Oral Delivery of Insulin via Polyethylene Imine-based Nanoparticles for Colonic Release Allows Glycemic Control in Diabetic Rats.

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Abstract

In this study, insulin-containing nanoparticles were loaded into pellet cores and orally administered to diabetic rats. Polyethylene imine-based nanoparticles, either placebo or loaded with insulin, were incorporated by extrusion and spheronization technology into cores that were subsequently coated with three overlapping layers and a gastroresistant film. The starting and coated systems were evaluated *in vitro* for their physico-technololgical characteristics, as well as disintegration and release performance. Nanoparticles-loaded cores showed homogeneous particle size distribution and shape. When a superdisintegrant and a soluble diluent were included in the composition enhanced disintegration and release performance were observed. The selected formulations, coated either with

enteric or three-layer films, showed gastroresistant and release delayed behavior *in vitro*, respectively. The most promising formulations were finally tested for their hypoglycemic effect in diabetic rats. Only the nanoformulations loaded into the three-layer pellets were able to induce a significant hypoglycemic activity in diabetic rats. Only the nanoformulation loaded into the three-layer pellets was able to induce a significant hypoglycemic activity in diabetic rats. Our results suggest that this efficient activity could be attributed to a retarded release of insulin into the distal intestine, characterized by relatively low proteolytic activity and optimal absorption.

Keywords: polymeric nanoparticles; multiple-unit formulation; insulin release; peptide oral delivery; Type 1 diabetes.

1. Introduction

Current therapy for diabetes mellitus relies on a correct diet, physical exercise, and oral hypoglycemic agents [1]. In case of disease progression or in Type 1 diabetes, insufficient insulin secretion or inadequate activity need to be considered. Therefore, replacement with exogenous insulin becomes mandatory for survival [1,2]. Unfortunately, nowadays the only administration route available for insulin is parenteral which implies one or more daily injections, with a significant reduction in quality of life and, sometimes, poor patient adherence to therapy [3,4]. Moreover, administration of insulin by subcutaneous injection may induce peripheral hyperinsulinaemia and portal hypoinsulinaemia. Under normal conditions, half of insulin produced by the pancreas is used for liver metabolism via the portal circulation, resulting in fine regulation of blood glucose levels and adequate metabolism of carbohydrates and proteins [2]. Thus, several research studies have been focused on the development of novel formulations of the hormone through alternative routes of administration [5]. Particularly, the oral route has been considered as possibly leading to a better glucose regulation exploiting the liver first-pass metabolism of insulin, thus preventing the risks of fluctuating blood glucose levels and possibly the resulting morbidity due to chronic microvascular complications [6]. Therefore, an oral formulation of insulin could revolutionize the management of insulin-dependent diabetic patients due to its potential clinical benefits. However, the oral bioavailability of insulin is very low and several efforts have been attempted to promote insulin bowel absorption, avoiding gastric or intestinal degradation by proteases.

Such attempts included formulations with protease inhibitors and/or absorption enhancers or mucoadhesive systems. Still, oral delivery of insulin remains an unmet need [3]. As a result of this, colonic delivery and release of insulin has gained increasing interest by researchers because of the longer transit time, prolonged localization of insulin on the gut mucosa, lower levels of proteases in the colon or mucosal P-glycoprotein and greater responsiveness to permeation enhancers compared to the more proximal regions of the gastrointestinal tract. Such features would point to the colon as an interesting target for insulin oral delivery but, so far, an efficient drug delivery system for this propose is still missing [3,6,7-9].

A few attempts to improve the oral delivery of insulin by means of nanoparticle-based vectors have been reported [10-12]. Several nanoparticle (NP) types have been designed to protect biological drugs, including insulin, against chemical and enzymatic degradation and to enhance the intestinal absorption through paracellular and transcellular pathways [10,11]. Structural characteristics of nanoparticles, including size and surface charge, have been shown to influence the insulin absorption by the enterocytes. In general, small particles, provided with a positive charge are absorbed more efficiently through the intestinal epithelium. This is due to the interaction of NPs bearing positive charges with mucin residues that are negatively charged at physiological pH. The consequent prolonged residence time and increased concentration gradient at the surface of the intestinal mucosa might therefore promote protein absorption [3,11]. *Ex-vivo* and *in vivo* studies have also proven the potential of colloidal nanoparticles in increasing insulin absorption throughout the colonic region, but the lack of an appropriate delivery system that ensures their safe transit through the upper gastrointestinal tract strongly limits their usefulness [12,13]. Therefore, a solid dosage form, including pellets and tablets, which could host drug-loaded nanoparticles and possibly undergo a subsequent coating process, might represent a valuable strategy to enhance stability and provide release versatility of these colloidal systems administered via the oral route [6].

The objective of the present study was to prepare, characterize and evaluate. both *in vitro* and *in vivo,* a novel nanoformulated, multiple-unit colon release system, i.e. coated pellets, as a possible oral nanocarrier for insulin. The novelty of this approach was the evaluation of the synergistic effect of colon release, muco-adhesive nanoparticles and the presence of a permeation enhancer, sodium glycocholate. The proposed multi-

approach strategy combines the well-known benefits of this multiple-unit formulation in terms of reproducible transit time through the gastrointestinal tract, the consequent absorption pattern with the advantages of colloidal nanoparticles [14]. For this purpose, a recently proposed three-layer release technology platform was applied, consisting of a flexible film composed of a neutral polymethacrylate Eudragit® NE and a superdisintegrant sodium starch glycolate Explotab®, added as a pore former, applied to a hydroxypropyl methylcellulose (HPMC) coating of reduced thickness in order to improve the efficiency of the erodible layer in delaying the drug liberation [14-16]. An outer gastroresistant layer was also added in order to neutralize the variable residence time in the stomach of the coated dosage form and allow its activation only following the entry into the duodenum. This time-dependent relies on the relative consistency of short intestinal transit time, the subsequent colon targeting and favoring the intestinal absorption of insulin at that level [17].

