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Title
Lipid nanoparticles as vehicles for oral delivery of insulin and insulin analogs: preliminary *ex vivo* and *in vivo* studies.

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Abstract

Aims
Subcutaneous administration of insulin in patients suffering diabetes is associated with the distress of daily injections. Among alternative administration routes, the oral route seems to be the most advantageous for long-term administration, also because the peptide undergoes a hepatic first-pass effect, contributing to the inhibition of the hepatic glucose output. Unfortunately, insulin oral administration has so far been hampered by degradation by gastro-intestinal enzymes, and poor intestinal absorption. Loading in lipid nanoparticles should allow to overcome these limitations.

Methods
Entrapment of peptides into such nanoparticles is not easy, because of their high molecular weight, hydrophilicity and thermo-sensitivity. In this study this objective was achieved by employing fatty acid coacervation method: solid lipid nanoparticles and newly engineered nanostructured lipid carriers were formulated. Insulin, and insulin analog - glargine insulin - were entrapped in the lipid matrix through hydrophobic ion pairing.

Results
Bioactivity of lipid entrapped peptides was demonstrated through a suitable *in vivo* experiment. *Ex vivo* and *in vivo* studies were performed by employing fluorescently labelled peptides. Gut tied up experiments showed the superiority of glargine insulin loaded nanostructured lipid carriers, which demonstrated significantly higher permeation (till 30% dose / mL) compared to free peptide. Approximately 6% absolute bioavailability in the bloodstream was estimated for the same formulation through *in vivo* pharmacokinetic studies in rats. Consequently, a discrete blood glucose responsivity was noted in healthy animals.

Conclusions
Given the optimized *ex vivo* and *in vivo* intestinal uptake of glargine insulin from nanostructured lipid carriers, further studies will be performed in healthy and diabetic rat models in order to establish a glargine insulin dose – glucose response relation.

1. Introduction

Subcutaneous administration of insulin in people with diabetes is associated with the distress of daily injections, with implications on quality of life. Moreover glucose control needs a personalized approach, and today different short and long acting insulin formulations, as well as insulin analogs, have been engineered in order to optimize glucose level after meals and during the night [1]. Among alternative insulin administration routes, the oral route seems to be the more advantageous for long-term administration [2], but it has so far been hampered by gastro-intestinal enzyme degradation and poor intestinal absorption, due to its high molecular weight. Although many oral insulin formulations have been studied, translation to a commercial preparation is still challenging [3].

One of the most fascinating approaches to overcome these obstacles is the employment of nanoparticles, to enhance insulin oral uptake and protect its peptide structure from enzymatic degradation. Uptake of particulate matter by the gastrointestinal tract (GIT) is now a widely accepted phenomenon, hence the debate has shifted to its exploitation for therapeutic delivery [4, 5]. In different studies lipid nano-formulations were employed as gut uptake enhancers [6, 7]. Solid lipid nanoparticles (SLN) are particles prepared from physiological solid lipids (triglycerides, partial glycerides, waxes and fatty acids - FA) with mean diameters ranging between 50–1000 nm [8]: sufficient data are available in the literature concerning their use in oral drug delivery [9, 10], and FA SLN are specifically described as oral absorption promoters [11]. Moreover, recently nanostructured lipid carriers (NLC) have been engineered from SLN, by employing mixtures of solid and liquid lipids, with improvement of drug entrapment and release properties [12]. Thus, insulin incorporation in FA SLN and NLC should protect against proteolytic degradation and enhance gut absorption, even if some concerns still remain, because of this peptide high molecular weight, hydrophilicity and thermo-sensitivity [13, 14].

An innovative solvent-free SLN production technique was developed and patented by our research group [15, 16, 17], named FA coacervation: only Food and Drug Administration (FDA) approved materials are employed, working temperatures are mild and, obtained nanoparticles can be freeze-dried without particle aggregation, allowing the perspective of a solid dosage form. In a previous report, we encapsulated insulin in stearic acid SLN by hydrophobic ion-pairing (HIP), a simple method based on the interaction between an anionic surfactant and a positively charged protein [18, 19]. In this experimental work this technique was improved, by using either bovine insulin, or glargine insulin, which shows different HIP ability, and modifying lipid composition of nanoparticles, in order to promote intestinal uptake. Obtained formulations were tested *ex vivo* and *in vivo* on healthy animal models.

2. Materials and Methods

2.1. Materials

Lactic acid, sodium dihydrogen phosphate and sodium monohydrogen phosphate were from A.C.E.F. (Fiorenzuola d'Arda, Italy); ethylendiaminotetraacetic acid (EDTA) and sodium chloride were from Carlo Erba (Cornaredo, Italy); 80% hydrolysed polyvinyl alcohol 9000–10000MW (PVA9000), dextran (MW 60000-90000), sodium stearate, Osmium Tetroxide, sodium dodecyl sulfate (SDS), fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), sodium 1-eptansulfonate, acetonitrile, bovine insulin and 6-coumarin were from Sigma Aldrich (Saint Louis, Missouri, MO, USA); trifluoroacetic acid (TFA), oleic acid and potassium dihydrogen phosphate were from Merck (Darmstadt, Germany). Glargine insulin was precipitated from commercial products (Lantus®, Abasaclar®) by dilution with phosphate buffer 0.1 M pH=7.40, followed by centrifugation (17.000 g, 5417 Eppendorf Centrifuge, AG, Hamburg, Germany), washing with distilled water and freeze-drying overnight. Distilled water was purified using a Milli-Q system (Millipore, Bedford, MO). All other chemicals were of analytical grade and used without further purification.

