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Article

Macromolecular confinement of therapeutic protein in polymeric particles for controlled release: insulin as a case study

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Sustained release systems for therapeutic proteins have been widely studied targeting to improve the action of these drugs. Molecular entrapping of proteins is particularly challenging due to their conformational instability. We have developed a micro-structured poly-epsilon-caprolactone (PCL) particle system loaded with human insulin using a simple double-emulsion w/o/w method followed by solvent evaporation method. This formulation is comprised by spheric-shaped microparticles with average size of 10 micrometers. *In vitro* release showed a biphasic behavior such as a rapid release with about 50% of drug delivered within 2 hours and a sustained phase for up to 48 h. The subcutaneous administration of microencapsulated insulin showed a biphasic effect on glycemia in streptozotocin-induced diabetic mice, compatible with short and intermediate-acting behaviors, with first transition peak at about 2 h and the second phase exerting effect for up to 48h after s.c. administration. This study reveals that a simplified double-emulsion system results in biocompatible human-insulin-loaded PCL microparticles that might be used for further development of optimized sustained release formulations of insulin to be used in the restoration of hormonal levels.

Uniterms: Human insulin/study. Microparticles. Poly-ε-caprolactone. Therapeutic proteins/study/action. Proteins/molecular entrapping.

INTRODUCTION

The World Health Organization (WHO) estimates that more than 180 million people worldwide are diabetic and this health problem is responsible for 5% of all deaths each year (Wild *et al.*, 2004; Roglic *et al.*, 2005). The Diabetes Mellitus (DM) is a group of chronic diseases which occur when pancreas does not produce enough insulin or there is a resistance to this hormone, and in some cases are observed production and action of insulin deficient condition increasing blood sugar level and risk of microvascular damage (retinopathy, nephropathy and neuropathy) (American Diabetes Association, 2013; WHO, 2008).

Insulin is a polypeptide hormone synthesized in the β-cells of Langerhans islets in the pancreas. This hormone is composed of two chains linked by disulfide bonds, chain **A** with 21 amino acids and chain **B** with 30 amino acids. Hormone replacement therapy using exogenous insulin is necessary for patients with DM, especially for patients with Type 1 DM. However, a perfect glycemic control is still a difficult task despite the number of insulin formulations in market with different pharmacokinetics characteristics (Veiseh *et al.*, 2015).

Therefore, the developments of pharmaceutical compositions, which reduce the number of insulin shots, are needed for both better glycemic control and for improving patient's quality of life. There are many

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approaches for sustained release development, but not all methods are applicable to biopharmaceuticals due to its high lability. The most common methods used in pharmaceutical biotechnology are: nano and micro-encapsulation, using solid matrixes as PLGA, PLA and PCL; liposomes,, which uses liquid matrixes such as phosphatidylcholine; derivatization with polymers, such as polyethylene glycol; the use of biocompatible gels, such as Pluronic F27; or the combination of two or more of these techniques (Calceti *et al.*, 2004; Almeida, Souto, 2007; Liu *et al.*, 2007a; Liu *et al.*, 2007b; Huynh *et al.*, 2009; Rastogi, Anand, Koul, 2009).

Several approaches have been proposed to make an insulin sustained release system but so far no medicine containing micro or nanoencapsuladed insulin has been available commercially. In our work, we ought to develop a system containing insulin confined into polymeric microparticles, which could be used for the sustained release of insulin *in vivo*. In this study, the PCL was the polymer of choice because it is of low-cost, biocompatible, biodegradable and it has been using in various formulations (Benoit, Baras, Gillard, 1999; Lamprecht *et al.*, 1999; Sinha *et al.*, 2004; Hasan *et al.*, 2007; Wang *et al.*, 2007). We report here a simple method based on double emulsification followed by simple vacuum solvent extraction for the production of PCL microparticles comprising bioactive insulin.