2. Materials and Methods

2.1. Materials:

Bovine insulin (MW 5,734 Da), polyethylene imine (MW 750 kDa), dextran sulfate (MW > 500 kDa), zinc sulfate, streptozotocin (STZ) and cellulose ester dialysis membrane tubing with a molecular weight cut-off (MWCO) of 1,000,000 Da (Spectra/Por® Biotech CE) were purchased from Sigma-Aldrich (St Louis, MO, US). All chemicals were used as received without further purification. Lactose was obtained from Prodotti Gianni (Milan, Italy). Microcrystalline cellulose co-processed with sodium carboxymethyl cellulose (Avicel® CL611) and hydroxypropyl methyl cellulose acetate succinate (Aqoat® LG, HPMCAS) were gifts from FMC Europe (Brussels, Belgium, distributed by IMCD Italia, Milan, Italy) and from Shin-Etsu (Tokyo, Japan, distributed by Seppic, Milan, Italy), respectively. Hydroxypropyl methylcellulose (Methocel® E50, HPMC) was kindly donated by Colorcon (Milano, Italy). Poly(ethylacrylate, methylmethacrylate) (2:1 monomer molar ratio) as 30% v:w aqueous dispersion (Eudragit® NE 30 D) of Evonik Röhm (Darmstadt, Germany) was a kind gift of Rofarma (Gaggiano, Italy). Polyethylene glycol (PEG 400) and size 4 hard-gelatin capsules were purchased from ACEF (Fiorenzuola D'Arda, Italy). Sodium glycocholate (NaGly) was obtained from and Tokyo Chemical Industry (Tokyo, Japan). Sodium starch glycolate (Explotab® CLV) was a gift from JRS Rettenmaier Italia (Castenedolo, Italy).

2.2. Synthesis of insulin-containing nanoparticles (nanoformulated insulin, NI)

22.1 mL of insulin solution (10 mg/mL in 0.01 M HCl), 10.8 mL of a 10% w/V dextran sulfate (DS) solution and 18.0 mL of 10 mM tris buffer, pH 9, were added under stirring (500 rpm) to 12.6 mL of polyethylene imine (PEI) solution (25 % w/V). Afterwards, the mixture was heated at 40 °C and maintained under stirring because of its high viscosity and 7.8 mL zinc sulfate solution (2 M) were added, dropwise. As a result of the addition of the stabilizer, the formation of the nanoparticles took place and the viscosity of the solution decreased. The product was stirred for 15 minutes at 40 °C. The product was finally dialyzed in Milli-Q® water with cellulose ester dialysis membrane tubing with a molecular weight cut-off (MWCO) of 1,000,000 Da.

2.3. Synthesis of placebo nanoparticles (NPs)

22.1 mL of HCl 0.01 M, 10.8 mL of a 10% w/V DS solution and 18.0 mL of 10 mM tris buffer, pH 9, were added under stirring (500 rpm) to 12.6 mL of polyethylene imine (PEI) solution (25 % w/V) and then treated as described above.

2.4. Nanoparticle characterization

2.4.1. Dynamic light scattering (DLS) and zeta potential measurements

The mean diameter and surface charge of the nanoparticles were assessed with a Zetasizer Nano ZS ZEN3600 from Malvern Instruments Ltd (Worcestershire, United Kingdom) operating at a light source wavelength of 633 nm and a fixed scattering angle of 173°. The sample concentration was chosen to keep attenuator values between 7-9. The refractive index of material was 1.524. The measurements were performed in triplicate, after dilution of the nanoparticles respectively with MilliQ® water and aqueous solution of sodium chloride (1 mM).

2.4.2. Transmission Electron Microscopy (TEM) analysis

Nanoparticles were visualized using 120 keV TEM (Jeol 1010, Tokyo, Japan). Two microliters of the sample, along with 2% w:V uranyl acetate solution, were deposited onto a piece of ultrathin 200-mesh copper grid (Ted-pella, Redding, CA, US) and left to dry in air before examination by TEM.

2.4.3. Determination of the entrapment efficiency (EE%) of insulin into the nanoparticles

The amount of insulin encapsulated into the nanoparticles was determined suspending the NI, corresponding to 30 μg/mL theoretical insulin concentration, in 0.05 M HCl and centrifuging at 12000 ×g for 20 min (ScanSpeed 1730R,

Labogene, Lynge, Denmark). After centrifugation, the amount of insulin in supernatant was measured by a reverse-phase, high-performance liquid chromatography (RP-HPLC) analysis with a method reported in Eur. Pharm. 8th Ed. for insulin and its degradation product A21-desamido insulin (A21) quantitation that was previously set-up [18]. Insulin quantitation was performed using a freshly prepared standard having identical insulin concentration and analyzed the same day of nanoparticles. The EE % is expressed as ratio percentage between the insulin amount in the suspension and the theoretical value of the insulin added.

2.4.4. In vitro insulin release from NI

In vitro release was assessed following incubation of the nanoparticle suspension containing theoretically 0.35 mg of insulin in 10 mL of 0.095 M phosphate buffer pH 6.8 prepared as indicatedin the Eur. Pharm. 8th Ed. at 37 °C under stirring (MIX15eco, 2mag, München, Germany; 600 rpm). Bovine albumin (0.2% w/V) was added to the dissolution medium to avoid non-specific adsorption of insulin to glass surface. At pre-determined time intervals, 1 mL of medium was withdrawn, filtered by 0.2 μm polyethersulfone (PES) membrane (VWR) and acidified with 40 μL of 1 N HCl before the analysis by RP-HPLC as previously described. Dissolution studies were performed in triplicate.

2.5. Preparation and coating of cores

All cores were prepared by extrusion and spheronization (E-S) process.

Powders (9.2 g) were mixed in a mortar for 5 min. Nanoparticle suspensions, either placebo or loaded with insulin, (70 g) were then added in small aliquots to the powder blend under continuous mixing, in the same mortar, over a total period of 8 h; during that time the most of water could evaporate to allow the moisture content suitable to the next step. The room was maintained at 30 °C with 25% relative humidity. The resulting wetted mass was extruded through an 850 μm sieve. Spheronization was performed in a spheronizer (Nica™ S320, GEA, Düsseldorf, Germany) with a cross-hatched plate (400 rpm, 5 min). Pellets cores were finally dried in a static oven at 40 °C for 24 h. As a reference, a pellet formulation containing the non-encapsulated peptide was prepared by mixing insulin powder with Avicel® CL611, NaGly, lactose and Explotab® CLV (9.4 g) and adding deionized water (3.5 g). The E-S was then performed as for nanoparticles loaded formulations.