2.2. Animals

Male Wistar rats (Charles River, MA, USA), weighing 250 g, were housed in standard facilities, handled and maintained according to our Institutional Animal Care and Use Committee ethical regulations. Rats were kept under controlled environmental conditions (24 ± 1°C, 50–60% humidity, 12 h light and dark cycles, lights on at 7:00 a.m.). Rats were given *ad libitum* access to food and water. The procedures conformed to the Ethics Committee of University of Turin's institutional guidelines on animal welfare (DL 26/2014 implementation of directive 2010/63 UE) as well as International Guidelines, and all efforts were done to minimize the number of animals and their discomfort. All experiments on animal models were performed according to an experimental protocol approved by the University Bioethical Committee and the Italian Ministry of Health (Aut. N. 32/2016-PR).

2.3. Fluorescence labelling

Details are provided in Supplementary Materials

2.4. Analytics

Details are provided in Supplementary Materials

2.5. Nanoparticles formulation and characterization

Details are provided in Supplementary Materials

2.6. Drug release studies in simulated intestinal medium (SIF)

SLN loaded with fluorescently labelled peptides were employed. 250 µL of SLN were diluted in 10 mL US Pharmacopoeia SIF and kept under stirring for several hours. At scheduled times, samples of 100 µL were withdrawn and centrifuged at 55.000 rpm for 5 minutes (Allegra® 64R centrifuge, Beckman Coulter, Palo Alto, CA, USA). Clear supernatants were frozen and kept for subsequent HPLC analysis.

2.7. Bioactivity of insulin loaded in nanoparticles

The bioactivity of insulin during nanoparticles manufacturing process was tested according to a method described in the literature [20, 21]. Peptide was extracted from nanoparticles: 1 mL suspension was dissolved in 2 mL ethanol, lipid was precipitated with 1 mL water; obtained mixture was centrifuged, reduced to 3 mL under nitrogen steam and filtrated (0.1 µm). As a reference, native peptide in PBS buffer was used. Both the extracted peptide and the reference were injected subcutaneously in separate groups of male Wistar rats (weight 250 g), that had been fasted for 12 h prior to the experiment, but allowed access to water *ad libitum*, at the dose of 2 IU/kg. At scheduled times after dosing, blood samples were collected by tail bleeding. The serum glucose level was determined by the enzymatic test Accuchek Compact (Roche Diagnostics, Monza, Italy).

2.8. Non-everted gut tied up experiments

Ex vivo absorption evaluation was carried out by permeation measurements in excised rat small intestine as described elsewhere [22] with some modifications. Male Wistar rats (250 g) were anaesthetized with isoflurane, sacrificed and exsanguinated. Freshly excised rat duodenum, jejunum and ileum tissue were washed with Krebs-Ringer buffer (KRB) and cut into pieces of 5-4 cm. 0.3 mL of the formulation under study were syringed into intestinal sacs; the filled tissues were incubated in oxygenated KRB (10 mL) at 37°C with smooth shaking. Sample solution (0.5 mL) was withdrawn from the serosal side at fixed time intervals up to 120 minutes and replaced with fresh buffer. Tests were performed in triplicate for each formulation under study on the three different intestinal segments from three different rats.

At the end of the experiments, tissues were washed with normal saline, and drug accumulation in the gut wall was investigated. Briefly, tissues were homogenized in distilled water, proteins were removed from the homogenates by dilution with methanol (100 µL homogenate + 300 µL methanol) and subsequent centrifugation (17.000 g, 5417 Eppendorf Centrifuge, AG, Hamburg, Germany), and clear supernatants were analyzed with HPLC.

2.9. Pharmacokinetics in healthy rats

Healthy male Wistar rats (weight 250 g) were employed (5 rats for each experimental condition). Nanoparticles loaded with fluorescently labelled peptides were used at 30 IU/Kg dose. Oral and duodenal administration were compared, in order to evaluate the variable of gastric emptying. Oral administration was performed through gavage, while duodenal administration by a surgically implanted duodenal cannula. Blood samples were collected, in heparinized tubes, at scheduled times after administration of formulations under study, through a catheter surgically positioned in the rat jugular vein [23]. Blood samples were centrifuged to isolate plasma. Plasma concentration of labelled peptide was determined through HPLC after deproteinization (100 µL plasma diluted with 300 µL methanol and centrifuged). On the same blood samples glucose level was measured through an enzymatic test Accuchek Compact (Roche Diagnostics, Monza, Italy).

An estimation of pharmacokinetic parameters was performed by a numerical integration procedure, the trapezoidal rule; the only assumption being that the terminal elimination phase of the drug could be described by a mono-exponential equation, following first order kinetics. Non-compartmental analysis for extravascular administration was performed by Kinetica 2000 4.1.1 (InnaPhase Corp, MA, USA)

2.10. Lymphatic uptake studies

Nanoparticles loaded with fluorescently labelled peptide were administered directly in the duodenal lumen of 500 g male Wistar rats under anesthesia, through an injection in the first portion of the duodenum [24]. The lymph was directed externally by cannulating the thoracic duct near its confluence with the cisterna chyli. The tubing was exteriorized through the main incision, which was closed in layers (muscle and skin). Lymph concentration of fluorescent peptide was measured as above.