MATERIAL AND METHODS

Chemicals

Poly-ε-caprolactone (MW 65,000 Da), poly (vinyl alcohol) (87-89% hydrolyzed, average MW 31,000-50,000Da) and streptozotocin (STZ) were obtained from Sigma-Aldrich (St. Louis, USA). Biosynthetic human insulin Humulin-R ® (recombinant human insulin, metacresol, gycerol and water for injection) was obtained from local drugstore and stored as directed until use. Type I water was used throughout. Other reagents were from analytical grade.

Preparation of insulin microparticles

PCL microparticles were prepared by a modified double-emulsion (water-in-oil-in-water) solvent evaporation/extraction technique (Benoit, Baras, Gillard, 1999; Damgé *et al.*, 2009). Briefly, 400 µL regular-acting human insulin formulation (Humulin R®) and 100 µL of 5 % PVA aqueous solution was emulsified in a mixed organic solution (5 mL of dichloromethane) containing PCL (100 mg) by an Ultraturrax® at 10,000 rpm for 1 min. Thereafter, the primary emulsion was poured into 25 mL of the 1.5 % PVA aqueous solution and homogenized with magnetic stirrer for 10 minutes. The resulting emulsion was stirred with a magnetic stirrer in low speed and the dichloromethane was purged out by evaporation under reduced pressure. The particles were washed out by centrifuging for 15 min at 14.000 x g at 4 °C and resuspending the particles in 1.5% PVA aqueous solution for three times.

Particle size distribution and morphology

Particle size distribution (mean diameter and polydispersity index) of microparticles was determined at 25°C by dynamic laser light scattering (Shimadzu SALD-2201, Shimadzu, Japan) over the range of 0.005 to 1,000 µm, in a 1.0 cm quartz cell after proper dilution in water. Particle distribution and morphology were also evaluated by conventional optical microscopy with particles dispersed in water over siliconized glass slide. Several viewing fields were registered and particle size measurement was performed using ImageJ software (version 1.42, National Institutes of Health, USA), computed from a set comprising a total of 10,000 particles randomly selected. Results are representative of measurements performed with three microparticles preparations (n=3), to confirm mean diameter obtained by DLS (data not show).

Morphological analysis by scanning electron microscopy (SEM)

The morphological analysis of the resulting microparticles was performed using Scanning Electron Microscopy (SEM). In brief, a suspension of the microparticles was dispersed onto a glass slide and plunge frozen in liquid nitrogen and freeze-dried. Dried samples were then coated with 10 nm gold layer at room temperature and observed in a JEOL6340 Field Emission SEM operating at 5.0 kV.

Insulin encapsulation efficiency and yield

The total amount of insulin entrapped within PCL microparticles was accessed by measuring the amount of nonentrapped protein in the external aqueous solution recovered after centrifugation and washing of the microparticles. Protein loading efficiency was expressed as the percentage of insulin entrapped with respect to the theoretical value (Equation 1). The yield was calculated

using the particles after washing and lyophilization. The value was the percentage of mass recovered as follow (Equation 2). Protein was quantified with the Bradford method (Bradford, 1976), using insulin for standard curve. Insulin for the standard curve was quantified by absorbance measurements at 280 nm (Gill, Von Hippel, 1989).

$$Efficiency\ Load = \frac{\left(\begin{matrix}Total\\Insulin\end{matrix}\right) - \left(\begin{matrix}Insulin\ quantified\ in\\the\ aqueous\ phase\end{matrix}\right)}{Total\ Insulin} \times 100 \quad \text{Eq. 1}$$

$$Yield = \frac{Mass\ after\ lyophilization \times 100}{\sum of\ non\text{-}volatile\ components} \quad \text{Eq. 2}$$

In vitro kinetic release assays

The washed microparticles were dispersed in 20 mL of PBS pH 7.4 and 0.02% NaN_3, distributed in centrifugal microtubes containing 1.0 mL of the microparticles suspension each and incubated statically at 37 °C (Guerreiro *et al.*, 2012). This assay was performed in sink conditions with the theoretical maximum insulin concentration below 0.05 mg/mL. At varying time intervals, samples were centrifuged for 30 minutes at 20,000 g at 4 °C, and the supernatant were assayed for total protein released using the Comassie colorimetric method (Bradford, 1976).