In order to obtain three-layer colonic systems, pellets were coated in a fluid bed (GCPG 1.1, Glatt®, Binzen, Germany). The following formulations were in turn sprayed: i) a hydro-alcoholic solution (1:9 w/w, water/ethanol) of Methocel® E50 (5% w/w) and PEG 400 (0.5% w/w) (10 g/min spray rate), ii) an aqueous suspension of Eudragit® NE 30D-Explotab® CLV (20% *vs.* solid Eudragit® NE) (1.4 g/min) and iii) a hydro-alcoholic solution (23% w/w, water/ethanol) of Aqoat® (5.8% w/w) (8 g/min). In the case of the coatings with the hydro-alcoholic solutions of both Methocel® E50 and Aqoat® the rotor insert was employed while for Eudragit® NE-based suspension a Wurster process was applied. In the case of gastroresistant formulations, pellets were directly coated with the hydro-alcoholic solution of Aqoat®, as previously described. The operating conditions for all coatings were: inlet air temperature, 40 °C; product temperature, 33-35 °C; airflow rate, 70 m^3/h; nozzle diameter, 1.2 mm; spray pressure, 2.0 bar; final drying time at 40 °C, 5 min. A curing step in a static oven at 40 °C was performed on intermediate systems coated with Eudragit® NE-Explotab® CLV and final enteric formulations for 24 h and 2 h, respectively. In all process steps, placebo mini-tablets (weight 10.5 mg, diameter 2.5 mm, height 2.3 mm) were added in the process chamber to reach the minimum capacity of the fluid bed.

2.6. Pellets characterization

2.6.1. Particle size and shape analyses

Particle size distribution of the pellets was determined by a set of analytical sieves (500, 600, 710, 850, 1000, 1180, 1400, 1700, 2000 µm) piled in a sieve shaker (Endecotts, Octagon 200, London, United Kingdom) operated for 5 min at an amplitude of 4. Mean diameter and aspect ratio were determined using an image analysis system. Digital photomicrographs (n=30, DinoCapture, Hsinchu, Taiwan) were analyzed by ImageJ software (Version 1.48, 19 April 2014, National Institute of Health, Bethesda, MD, US) that allow calculation of pellets dimensional and shape descriptors. The aspect ratio (AR) was calculated as follows:

$$Aspect\ ratio\ (AR) = \frac{Major\ Axis}{Minor\ Axis}$$

AR indicates the particle's fitted ellipse.

Yield % was calculated as ratio between the mass of obtained cores (500-2000 µm range) and the weighed amount of excipients.

2.6.2. Disintegration test

Disintegration test was performed in a dissolution apparatus 2: 300 mg of pellets in the 850-1000 µm range were poured in a vessel containing 1000 mL of pH 6.8 phosphate buffer (37.0 ± 0.5 °C), and stirred at 100 rpm for 30 min. Then, pellets retained by a 400 µm net were dried (40 °C, 24 h) and weighted; finally, the percentage of mass loss was calculated [19].

2.6.3. Insulin assay and in vitro release

In order to assess insulin content, approximately 10 mg of cores of pellets, exactly weighed, were added to 0.05 M HCl, stirred at 350 rpm for 10 min, sonicated for 10 min and centrifuged (12000 rpm, 20 min). The supernatant was analyzed by RP-HPLC analysis as described in section 2.4.3. *In vitro* release from nanoparticles, uncoated cores and coated pellets was assessed following incubation in 10 mL of phosphate buffer (pH = 6.8) containing 0.2 % bovine albumin at 37 °C under stirring. At pre-determined time-points, 1 mL of medium was withdrawn and replaced with fresh buffer solution, centrifuged and analyzed by RP-HPLC as previously described in section 2.4.3. In the case of three-layer and gastroresistant formulations, coated pellets were tested in HCl 0.1 M for 2 h prior to the buffer stage.

2.6.4. Re-dispersion studies

25 mg of cores (PNI02) were incubated in 5 mL of 0.1 M HCl at 37 °C under stirring (600 rpm) for 5 hours. After incubation, an aliquot (2 mL) was filtered with 0.45 µm membrane and analyzed by DLS. The choice of the medium is occurred by checking the non-influence of other excipients in analysis result and the stability of the nanoparticles in this solution. The study was conducted in triplicate.

2.7. In vivo studies

2.7.1. Diabetic rat model

For the *in vivo* experiments, Sprague Dawley rats, purchased by Charles River Laboratories (Calco, Italy), were maintained in a fully-equipped facility in appropriate cages and provided with a proper environment. Diabetes was induced by two intraperitoneal injections of 50 mg/kg streptozotocin diluted in 0.1 M sodium citrate buffer pH 4.5 (one injection per week for two weeks). Seven days after the first administration, all rats were weighted and glycaemia was measured by puncturing the tail vein with an 18-gauge needle and collecting the blood droplet in the test strip of a blood glucose meter (Contour Link, Bayer, Milan, Italy). After the first injection, around 40 % of treated rats developed glycemic values over 350 mg/dL, indicative of diabetes onset [20]. All animals were treated again and, after

further seven days, we obtained 75-80 % of stably diabetic rats (glucose concentration ranging between 350 and 600 mg/dL).

2.7.2. Administration of insulin formulations to diabetic rats

Three groups of diabetic rats have been in turn injected subcutaneously with a solution of free insulin (n=3, 0.07 mg/kg in phosphate buffer pH 6.8), and orally by gavage with a peptide solution (n=5, 1.33 mg/kg in 10^{-3} M HCl) as well as with a nanoparticle formulation (n=6, NI, 1.33 mg/kg). All other rats have undergone a surgical gastrostomy, upon anesthetization by intraperitoneal injection of 500 mg/kg of Avertin, for the insertion into the stomach of one capsule containing pellets formulations loaded with insulin (1.33 mg/kg): GPNI (n=5, 53 mg), GPI (n=3, 55 mg) CPNI (n=4, 168 mg), CPI (n=4, 63 mg). After insertion of the capsule, the stomach was sutured with 4-0 resorbable running suture, and the abdomen closed by 2-0 silk interrupted stitches. The gastrostomy procedure was chosen due to the impossibility to obtain a suitable oral administration of the free pellets in aqueous solution: low stability and high electrostatic interaction with the gavage tube were observed. One group of 5 rats was left untreated. The blood glucose levels were monitored by collecting blood droplets as described above at 1, 2, 3, 4, 5, 6, 7, 8, and 48 h post-treatment. Rats were used in accordance with an experimental protocol subjected to the direct approval of the Italian Ministry of Health.