2.11. Fluorescent peptide uptake in gut sections

After 1.5 h, the rats undergoing the above mentioned lymphatic uptake studies were sacrificed, and the section of each loop was removed and extensively washed using normal saline solution. Subsequently, the small intestinal tissue sections were embedded in Optimum Cutting Temperature (OCT) for cryostat sections at controlled temperature (−15 ± 1 °C). Five micrometer-thick sections were prepared by cryostat microtome (Reichert-Jung/Leica, Frigocut 2800 N), labelled with 4′,6-diamidino-2-phenylindole (DAPI) staining, and mounted on the slide glasses. The tissue sections were visualized using DM2500 fluorescence microscope (Leica, Wetzlar, Germany).

2.12. Blood glucose responsivity studies

Glucose responsivity studies were performed on healthy male Wistar rats (weight 250 g). Nanoparticles loaded with non labelled peptide were administered orally by gavage at a dose of 30 IU/kg. 4 animals were employed for each experimental condition. The animals were fasted for 12 h prior to the experiment, but allowed access to water *ad libitum*. Blood samples were collected, by tail bleeding, 10 min before (basal), and at scheduled times, up to 6 hours after administration. In separate experiments, oral glucose tolerance test (OGTT) was also performed by administration of a 2 g/Kg glucose bolus by gavage, simultaneously to the formulations under study [25]; plain glucose bolus was used as control. Blood samples were collected, by tail bleeding, 10 min before (basal), and at various time points, up to 2 h after bolus. In both experiments, glucose level was measured through an enzymatic test Accuchek Compact (Roche Diagnostics, Monza, Italy)

3. Results

Data concerning lipid nanoparticles formulation and characterization are shown in Supplementary Materials (Table 1s, Figures 1s, 2s). Drug recovery is nearly 85% for SLN formulation and 65% for NLC formulation. Drug release from labelled peptide loaded SLN into SIF medium (pH=6.5) was evaluated in order to investigate the influence of different ion pairing on drug release (Figure 1). It can be noted that the improved ion pairing ability of glargine insulin, due to its different aminoacid sequence, leads to burst release avoidance, that is typical of insulin ion pair.

The bioactivity of entrapped peptides was evaluated according to a literature method [20, 21]. Blood glucose level was monitored within different time frames for insulin and glargine insulin, owing to differently delayed hypoglycaemic effect after subcutaneous injection. All the peptides entrapped in SLN and NLC showed a pattern similar to free peptide (Figure 2).

Preliminary *ex vivo* experiments with gut tied up method were performed in order to evaluate the permeation ability of SLN and NLC. Nanoparticles were fluorescently labelled with 6-coumarin. Free probe in suspension was also employed as control. Significant permeation was only detected for NLC in the duodenum (Figure 3).

Basing on experiments with 6-coumarin, only NLC were employed for the following *ex vivo* studies performed with labelled peptides. In such experiments NLC loaded with labelled insulin gave no permeation, while TRITC-glargine insulin NLC permeation was large (Figure 4). While free drug didn't permeate significantly, loading in NLC was effective in promoting drug uptake, especially in duodenum, and to a lesser extent in jejunum and ileum. Permeation data correlate with accumulation in the gut wall. Noteworthy, the permeation of labelled peptide loaded in NLC was quantitatively much higher than that of 6-coumarin labelled NLC (Figure 3).

Consequently, optimized glargine insulin loaded NLC underwent *in vivo* pharmacokinetic studies. TRITC-glargine insulin was employed. Duodenal and gavage administration were compared, in order to evaluate the variable of gastric emptying on drug uptake. A major absorption peak was shown at 30 min after administration (Figure 5). Pharmacokinetic analysis (Tables 2s, 3s, Supplementary Materials) showed a bioavalability of 6.10% and 4.50% for duodenal and gavage administration, respectively. Lymphatic uptake studies were also performed by cannulating the thoracic duct. A concentration of 2.0 µg/mL mg^{-1} was recovered in the lymph 1.5 h after duodenal administration, indicating that lymph can play an important role in the intestinal uptake of the formulation under study. Fluorescence images of the gut wall showed the translocation of labelled glargine insulin across the gut villi (Figure 6).

Glucose responsivity was evaluated in healthy rats (Figure 7). Unlabelled glargine insulin was used. For healthy rats a slight, but significant glucose reduction was obtained 150 min after administration. Accordingly, in OGTT significant differences from controls were recorded at 90 min after administration. A more delayed glucose reduction was detected at 360 min after administration (Figure 7).

4. Discussion

HIP approach to load peptides in nanoparticles [27] was employed to prepare insulin loaded SLN by our research group [19]. In this experimental work we exploited this method, both with insulin and its analog (glargine insulin), assessing the maintainance of peptides biological activity, through an *in vivo* assay. Drug encapsulation efficiency (EE%) depends on ion pairing ability of the molecule under study (Table 1s, Supplementary Materials). Thus, glargine insulin is better associated to lipid nanoparticles compared to insulin, and it doesn't give burst release effect in SIF medium (Figure 1). This is very important, because fast release in the intestinal lumen exposes the peptide to proteolityc enzymes, and, thus, gut permeation of released peptide is inhibited.