Pharmacological evaluation *in vivo*

Pharmacological evaluation of the hypoglycemic effect of insulin was performed as previously reported (Finotelli *et al.*, 2010) with Swiss male mice (8 weeks; 27 g ± 1 g), housed in a temperature-controlled room with a 12 h light-dark cycle and given free access to water and normal chows. Type 1 diabetes was induced by one intraperitoneal (ip.) injection of STZ (200 mg/kg) dissolved in fresh citrate buffer (100 mM, pH 4.5). After STZ administration (5 days), blood was drawn from mice by tail snip and glucose levels were measured using a glucometer (Accu-Chek® Active - Roche). After STZ treatment, animals, which show more than 300 mg/dL, were considered diabetics. Mice were separated in three groups: i) control: receiving placebo MP (n= 3); ii) receiving soluble regular human insulin (n=5; 5 U/kg); and iii) test: receiving insulin-loaded microparticles (n=5; 10 U/kg). Food was suspended 4 h before the experiments, and all mice were fasted throughout the experiments and kept at 22±2 °C. Mice were administrated by subcutaneous route (s.c.) using a standard 29 gauge needle (BD™). The glycemia was monitored following the recommendations of the Mouse Metabolic Phenotyping Center (MMPC) Consortium from the National Institutes of Health (NIH) (Ayala *et al.,* 2010) by using whole blood from the tail tip of conscious, unrestrained mice using pre-calibrated point-of-care glucometers (Accu-Chek® Active, Roche Diagnostics, Germany; Serial No GN08146937) before injection and at repeated intervals after administration. The pharmacological evaluation over a prolonged time was not possible due to the limitations in the extent of animal starvation. This protocol was approved by the Institutional Bioethics Committee on Animal Care and Experimentation at UFRJ.

Data analysis

Data analysis of insulin release *in vitro* and insulin effects over plasma glycemia was performed to obtain the rates by non-linear least squares fitting, using double exponential equation as follow:

$$C_t = C_0 + A_1*\exp(-k_1*t) + A_2*\exp(-k_2*t) \quad \text{Eq. 3}$$

where $\mathbf{C_t}$ and $\mathbf{C_0}$ are the concentration of insulin or glucose at time **t** and **zero**, respectively; **A** and **k** are the event amplitude (concentration changes) and kinetic rates for phases 1 and 2, respectively.

Statistical analysis

Statistical analysis was performed with the t-test. The significance of the differences in the pharmacological evaluation was calculated and values of $p<0.05$ were considered to be significant. The analyses were performed using the SigmaStat as implemented in SigmaPlot 11 (Systat Software Inc).

RESULTS AND DISCUSSION

Characterization of the microparticles

We have prepared insulin-loaded microparticles using PCL and a simple w/o/w emulsification system. Our preparation yielded microparticles with average size of 9.8±0.6 μm, as evaluated by dynamic light scattering measurements (Figure 1).

Optical microscopy imaging shows uniform, individualized spherical microparticles, with average sizes of 10.8 ± 4.6 μm (Figure 2), in agreement with DLS measurements. The particles have micrometric sizes, as demonstrated by the morphological evidences shown in the optical microscopy, because this data reveal compartmentalized microparticles. The insulin concentration in supernatant of the washing pool indicates efficiency of 64.5±6.8%. After freeze-dried samples