3. Results

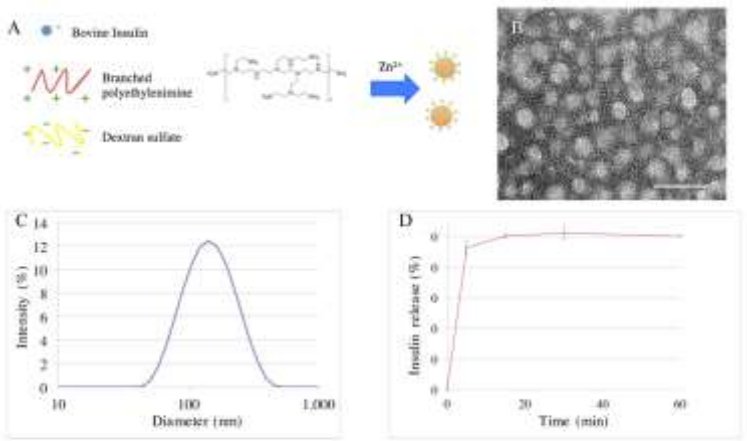
3.1. Nanoparticle synthesis and characterization.

Insulin-loaded polymeric nanoparticles were synthesized according to previously published protocols with some modifications with the aim of scaling up the amount of synthesized nanoparticles and reduce the amount of excipients to include in the final pellet formulation [21,22]. Briefly, insulin and dextran sulfate solutions were added to a PEI solution under heating at 40 °C. The driving force for the formation of the nanoparticles was the opposite charges of PEI and dextran sulfate resulting in the insulin entrapment into the polymeric matrix. The weight ratio between the two polymers was optimized in order to control the particle size and zinc sulfate solution was added dropwise to stabilize the nanoparticles (Fig. 1A). The resulting insulin-containing nanoparticles (NI) were washed by dialysis against deionized water that was chosen as the dialysis medium

instead of recommended 5% (w/v) mannitol solution [21]. The amount of NI in the final dialyzed product was 72% of the starting amount. Compared to previously reported preparation, bulky stabilizer exclusion, along with the 5-fold increase of insulin loading, resulted in a 35-fold reduction of the material amount required to form the pellets [21,22]. Furthermore, added solutions were at the minimum volumes needed to ensure the reagents solubility. With this adjustment, concentrated colloidal suspension was obtained, more suitable for following pellets inclusion.

Transmission electron microscopy (TEM) images of the negatively stained NI (Fig. 1B) showed spherical and nearly monodisperse 30 nm nanoparticles. However, the mean hydrodynamic size of NI as determined by DLS was 177.1 ± 12.3 nm, probably due to the high solvation efficiency of the polymeric matrix, and the zeta potential was +28.9 ± 0.5 mV. The entrapment efficiency (EE%) determined after extraction in acidic solution and measured by RP-HPLC was 62.3 ± 3.5%, while the % of the degradation product A21-desamido insulin was 1.2 ± 0.1%. Although slightly lower than previous reports, EE% was in the range of interest to conduct the subsequent loading steps. In addition, the A21% levels below the limits reported in the bovine insulin monograph of the Pharm. Eur. 8th Ed. confirmed that no major hydrolytic degradation had occurred during the preparation of colloidal nanoparticles.

Insulin nanoparticles subjected to release test showed a fast peptide dissolution at pH 6.8 (over 90% within 5 min). This behavior is consistent with that of similar nanoparticles reported in the literature [21].



Diameter (nm) - intensity	177.1 ± 12.3 PDI 0.244 ± 0.023
ζ-potential (mV)	28.9 ± 0.45
Entrapment efficiency (%)	62.3 ± 3.5

Figure 1. Schematic representation of NI synthesis (A). TEM image of NI, scale bar: 100 nm (B). Hydrodynamic size of NI measured by DLS (C). Dissolution profile of NI at 6.8 pH (mean ± SD, n = 3) (D).

3.2. Core preparation and characterization

Table 1. Compositions of cores under investigation.

	% (w/w)						
Formulation	Insulin	NI*	Placebo NPs*	Avicel® CL611	Sodium glycocholate (NaGly)	Lactose	Explotab® CLV
PN01	---	---	50.0	50.0	---	---	---
PN02	---	---	50.0	26.4	---	15.7	7.9
PNI01	---	43.2	---	43.2	13.6	---	---
PNI02	---	43.2	---	22.7	13.6	13.8	6.7
PI02	2.3	---	---	39.1	23.4	23.7	11.5

* the percentage refers to the solid nanoparticles

In order to achieve a prompt release of the nanoparticles at the desired site, an appropriate core formulation was designed. A cellulose derivative, Avicel® CL611, a co-processed microcrystalline cellulose and sodium carboxymethyl cellulose, was identified as suitable spheronization agent due to the well-known advantages in terms of improved disintegration and dissolution performance over the traditional formulations based on microcrystalline cellulose [19,23]. Moreover, a superdisintegrant agent (Explotab® CLV) and a soluble diluent (lactose) were added in an alternative formulation in order to further improve the pursued fast-disintegration properties (Table 1). Only in the case of pellets loaded with NI, sodium glycocholate (NaGly), an absorption enhancer with protease inhibition properties, was considered [24,25]. The latter functional agent was indeed demonstrated to promote the oral absorption of a pancreatic hormone in rats administered with a minitablet formulation at 1:10 protein/adjuvant ratio [26]. In addition, NaGly, due to its high solubility in aqueous media, could help to prevent the undesired matrix formation when pellets interact with water, thus possibly aiding their disintegration. Based on preliminary extrusion and spheronization trials, a 1:1 Avicel® CL611/placebo nanoparticles binary mixture was acknowledged as starting formulation suitable to lead to a dough with

appropriate plasticity and pellets with adequate morphological characteristics (Table 1). When insulin-containing formulations or all other systems containing Explotab® CLV and lactose were used, the process appeared facilitated probably due to the concomitant decrease of nanoparticle amount and increase in the total solid mass attributable to the presence of the NaGly powder. In all cases, the possible issue associated with the large amount of colloidal suspension needed to obtain the required insulin dose for the *in vivo* studies, i.e. 4:1 w/w water vs. total solid mass, was overcome by adding the liquid in small aliquots and allowing the subsequent evaporation that limited the moisture content of the extrudable mass.

Table 2. Physico-chemical properties and process yield of cores prepared by extrusion and spheronization.