The permeability of lipid nanoparticles, as well as of the delivered peptide, through rat small intestine was evaluated *ex vivo* with the non-everted intestinal sac method. Despite some shortfalls (e.g. interruption of normal blood flow, lack of a nervous system) this method is widely used to

study passive absorption of molecules [28]. Furthermore the presence of a mucus layer, the expression of transport proteins and drug metabolism allows this model to provide additional useful data [29]. In our experiments, the permeation of lipid nanoparticles was quantitatively low, restricted to duodenum and only to NLC formulation (Figure 3). On the other hand, permeation of the peptide loaded in NLC was negligible in the case of insulin, and relevant in the case of glargine insulin, with a major uptake in the duodenum section (Figure 4). A mechanism could be hypothesized for this phenomenon. NLC act as a promoter of intestinal uptake, owing to tight junction opening by specific components of the lipid matrix, such as oleic acid [30]. Consequently, nanoparticulate matter should be taken up by paracellular route [31], but only up to a low extent, because nanoparticle size is larger than paracellular interspace. Limitation to duodenum should be due to the thinner mucus protecting layer in this section [32, 33], allowing a more efficient action on tight junctions. Insulin suffers burst release from lipid nanoparticles in the intestinal lumen (Figure 1), thus it is not taken up when delivered in NLC. Glargine insulin instead is well associated to NLC in SIF (Figure 1): when NLC come in contact with the inner mucus layer in the gut, probably the peptide is displaced from HIP by the mucin protein present nearby the mucosa, and released. Since the peptide size is much smaller than NLC, and compatible with the paracellular route, its uptake is higher than the uptake of the nanoparticles themselves.

In vivo pharmacokinetic studies involve a more complex system, including gastric emptying, intestinal motility, lymphatic uptake through the Peyer's patches, biodistribution and elimination from the bloodstream of the absorbed peptide. The effect of gastric emptying was evaluated by comparing gavage and duodenal administration: the main plasmatic peak was not influenced by the way of administration, but the pattern of plasmatic curve is almost flat 2 h after gavage. This could be interpreted as a low, but delayed uptake due to prolonged gastric emptying. In fact 6 h after gavage a glucose level reduction was noted in healthy rats, both with labeled (Figure 5) and native (Figure 7) glargine insulin. The role of lymphatic system in glargine insulin uptake was demonstrated in a suitable experiment, and the translocation of labeled drug through intestinal villi was observed in fluorescence microscopy (Figure 6). The approximated bioavailability (Table 3s, Supplementary Materials) is low, especially if compared to the bioavailability of small molecular weight drugs, but relevant if referred to an high molecular weight peptide, such as insulin and its analogs, which, furthermore, undergo a very rapid elimination from the bloodstream [34], in front of a relatively slow absorption by the oral route. In healthy rats the hypoglycemic effect is evident and reproducible, but not broad (Figure 7): nevertheless a correlation between plasmatic peptide concentration and blood glucose level could be hypothesized basing on literature data [34].

Among the alternative administration routes, the oral route, if effective, should be considered as the most suitable for insulin delivery, because the peptide undergoes a hepatic first-pass effect, by which it contributes to the inhibition of the hepatic glucose output, thus avoiding side effects from peripheral hyperinsulinemia [2]. This strategy needs the employment of suitable vehicles, able to overcome peptide degradation and poor uptake by the gut, such as the here proposed NLC. However, lipid nanoparticulate vehicles are associated to uptake through the Peyer's patches and the gut lymphatic system, which bypasses the portal system, avoiding the first-pass effect [6]. In this experimental work, we demonstrated the involvement of gut lymphatic system in glargine insulin uptake from NLC through a thoracic duct cannulation experiment. Nevertheless, basing on gut tied up experiments, paracellular uptake through opening of tight junctions between enterocytes can be hypothesized as the major absorption mechanism: in this case peptide taken up through the enterocytes should undergo the portal system and hepatic first-pass.

Different strategies have been attempted in order to increase insulin bioavailability through alternative administration routes [35], especially through the oral administration [36]. Various insulin conjugates have been developed to this aim. Insulin–Transferrin conjugate was detected in the serum 4 h after oral administration, indicating successful overcome of GI biological barriers, due to the presence of Transferrin receptors in the gut wall [37]. Hexyl-insulin-monoconjugate-2 (HIM2) - a conjugate between recombinant insulin and a short oligomer of polyethylene glycol

(PEG) and hydrophobic (alkyl) groups - prevents enzymatic degradation, and confers amphiphilic character, resulting in better uptake by the oral route [38]. On the other hand, the employment of nanoparticulate systems as oral insulin permeation enhancers has been considered as an innovative and fascinating approach. Chitosan induces the re-distribution of claudin-4 (CLDN4) from the cell membrane to the cytosol, followed by lysosome degradation, thus weakening the tight junctions and enhancing paracellular permeability transiently. Consequently, chitosan nanoparticles have been often employed to permeate enterocytes layer in the gut. Indeed, encapsulation of insulin into dextran sulfate/chitosan nanoparticles improved its pharmacological availability (5.6 and 3.4% for the 50 and 100 IU/kg doses, respectively) after oral administration [39]. Ligand conjugated polymeric nanoparticles have been used for insulin oral delivery, owing to targeting of specific receptor on gut wall. Insulin loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles were surface modified with concavalin, a lectin which targets lymphoid tissue in the Peyer's patches, resulting in blood glucose level reduction within 4h after oral administration [40]. Insulin loaded PLGA nanoparticles targeted to the neonatal Fc receptor (FcRn), which mediates the transport of immunoglobulin G antibodies across epithelial barriers, were efficiently transported across the intestinal epithelium, exerting an hypoglycaemic effect at a low dose (1.1 UI/kg) after oral administration to mice [41]. However, despite comparison between different technological approaches is difficult, because of the different experimental conditions and models (healthy or diabetic animals) employed, in this work similar results in insulin uptake were reached with a simple and reproducible lipid nanoparticulate vehicle, based on biocompatible lipids, and without involving any chemical reaction and complex purification procedure. Even if in our setting (healthy rats) the effect on blood glucose is still modest, the presence of a relevant plasmatic glargine insulin peak could be effective on important physiological parameters for diabetic subjects, such as arterial stiffness, which predicts for cardiovascular complications. In fact, although insulin is a vasodilator, concomitantly it can also constrict the arteries through the autonomic nervous system. The balance of the sympatico-excitatory and the vasodilator effects can occur already at modestly elevated plasma insulin levels [42]. In particular, diabetic subjects with albuminuria have advanced autonomic neuropathy, which can diminish the vasoconstrictive effects of insulin: as a result, the vasodilatory effects of insulin will be dominant in this setting [42].

