded nanoparticles might increase the residence time of insulin in the GIT, especially next to the apical surface of the absorptive cells, reducing the aggression by proteolytic enzymes. This can be explained by the attraction of the electropositive nanoparticles due to Eudragit® RS and the electronegative mucus layer which covers the intestinal epithelium. Indeed, as previously reported,[4,33] when insulin-loaded nanoparticles were labeled with fluorescein isothiocyanate, a thin fluorescent film was observed in close contact with the apical pole of enterocytes. Similar observations were made with another mucoadhesive polymer composed of alginate nanoparticles recovered by chitosan alone[26] or with chitosan, PEG 4000, and albumin.[28] However, insulin either free or formulated with nanoparticles could also be absorbed by a paracellular pathway.[4] Indeed, at the top of intestinal villi, there is a constant physiological desquamation of cells, promoting the uptake of large molecules or particles. Already, in 1987, a paracellular uptake of polyisobutylcyanoacrylate nanocapsules was described in the rat intestine by Aprahamian et al.[39] and confirmed later by Damgé et al.[40,41] and Pinto-Alphandary et al.[42] Finally, insulin-loaded nanoparticles could be also absorbed by transcytosis via Peyer's patches, lymphoid follicles which are the most numerous in the ileum, arriving then in the lymph vessels or underlying capillaries.[4,33]

Insulin could be also released from the nanoparticles in the intestinal lumen in close contact with enterocytes. There, it could be absorbed as a free peptide either by a paracellular pathway or a receptor-mediated pathway.[4] Indeed, the absorption of small fractions of insulin prior to its degradation cannot be discarded. As demonstrated by Bendayan et al.,[43] insulin, formulated with antiproteases and surfactants, reduced glycemia for several hours after introduction in the intestinal lumen of normal and diabetic rats. This effect was explained by a receptor-mediated transport of insulin through the epithelial cells via the Golgi apparatus and intercellular spaces, reaching the blood circulation.[44] Indeed, insulin receptors described on epithelial cells along the intestinal tract[45] could play a role in insulin uptake via a receptor-mediated pathway.[4] Nevertheless, according to the *in vitro* results, the most probable way of uptake of insulin through CaCo 2 cells, an intestinal cell line, seems to be a paracellular route.[46]

Regarding these possible routes of insulin absorption, the higher biological efficacy and duration of action of aspart-insulin-loaded nanoparticles compared to regular insulin nanoparticles may be explained by several hypothesis. At first, aspart-insulin is presented more as a monomeric as an hexameric form, which should be better absorbed by the intestinal mucosa. Secondly, to induce the biological response, insulin binds to receptors also located on its target organs (liver, muscle, adipose tissue). Thus, one hypothesis may be that the insulin analogue has a different affinity with insulin receptors and/or an abnormal signal transduction mechanism. As reported by Kurtzhals et al.,[47] the affinity of

aspart-insulin and human insulin to insulin receptor was quite similar, in accordance with the B26–B30 region being of little importance for insulin-receptor recognition. In contrast, the time course for dissociation of aspart-insulin from the insulin receptor, determined using CHO-hIR cells, a cell line which overexpresses human insulin receptors, was slightly slower when compared to human insulin.[47] This could lead to a saturation of the receptors when the dose of aspart-insulin increases to 100 IU/kg and in consequence a blockage of the binding of insulin molecules. Such a supra-maximal dose-induced blockade of receptors followed by an inhibition of the biological effect has already been reported for cholecystokinin octapeptide.[48] The slower insulin receptor off-rate could also contribute to a prolonged effect of oral administered insulin-loaded nanoparticles (50 IU/kg) on OGTTs, due to a longer occupation of the receptors. As illustrated on Figure 4, aspart-insulin-loaded nanoparticles improved considerably glucose tolerance from 6 up to 24 h at least after oral administration. In contrast, regular insulin-loaded nanoparticles exerted a similar effect though of lesser intensity only after 4 and 8 h. This is in agreement with a better postprandial control of glycemia by insulin analogues compared with soluble human insulin.[35,36] In the present study we have also noted two peaks in plasma insulin concentrations, one after 6 h and another after 12 h which could be attributed to the release of insulin from nanoparticles during the process of degradation. These peaks of plasma insulin concentrations could also contribute to the prolonged effect of aspart-insulin on OGTTs.

In conclusion, this study shows for the first time, that by modifying the configuration of the insulin molecule, it is possible to considerably improve the biological actions of oral insulin formulated with a blend of poly(ε-caprolactone) and Eudragit® RS nanoparticles. These results also confirm that our formulation may be applied, in preference, to the treatment of type 2 diabetic patients who respond not enough to oral anti-diabetic agents and who need a long-acting insulin for a correct control of glycemia.

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