Batch	d_{geo} (µm)	σ_{geo} (µm)	AR	Yield (%)	Insulin recovery %	A21%
PN01	913	1.18	1.36 ± 0.18	59.9	-	-
PN02	804	1.21	1.38 ± 0.20	40.1	-	-
PNI01	809	1.21	1.21 ± 0.18	41.3	102.1 ± 1.6	1.8 ± 0.2
PNI02	1137	1.28	1.16 ± 0.10	61.9	88.8 ± 3.7	0.9 ± 0.3
PI02	1066	1.16	1.29 ± 0.15	74.4	91.8 ± 1.1	2.2 ± 0.3

All the process yields were > 40% and, taking into account the low amounts of processed material, were considered satisfying. All batches showed a mean particle size comprised within the desired 710-1400 µm range and a nearly spherical shape (Table 2, Fig. 2). Only in the case of formulation batches PNI02 and PI02 a slightly higher mean diameter was observed. No differences in size or shape were observed between placebo and insulin-loaded pellets, which was not surprising considering that the final core product structure was not significantly affected by the low amount of protein payload within the colloidal suspension.

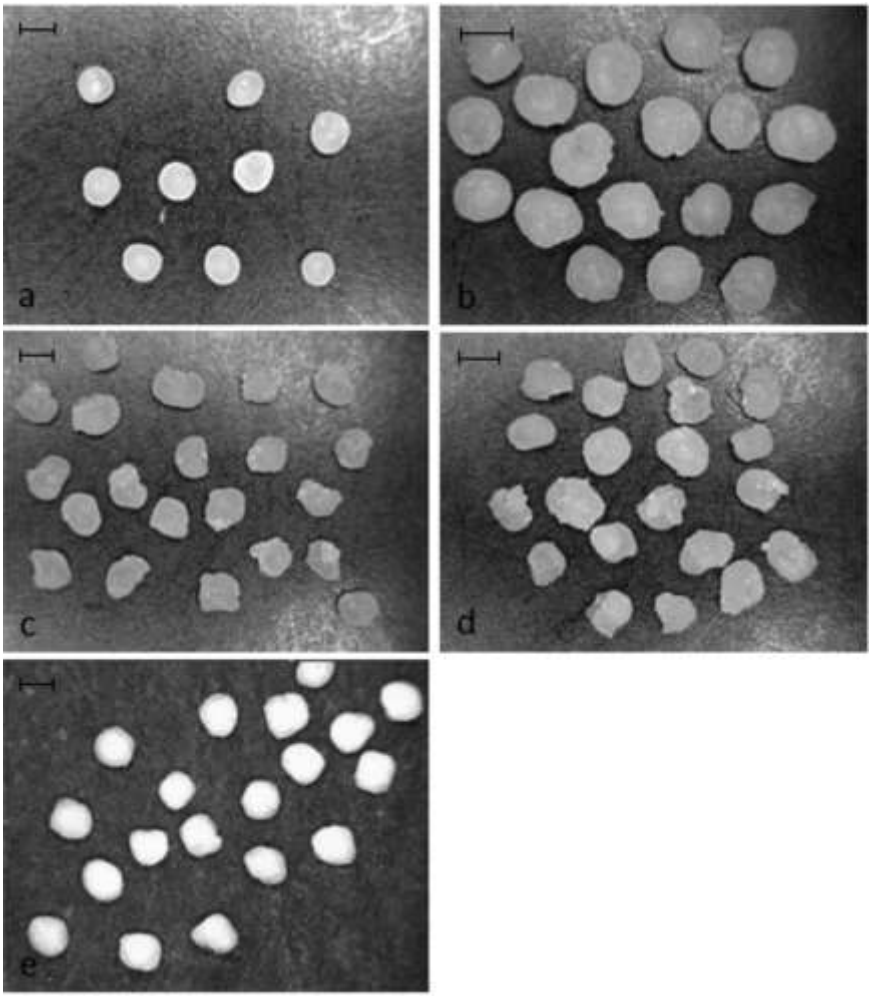


Figure 2. Digital photographs of core formulation under investigation: a) PN01, b) PN02, c) PNI01, d) PNI02 and e) PI02. Scale bar: 1 mm.

The protein content in the pellets was ≥ 90% of the theoretical amount and, despite the long wetting and evaporation phases, the % of A21 remained below the limits indicated in the monograph of bovine insulin reported of Pharm. Eur. 8th Ed., *i.e.* 3%. The overall results suggested that extrusion and spheronization might be a promising technology for loading a nanoparticle suspension into pellets.

3.2.1. In vitro studies on cores

Pellets containing the insulin-loaded nanoparticles were subjected to *in vitro* studies aiming to assess the disintegration and insulin release performances from the two different formulations.

PNI02 formulation showed enhanced disintegration compared to PNI01, i.e. 26.4 vs. 10.8%. This behavior could be ascribed to the presence of the superdisintegrant Explotab® and that of lactose acting as pore former compared to the formulation where the excipients were only Avicel® CL611. However, comparing such performance with that of a reference formulation containing paracetamol as analytical tracer instead of insulin nanoparticles and Avicel® CL611/ lactose / Explotab® CLV in the same ratios as PNI02 formulation, a ≥ 75% disintegration was obtained. It can be thus inferred that the presence of nanoparticles considerably contributed to the formation of a slow-eroding matrix.

To confirm this result, re-dispersion studies were performed on batch PNI02, which appeared the most promising system from *in vitro* disintegration and dissolution tests. These studies were set up in order to evaluate the presence of NI after exposure of the solid dosage form to aqueous fluids. Samples of this medium were collected and analyzed by DLS.

Nanoparticles with a mean diameter of 146.1 ± 1.0 nm (n=3) were detected. Comparing the size of NI, before and after E-S process, it was noticed that the mean diameter was slightly lower than the initial value (177.1 ± 12.3 nm), although the size distribution appeared to be maintained as evaluated by DLS polydispersity index (0.296 ± 0.005). To figure out the cause of this reduction, the same test was performed on 25 mg of a dried dough obtained by using the same components and operating conditions of the cores of PNI02 batch avoiding extrusion and spheronization steps. Also in this case, a decrease of mean diameter occurred, suggesting that it may depend on the mechanical stress during the dough formation. It can be thus inferred that cores under investigation might convey and liberate the colloidal system upon exposure to aqueous media.

3.2.2. Preparation and characterization of coated pellets

Pellets batches PNI02 and PI02 were selected as core formulations for preliminary *in vivo* testing on the basis of the *in vitro* results in terms of enhanced disintegration and dissolution properties. The cores were therefore coated either with a hydroalcoholic solution of Aqoat® or in turn with a Methocel® E50-, Eudragit® NE / Explotab® CLV- and Aqoat®- based formulations in order to prepare gastroresistant (G) and three-layer colonic (C) systems, respectively (Table 3). In the three-layer system, the hydrophilic layer based on Methocel® E50

was demonstrated to delay the drug release by a swelling / erosion mechanism, while the Eudragit® NE / Explotab® CLV film was aimed at prolonging the duration of the lag phase as imparted by the underlying HPMC coat. Finally, an outer gastroresistant film was added to the system to overcome the unpredictable gastric residence time, of the system thus allowing its activation only at the duodenum and the consequent colonic release based on a time-dependent approach [17,26].