Indeed, lipid nanoparticles have been considered for oral insulin delivery owing to their biocompatibility, sustained release properties and favourable uptake by the Peyer's patches [6, 7]. Compared to analogous approaches exploiting insulin loaded SLN for oral administration [43], the here proposed nanoformulation shows relevant novelties. A solvent-free and easy to scale-up technique is employed [19], which coupled with an HIP approach, allows to obtain higher entrapment efficiencies for an hydrophilic peptide, preserving its biological activity. The formulation was significantly improved, compared to previously engineered insulin loaded SLN by coacervation [19]: NLC were formulated by mixing solid and liquid fatty acids, with an increased gut uptake. This was probably due to tight junctions opening by a component of the formulation (oleic acid), leading to paracellular permeation across the enterocytes. Despite elevated free fatty acid levels in plasma and excessive lipid accumulation in non-adipose tissue (liver and muscle) are related to obesity-associated insulin resistance in type 2 diabetic subjects, clinical evidence showed that insulin therapy decreases fatty acid levels and improves dyslipidemia in these patients, and experimental data from diabetic animal models showed that insulin therapy reduces lipid content in liver and muscle, owing to a precise molecular mechanism [44]. Thus, it can be hypothesized that employment of fatty acid nanoparticles as carriers for oral insulin delivery should be exempt from lipotoxicity. Finally both insulin and an insulin analog, glargine insulin, were loaded within nanoparticles: in this way also an insulin analog from the first-generation basal insulins (insulin determir, glargine insulin), with longer half-lives and reduced within-subject variability, is purposed by the oral route for the first time. Indeed, glargine insulin significantly reduces the frequency of hypoglycemic episodes, even if it does not provide reliable full 24-h coverage, and considerable residual within-patient variability and hypoglycaemia risk remain [45].

Some limiting aspects should be improved in further studies, such as the optimization of the hypoglycemic effect as a function of the glargine insulin administered dose. The effect of the lipid nanoparticles suspension administered volume should be considered, too, as well as the possibility to obtain solid oral dosage forms, following freeze-drying or spray-drying of nanoparticles suspension. In fact, the administered volume should affect the gastric emptying rate. Moreover, experiments on diabetic rats should be also performed. Indeed they are less insulin resistant compared to healthy ones, because of long lasting hyperglycemic condition: thus, in these animals the hypoglycemic effect following insulin administration will no more be counterbalanced by physiological mechanisms devoted to avoid hypoglycemia. In fact, occurrence of hypoglycaemia is an important safety issue for insulin administration in diabetic subjects. To this aim, insulin degludec, a novel ultra-long-acting basal insulin with a half-life of approximately 25 h and a duration of action greater than 40 h, results in reduced within-patient variability and better safety in reducing hypoglycaemic events, compared to glargine insulin [45]. Moreover, recent investigation disclosed the potential of glucose transporter inhibitor-conjugated insulin, a modified peptide, which is able to modulate insulin availability basing on blood glucose level [46]. In perspective, the formulation technique described in this manuscript, which can be easily adapted to load insulin analogs and conjugates, can be employed with alternative insulin analogs, as well as with glucose modulated peptides, in order to meet a better glycaemic control in diabetic subjects.

5. Conclusions

In this experimental work lipid nanoparticles loaded with insulin and insulin analog glargine insulin were prepared. Solvent free fatty acid coacervation method and HIP technique were employed to load the peptides within nanoparticles, preserving their biological activity. *Ex vivo* studies allow to discriminate factors influencing peptide uptake by the gut. Glargine insulin loaded NLC, showing optimized uptake *ex vivo*, underwent *in vivo* pharmacokinetic studies, assessing the presence of a plasmatic peak of the labelled peptide. Accordingly, a discrete blood glucose reduction was observed after gavage administration in healthy rats. Obtained results will drive further studies, involving the establishment of a dose-response relation *in vivo* and a deeper investigation of hypoglycaemic effect in diabetic rats.

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Conflict of interest

The authors declare no conflict of interest.