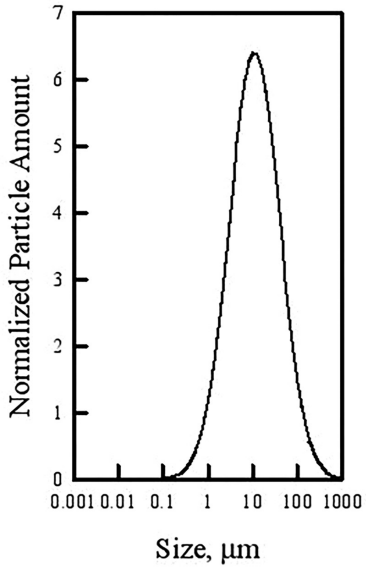

FIGURE 1 - Insulin-PCL particles size distribution. Particles were evaluated by DLS after dispersion in water. Mean particle diameter = 9.8 ± 0.57 µm. Details in the *Material and Methods* section.

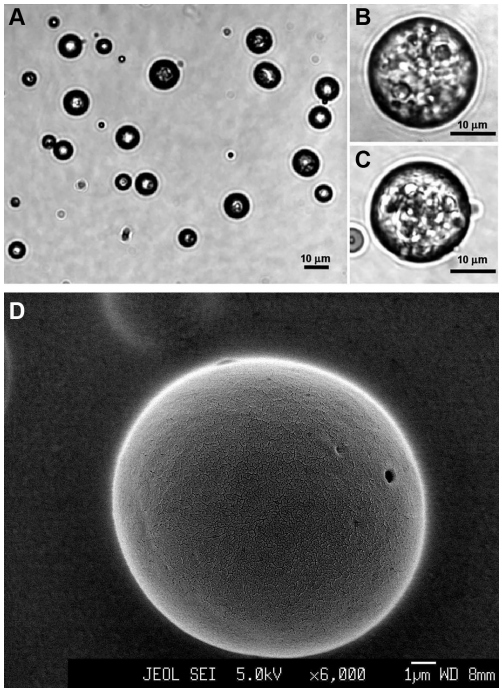

FIGURE 2 - Morphological characterization of insulin-loaded PCL microparticles. Optical microscopy (**A, B and C**). Scanning Electron Microscopy (**D**). Details in the *Material and Methods* section.

were weighed and yield calculated for the process was 88.4±4.2%.

In vitro release

Figure 3 shows the release kinetics *in vitro* for microencapsulated insulin formulation. In this result was possible to observe a burst effect with about 50% of insulin being released in the first 2 hours. The kinetics of *in vitro* release was shown biphasic with an initial burst effect in the first 2 h and a second phase of release, slower, approximately four days.

In vivo release

Were performed *in vivo* experiments using diabetic mice to evaluate the activity of insulin released over time.