The adopted process operative conditions and the coating levels needed to achieve gastroresistance or a delayed release after a lag-phase suitable for colonic delivery were previously set up with an analogous formulation containing paracetamol as analytical tracer (data not shown). In particular, gastroresistance criteria were accomplished with an Aqoat® coating level of 12-13 mg/cm^2. When using colonic systems, an *in vitro* lag time of approximately 60 min corresponded to 85-90 mg/cm^2 of Methocel® E50, 3.0-4.0 mg/cm^2 of Eudragit® NE-Explotab® CLV 20% and 12-13 mg/cm^2 of Aqoat® [26]. Particularly, a similar multiple-unit system with an *in vitro* lag phase of the latter duration showed an insulin peak in rats and a corresponding drop in blood glucose levels 6 h post-dose following oral administration. Based on typical gastrointestinal transit times reported in the literature for rats, the delivery system, after this lag time, was expected to be able to arrive, mostly intact, to the ileo-colonic region [27].

Table 3. Physico-technological characteristics of gastroresistant and three-layer colonic pellets.

Formulations	d_m (µm)	SD (µm)	AR	Methocel® E50 mg/cm^2	Eudragit® NE-Explotab® CLV 20% mg/cm^2	Aqoat® mg/cm^2
GPNI	1355	114	1.14	-	-	13.1
CPNI	1865	102	1.07	89.9	4.0	13.0
GPI	1392	121	1.25	-	-	11.9
CPI	2070	149	1.24	85.1	3.1	12.5

All coated batches showed a mean particle size in the 1.3-2.1 mm range and coating levels in line with what expected based on preliminary set up results. The shape was closer to roundness than the starting cores, as a result of the subsequent coating steps.

When subjected to *in vitro* release tests all formulation met gastroresistance criteria (< 10% release after 2h in 0.1 M HCl), albeit the release test was conducted under more vigorous stirring, performed by magnetic stirrer, compared to the compendial method (Fig. 3).

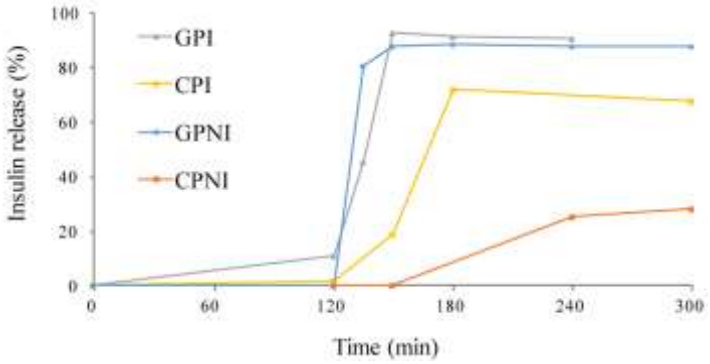


Figure 3. *In vitro* release of coated pellets

When exposed to a pH 6.8 buffer, all enteric systems showed a prompt release due to core disintegration following dissolution of the enteric coat. The three-layer colonic systems containing the NI showed a lag-phase preceding the onset of release. The time corresponding to 10% release, $t_{10\%}$, was 65 min. Moreover, the CPI pellet formulation used as referenceshowed a shorter lag time that was demonstrated to be caused by the adopted release testing conditions (data not show).

3.3. In vivo hypoglycemic effect

The NI loaded into the pellets were tested *in vivo* in diabetic rats. The rat model was chosen since the gastrointestinal transit time is comparable to that of humans [28]. The animals that reached stable glucose values in blood of at least 350 mg/dL following streptozotocin administration were subjected to one of the following treatments: 1) oral administration of 1.33 mg/kg insulin (OI) or as NI, 2) subcutaneous injection of 0.07 mg/kg insulin (SCI), 3) insertion through gastrostomy of different amounts of pellets in capsules (GPI, GPNI, CPI, CPNI) to allow administration of 1.33 mg/kg. A group of non-treated rats was used as control (NT). Fig. 4 shows the % variation of glycaemia from 1 to 48 h post-treatment. The baseline for each

experimental group was the mean value of blood glucose measured before the treatment, considered as 100%. In the first 8 h post-treatment, physiological oscillation of glucose blood levels was observed in NT rats, which however did not exceed 30% of the initial value. Orally administered insulin, either free or in NI had no significant effect on blood glucose levels, while the subcutaneous injection of insulin caused a rapid and marked reduction of the glycemia to 25% of the initial values at 1 h post-treatment. However, blood glucose levels returned to the initial level when measured after 8 h. The treatment with GPI or GPNI did not show any significant effect on the glucose blood levels as compared to NT during the first 8 hours. In contrast, the rat treatment with CPNI was able to induce an immediate, significant decrease in blood sugar levels as compared to the NT, with a significant fall in glycaemia, as much as 50% of the initial value, at 6 h post-treatment, and a subsequent further decrease of up to 30% at 8 hours. On the other hand, the treatment with CPI did not exert any significant hypoglycemic effect. At longer times (48 h), NT and SCI-, OI-, NI-, and GPI-administered rats had blood glucose equal the initial values, while in rats administered with CPNI the glucose concentration remained at 45% of the starting level and significantly different from those of all other experimental groups. At 48 h post-treatment, a slight decrease of glycaemia was observed upon administration of CPI, while in GPNI-treated rats glucose values became about 70% of initial values and significantly lower than those of controls.