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Figures

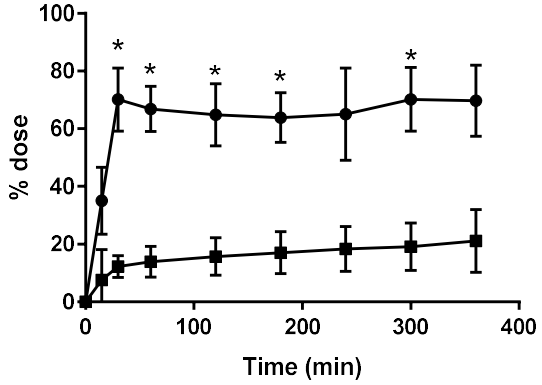

Fig. 1

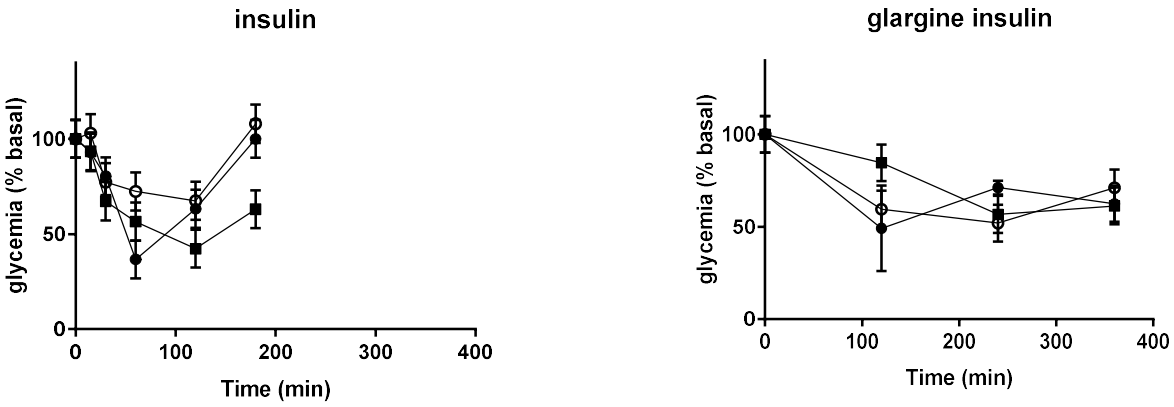
insulin
glycemia (% basal)
100
50
0
0
100
200
300
400
Time (min)
glargine insulin
glycemia (% basal)
100
50
0
0
100
200
300
400
Time (min)

Fig. 2

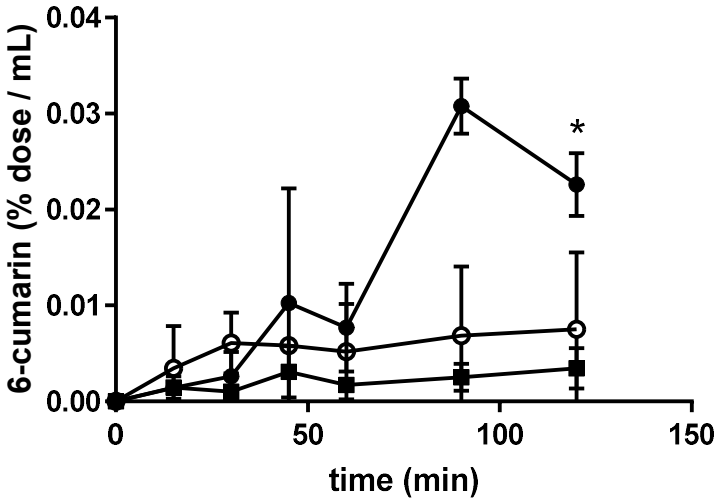

Fig. 3

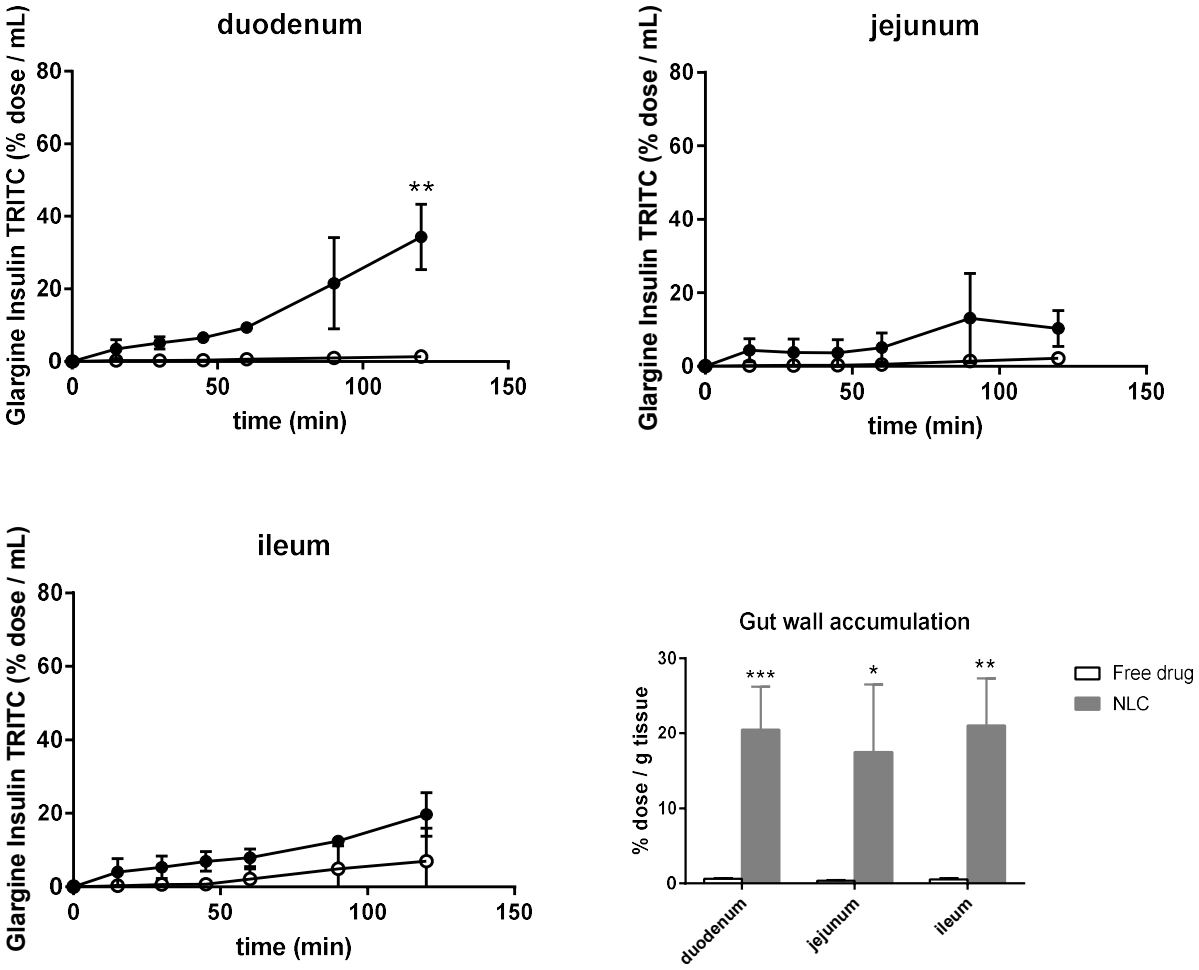
duodenum
Glargine Insulin TRITC (% dose / mL)
80
60
40
20
0
0
50
100
150
time (min)
**
jejunum
Glargine Insulin TRITC (% dose / mL)
80
60
40
20
0
0
50
100
150
time (min)
ileum
Glargine Insulin TRITC (% dose / mL)
80
60
40
20
0
0
50
100
150
time (min)
Gut wall accumulation
% dose / g tissue
30
20
10
0
***
*
**
duodenum
jejunum
ileum
Free drug
NLC