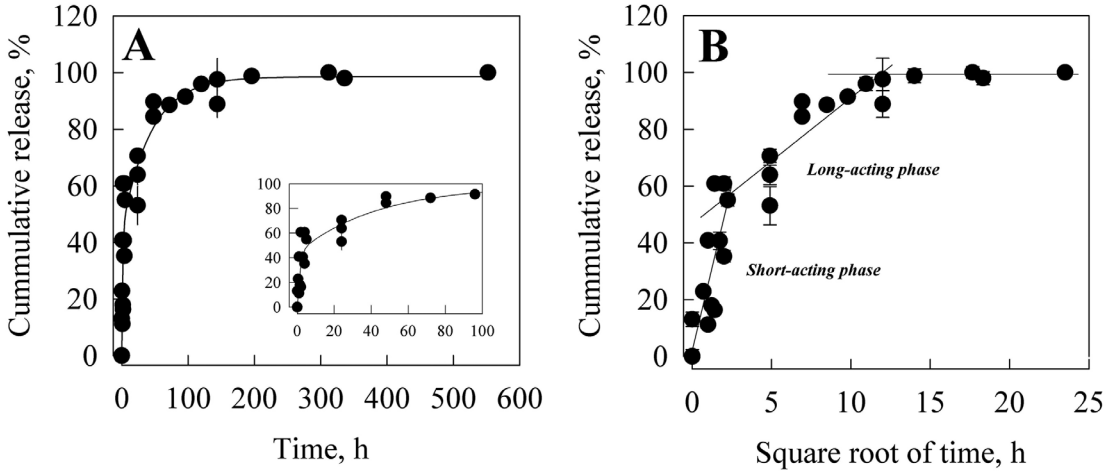

FIGURE 3 - Kinetics of human insulin release from PCL microparticles. A) Cumulative release in a linear time scale. Inset: detail view of kinetics at initial range. Solid lines correspond to best fitting using Equation 1. Results are depicted in Table I and II. B) Higuchi plot. Solid lines are solely to depict human insulin releasing phases. Data represents measurements in triplicate from kinetics performed with three microparticles batches.

TABLE I - Kinetic parameters from the *in vitro* release of human insulin from the PCL microparticles. Parameters were obtained from data in Figure 3

	MP: insulin
Rate constant k_1	$0.76 \pm 0.39\ h^{-1}$
Rate constant k_2	$0.023 \pm 0.009\ h^{-1}$
Amplitude A_1	44.9 ± 9.9 %
Amplitude A_2	55.6 ± 9.8 %

TABLE II - Kinetic parameters from the pharmacological evaluation of the glycemic effect of MP-insulin. Parameters were obtained from data in Figure 3

	MP: insulin	Soluble insulin
Rate constant k_1	$2.58\ h^{-1}$	$2.59\ h^{-1}$
Rate constant k_2	$0.047\ h^{-1}$	$0.18\ h^{-1}$

Figure 4 shows that the formulation was able to reduce blood glucose levels of mice for 24 hours significantly different from that observed in the group of mice that received the commercial formulation of insulin R.

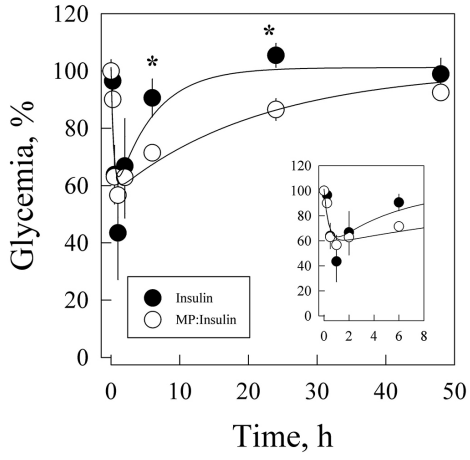

FIGURE 4 - Pharmacological evaluation of MP-insulin in diabetic mices. Streptozotocin-induced mices were administrated s.c. with regular human insulin solution (closed symbols; n=5) or insulin-loaded PCL microparticles (open symbols, n=5) and the glucose level was evaluated. Symbols represent mean ± SD. *Inset*: glycemia change at initial time range. * $p<0.05$ (t-test). Lines are non-linear regression with Eq. 1. Details in the *Material and Methods* section.

The development of formulations with insulin sustained release systems have been previously reported in the literature (Liu *et al.*, 2007a, 2007b; Damgé *et al.*, 2009; Han *et al.*, 2009; Huynh *et al.*, 2009; Kim *et al.*, 2009; Klingler, Müller, Steckel, 2009; Ungaro *et al.,* 2009). Almost recent studies use nanoparticles to increase the insulin effect. However, it was reported the nanoparticles may move from injection sites to the blood vessels leading to the capture of these by the liver and spleen and consequent loss of action (De Jong, Borm, 2008). Despite of all efforts, only a few systems for insulin pulmonary delivery reached the market, and one of these was removed after showed some side effects such as the formation of amyloid deposits in the lungs of patients (Lasagna-Reeves *et al.*, 2010). The encapsulation efficiency of 64.5% is lower than the expected for the method of double-emulsion and solvent extraction using emulsion type w/o/w since other authors using a similar methodology obtained higher efficiency (Damgé, Maincent, Ubrich, 2007; Peng *et al.*, 2012). However, the encapsulation efficiency *per se* is not an exclusion criterion in the development pipeline, once assured proper pharmacological profile and maintenance of physical-chemical stability of the peptide in the formulation. In preliminary studies we tried to use dialyzed insulin, but the formulations without excipients (metacreol, glycerol etc.) did not show satisfactory data (data not shown) indicating that our method, to keep these excipients, was able to better preserve this hormone.