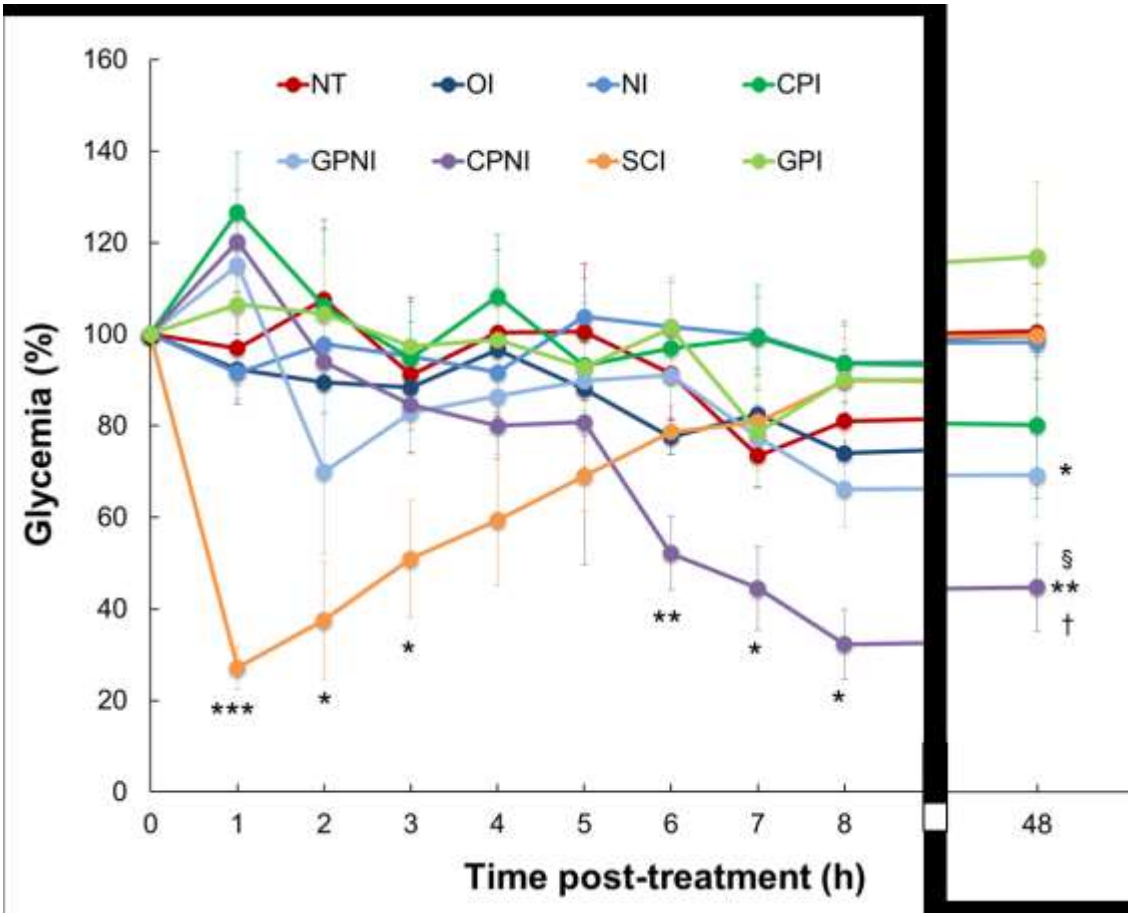


Figure 4. Time course of the i*n vivo* activity of different insulin formulations, expressed as percentage variation of rat blood glucose levels after treatment. Diabetic rats were exposed to oral (OI) or subcutaneous (SCI) administration of insulin, oral administration of insulin nanoparticles (NI), or insertion through gastrostomy of capsules containing gastroresistant pellet-formulated insulin (GPI) or insulin-nanoparticles (GPNI), colon-release pellet-formulated insulin (CPI) or insulin-nanoparticles (CPNI); NT: untreated rats. Mean ± SE of 3-6 animals for group. ***p<0.0005; **p<0.01; *p<0.05 vs NT; §p<0.0005 vs OI, NI, GPI and SCI; †p<0.05 vs GPNI and CPI (one-tailed Student t-test).

4. Discussion

Preparation method of nanoparticles allows a considerable amount of insulin loaded and to a high concentration of nano-suspension: these factors are both favorable for the preparation of NI loaded pellets. Extrusion/spheronization of a formulation containing Avicel® CL611, lactose and Explotab® CLV wetted by nano-suspension leads to cores with technological and biopharmaceutical characteristics suitable for the following coating steps and for allowing a rapid NI release. Finally, in vitro performances of three layers coated pellets pledged a delayed release to promote colon targeting.

Orally administered insulin, either “free” or formulated in nanoparticles (NI) were not effective in reducing blood glucose levels in diabetic rats at 8 h after treatment. That was expected since insulin is rapidly degraded in the stomach. On the other hand, the subcutaneous injection of insulin, as used in clinical practice, caused a rapid and significant hypoglycemic effect with a subsequent gradual return to the initial blood glucose levels at 8 h. Curiously, at 1 h post-treatment all rats treated with the different capsules (GPI, GPNI, CPNI, or CPI) showed a temporary increase in blood glucose concentrations, possibly due to the effect of surgery and anesthesia, which is known to cause stress-related acute hyperglycaemia in fed rats [29]. The treatments with GPI or GPNI, enteric coated pellets which were supposed to release the nanoformulated insulin in small intestine, in the duodenum and the jejunum, did not significantly reduce the blood glucose levels as compared to NT in the first 8 hours ($p>0.05$). This may be due to the degradation of insulin by the high proteolytic activity of pancreatic peptidases but also to the effect of the insulin-degrading enzymes inside the cytosol of the small intestine enterocytes which can internalize the peptide by specific insulin receptors [30,31]. In contrast, the rat treatment with CPNI induced a significant decrease in blood glucose levels as compared to the NT group. The reduction was significant at 6 h post-treatment with as much as 50% of the initial values, with a subsequent further decrease of up to 30% at 8 hours. This result suggested that, unlike the gastroresistant pellet (GP) which releases the insulin into active small intestine, the three-layer colon-release pellet (CP) passed this tract without being degraded and released the NI in the distal part of the small intestine or in the colon. There are several reasons which make the colon as a desirable tract for insulin delivery and release: 1) the enzymatic activity, such as pancreatic endopeptidases, is remarkably lower and most of proteins, including insulin, would be available to intestinal absorption; 2) the microvilli are less developed in this tract as compared to the small intestine and their membrane-associated peptidases are limited making such microvilli more susceptible to permeation; 3) the P-glycoprotein is less expressed in colonic mucosa, and colon enterocytes and M-cells easily internalize nanoparticles; 4) there is a prolonged localization of insulin on the colonic mucosa due to slow transit time, thinner unstirred water layer (UWL) adjacent to mucosa, and slow turnover of colon mucosal film [5]. The lack of significant decrease in glycaemia the rats treated with CPI indicates that the delivery of insulin as such to the colon by pellets is not sufficient to exert the hypoglycemic effect. A drug delivery system is required to properly address and release insulin to the colonic mucosa.