Fig. 4

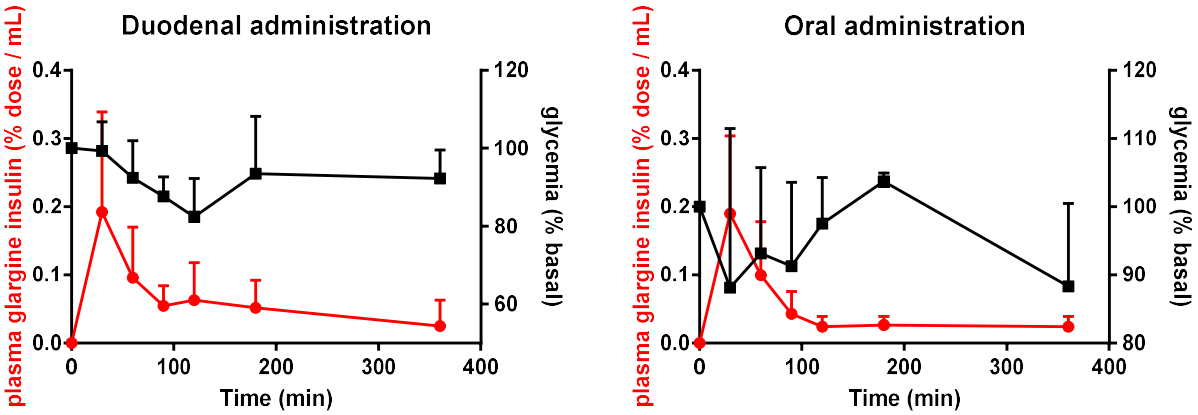
Duodenal administration
plasma glargine insulin (% dose / mL)
glycemia (% basal)
Time (min)
0.0
0.1
0.2
0.3
0.4
60
80
100
120
0
100
200
300
400
Oral administration
plasma glargine insulin (% dose / mL)
glycemia (% basal)
Time (min)
0.0
0.1
0.2
0.3
0.4
80
90
100
110
120
0
100
200
300
400