Regarding the release profile *in vitro* was observed that the preparation have a release kinetic over three days, and the first day of a release of most of the material. The kinetics of release stresses of the first 2 hours was also observed in experiments using diabetic mice, and the hypoglycemic effect of the formulation was more than 24 hours of experiment. Interestingly, despite having a release kinetics *in vitro* faster than those described by other authors working with similar formulations developed here, our *in vivo* release data shows a long-lasting action, suggesting a more effective stabilization. We believe that this stability is due in part because of the use of preformulated regular-acting insulin from commercial source (insulin and adjuvants) to prepare the microparticles, since experiments using non-formulated insulin (without excipients) did not result in satisfactory encapsulation efficiency as well as in an erratic release profiles (data not shown). These data demonstrate that the encapsulation efficiency and quality of the resulting product for protein products in a biologically active form is dependent of the formulation process, including stabilization of the proteic active pharmaceutical ingredient. In fact, glycerol and metacresol are important excipients for the stability of commercial insulin formulations.

Glycerol is commonly used as an osmotic agent, but this adjuvant also can prevent the formation of high-molecular-weight protein and aggregates in the

compositions with insulin (Brange, Langkjaer, 1992). Phenol derivatives are used because of their antimicrobial effects as preservatives, but they are capable of reducing deamidation processes, which are the main route of degradation of insulins during storage (Whittingham *et al*., 1998). In additional, phenolic derivates can stabilize interactions between monomers and insulin dimers and hexameric conformation (Crommelin, Sindelar, Meibohm, 2008). In this work we have not investigated the effects of the excipients alone since both are important for the stability of the compositions because they act in different ways, but further studies may be carried out in order to evaluate the stability of the insulins in polymer matrices in the presence of pharmaceutical adjuvants.

Another important finding in our study is the reduction in blood glucose in animals treated in the first points of the test. Thus, although high dose of 10 IU versus 5 IU in control group, the microencapsulated formulation was able to prevent episodes of hypoglycemia in diabetic mice, a major side effect of commercial insulin formulations. Another important application of this system might be the prevention of amyloid insulin formation, since studies reporting insulin aggregates at the application site are frequent (Swift *et al.*, 2002; Nagase, 2009; Lasagna-Reeves *et al.*, 2010). In contrast, polymer particles similar to those developed in this work have been used to inhibit markedly amyloidogenic proteins (Cabaleiro-Lago *et al.,* 2010; Guerreiro *et al.*, 2012).

CONCLUSION

Despite all efforts for developing intelligent drug delivery systems, the characteristics of complex peptides, like insulin, added to the specific needs for diabetes treatments make this a challenge that seems to have a distant end. Our work has shown a simple and scalable way to produce longer lasting insulin formulation with no hypoglycemic effect in the tested dose, which can improve the quality of the therapeutic intervention.

ABBREVIATIONS

PCL, poli-ε-caprolactone; MP, microparticles; w/o/w, water-in-oil-in-water emulsion; s.c., subcutaneous; STZ, streptozotocin; PVA, poly(vinil alcohol).

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CONFLICT OF INTEREST

"Prof. Luis Mauricio T. R. Lima, Prof. Luiz H. Guerreiro, Prof. Eduardo Ricci-Junior and Camila Moreira Mascarenhas are applicant of a patent regarding insulin controlled release. "Sistema polimérico de confinamento de insulina, processo e uso de dito sistema". Camile Moreira Mascarenhas, Eduardo Ricci Júnior, Luís Mauricio T. R. Lima, Luiz Henrique Guerreiro Rosado. PI 1103164-6 A2.

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