Therefore, insulin nanoformulation plays a key role in reaching this goal. It has been demonstrated that polyethylenimine, as other cationic polymers (e.g. chitosans-CS), is able to loosen tight junctions (TJ) resulting in increased permeability of intestinal epithelium cells, which leads to paracellular permeation of molecules [32]. Many studies have already demonstrated the ability of different kinds of CS nanoparticles to increase the intestinal permeation of conjugated insulin through TJ opening [33]. In particular, Lin and colleagues suggested a mechanism for the paracellular transport of insulin to the blood circulation based on CS nanoparticles, whose stability and release of loaded insulin greatly depended on the environmental pH. In this model, the neutral pH, typical of the jejunum and the ileum, favors the degradation of the nanocomplex resulting in the complete release of CS and insulin. CS interaction with the TJ proteins enhances the paracellular pathway, promoting the absorption of greater amounts of insulin [34]. An equivalent mechanism could be suggested also for the NI released from pellets into the colon. Indeed, the pH values of this tract, from 6.5 in the ascending colon to 7 in the descending colon, could promote nanoparticle instability and cause the release of insulin (Fig. 1). Considering that the entire nanocomplex was not allowed to permeate across the TJ, even under the effect of paracellular enhancers, a few conclusions can be drown: 1) the released NI undergoes degradation in the colon lumen; 2) the released insulin that is not subjected to significant proteolytic activity in this tract of the gut and the PEI fragments both reach the mucosal surface; 3) PEI is supposed to interact with the TJ and opens the paracellular pathway, thus promoting a significant insulin absorption [35]. In addition, the presence of sodium glycocholate, that is known to promote insulin passage through the colonic mucosa by different mechanisms, including the rearrangement of the phospholipid bilayer and increase of the relevant fluidity, could have a synergistic effect on the overall protein absorption.

At 48 h, NT and SCI-, OI-, NI-, and GPI-administered rats had the blood glucose values return to initial levels while in rats administered with CPNI the blood glucose levels remained persistently and significantly reduced, as compared to the initial values. Such a long-lasting effect could be ascribed to the mucoadhesive properties of the nanocomplex, involving ionic interactions between the positively charged PEI and the negative charges of the mucosal surface. It may be hypothesized that, even if most of the nanoparticles were degraded in the colon lumen, part of them still interacted with the membrane-bound mucins on the intestinal epithelium,

releasing the insulin beyond UWL, in the proximity of the absorptive cells. The UWL, forming a diffusion barrier between the luminal contents and the epithelium, probably maintained an adequate concentration of insulin in proximity of the enterocytes, maintaining a prolonged insulin absorption over the whole period.. On the contrary, most of the other administered formulations were eliminated previously. These results suggest a two-phase mechanism in rats administered with CPNI. During the first phase, a massive absorption of insulin takes place within the initial hours from the treatment (i.e. 6-8 h) as an effect of the rapid release of the drug from the nanoparticles into the colon lumen followed by the PEI-mediated opening of the paracellular route. During the second phase, a long-lasting absorption of the insulin released in proximity of the colon epithelium by the nanoparticles is triggered by active interaction with the negatively charged mucus layer.

At 48 h post-treatment, a slight decrease of blood glucose levels, not significantly different from that observed in NT and orally treated rats, was observed upon administration of CPI, while in GPNI-treated rats, glucose values were still about 70% of the initial values and significantly lower than those of controls. It is likely that the hypoglycemic effect could be attributed to the release of insulin by those nanoparticles which escaped luminal degradation and reached the mucosal surface. In this case, a certain amount of the protein, protected from the proteolytic activity due to the presence of the secretory mucin barrier [36], is still available for the intestinal absorption. This likely occurs through the PEI-activated paracellular pathway.

The colonic delivery of nanoformulated insulin holds a significant potential in clinical practice. This study provides the proof of the concept that the oral delivery of insulin targeted at colonic release and absorption may survive the hostile environments of gastric acidity or the peptidase rich small bowel. Moreover, blood glucose remained nearly half of the initial levels at 48 h post-administration of CPNI, thanks to slow and continuous release of insulin in the colon. This fact is particularly significant, since it may indicate that a prolonged control of glycaemia may be reached with a possible reduction in the overall insulin requirements of the patient resulting in a possible reduction in the number of daily injections. This would result in improved quality of life and potential reduction in long-term complications related to unstable glycemic control. Another goal reached by this novel insulin nanoformulation is the restoration of a physiological delivery of insulin into the portal vein, with a proper liver metabolism. This avoids fluctuating blood glucose levels and allows a more

precise control of glycaemia. No fluctuation was observed in blood glucose levels of CPNI-treated rats, in favor of a progressive regular fall of the glycaemia [2,6]. In addition, a low between-subjects variability in blood glucose levels was observed in rates treated with CPNI, suggesting a reproducible response of glycaemia to this novel insulin nanoformulation. The clinical impact of this phenomenon could be important considering that, currently, a major limitation encountered in clinical and preclinical trials with oral insulin delivery so far was the highly variable response of glycemic control [37]. Furthermore, mimicking the physiological insulin circulation in the portal vein and the extraction by the liver, this formulation might obviate the drawbacks of insulin administered subcutaneously: systemic hyperinsulinemia with subsequent possible hypoglycaemia, local lipoatrophy with consequent day-to-day variability of subcutaneous absorption, weight gain, atherogenesis and enhanced lipogenesis. The peripheral administration of insulin is also implicated in the worsening of insulin resistance which makes the adjustment of insulin dosing difficult in, for instance, diabetes Type 2 patients.

Finally, hepatic insulin extraction after colon absorption as opposed to that after peripheral administration might improve the liver inflammation and the oxidant production involved in the pathogenesis of hepatic steatosis. Therefore, the multitasking nanodevice described in this study for oral delivery of insulin is promising, although further research is needed to better clarify, for example, the efficacy in glycemic control immediately after a meal and the variations caused by oral insulin on various others metabolic and diabetes indicators, such as glucagon, insulin-like growth factor 1 (IGF-1), fructosamine and glycated hemoglobin.

5. Conclusion

Oral insulin delivery remains a clinical challenge, and despite a growing body of publications on this topic, there is still no oral formulation for insulin. In this study, a new approach for the oral administration of insulin is proposed targeting the colon as the release and absorption site. The synergistic effect due to the nanoformulation of insulin and the encapsulation in a triple-layer pellet system for colon-release delivery resulted in a significant and long-lasting hypoglycemic effect. The impact of our multitasking macromolecule delivery system for oral insulin in controlling diabetes is clinically appealing, since it represents an oral route

for insulin administration, with a prolonged hypoglycemic activity and a more physiological insulin metabolism. However, further research is needed to promote this novel nanoformulation into clinical trials.

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