Fig. 5

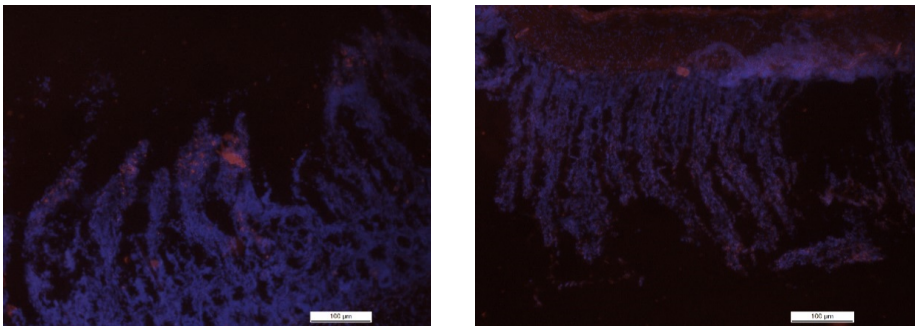

Fig. 6

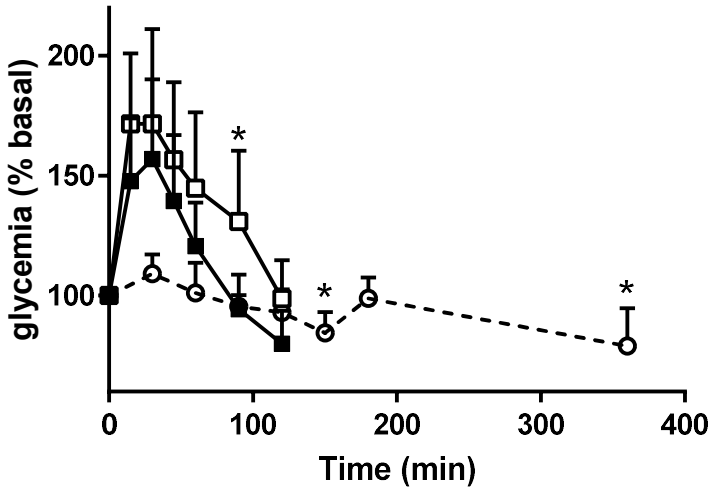

Fig. 7

Figure captions

Fig.1 Release profile of labelled peptides from SLN towards SIF medium. SLN loaded with fluorescently labelled peptides were diluted in US Pharmacopoeia SIF and kept under stirring; at scheduled times, samples were withdrawn, centrifuged and analysed with HPLC.
(●) FITC insulin; (■) TRITC glargine insulin. FITC insulin *vs* TRITC glargine insulin, * $P < 0.05$ (un-paired t-Test, two tailed) (N=3)

Fig. 2 Bioactivity of peptides loaded in lipid nanoparticles. Insulin and glargine insulin were extracted from nanoparticles. Both the extracted peptide and native peptide in PBS as reference were injected subcutaneously (2 IU/kg) in male Wistar rats fasted for 12 h prior to the experiment. At scheduled times, blood samples were collected by tail bleeding. The serum glucose level was determined by the enzymatic test Accuchek Compact.
(○) free peptide; (■) peptide from SLN; (●) peptide from NLC. (N=3)

Fig. 3 Labelled nanoparticles *ex vivo* permeation in gut tied up experiments. The formulations under study were syringed into intestinal sacs obtained from freshly excised rat duodenum, jejunum and ileum tissue. The filled tissues were incubated in oxygenated buffer at 37°C with smooth shaking. Sample solution was withdrawn from the serosal side at fixed time intervals up to 120 minutes and replaced with fresh buffer. The samples collected were analysed with HPLC.
Duodenum permeation; (○) free 6-coumarin; (■) SLN; (●) NLC; NLC *vs* SLN, * $P< 0.05$ (un-paired t-Test, two tailed). Results are expressed as % dose / mL. (N=3)

Fig. 4 Labelled glargine insulin *ex vivo* permeation in gut tied up experiments. The formulations under study were syringed into intestinal sacs obtained from freshly excised rat duodenum, jejunum and ileum tissue. The filled tissues were incubated in oxygenated buffer at 37°C with smooth shaking. Sample solution was withdrawn from the serosal side at fixed time intervals up to 120 minutes, replaced with fresh buffer and analysed with HPLC. At the end of the experiments tissues were homogenized in distilled water, proteins were removed from the homogenates by dilution with methanol and subsequent centrifugation. Clear supernatants were analyzed with HPLC.
(○) free drug; (●) NLC. Duodenum permeation: NLC *vs* Free drug, ** $P < 0.01$. Gut wall accumulation: b) NLC *vs* Free drug, * $P < 0.05$ jejunum; ** $P < 0.02$ ileum; *** $P < 0.01$ duodenum (un-paired t-Test, two tailed). (N=3)

Fig. 5 *In vivo* pharmacokinetics in healthy rats of labelled glargine insulin loaded NLC. Nanoparticles loaded with fluorescently labelled peptides were administrated (30 IU/Kg) to healthy male Wistar rats. Oral administration was performed through gavage; duodenal administration was performed by a surgically implanted duodenal cannula. At scheduled times, blood samples were collected through a catheter surgically positioned in the rat jugular vein. Blood samples were centrifuged and plasma concentration of labelled peptide was determined through HPLC after deproteinization. On the same blood samples glucose level was measured through an enzymatic test Accuchek Compact.
(■) glycemia (% basal); (●) plasma glargine insulin (% dose / mL). (N=5)

Fig. 6 Fluorescence microscopy of duodenum (left) and jejunum (right) wall after duodenal administration of labelled glargine insulin (red) loaded NLC. Nuclei of intestinal cells are stained in blue. Five micrometer-thick cryostat sections of duodenal tissue were labelled with 4′,6-diamidino-2-phenylindole (DAPI) staining, and mounted on the slide glasses. The tissue sections were visualized using DM2500 fluorescence microscope.

Fig. 7 Blood glucose responsivity assays. Nanoparticles loaded with non labelled peptide were administered orally by gavage (30 IU/Kg) to healthy male Wistar rats fasted for 12 h prior to the experiment. Blood samples were collected, by tail bleeding, 10 min before (basal), and at scheduled times, up to 6 hours after administration. Oral glucose tolerance test (OGTT) was performed by administration of a 2 g/Kg glucose bolus by gavage, simultaneously to the formulations under study; plain glucose bolus was used as control. Blood samples were collected, by tail bleeding, 10 min before (basal), and at various time points, up to 2 h after bolus. In both experiments, glucose level was measured through an enzymatic test Accuchek Compact.
(○ dashed line) glycemia reduction in healthy rats 30 IU/Kg; (□ continuous line) OGTT untreated; (■ continuous line) OGTT 30 IU/Kg. Glycemia reduction in healthy rats: treated *vs* basal, * $P < 0.05$ (un-paired t-Test, two tailed). OGTT: treated *vs* controls, * $P < 0.05$ (un-paired t-Test, two tailed). (N=4)