Reduction of the *in vivo* burst release of insulin-loaded microparticles

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A B S T R A C T

The main objective of this work was to evaluate the behavior of composite microparticles (microparticles containing nanoparticles) for parenteral delivery with a view to reducing the initial burst release of short half-lives peptides/proteins (using insulin as a model drug). These composite microparticles were prepared with hydrophobic and biodegradable polymers [Poly (ε-caprolactone), poly (lactic-co-glycolic) acid] by the double emulsion extraction technique. Particles were administered subcutaneously (1 IU/kg of insulin) as a single dose to diabetic rats (streptozotocin-induced) and serum insulin levels were monitored. The results show a significant reduction of insulin release from composite microparticles both *in vitro* (around 19% after 15 min) and *in vivo* (around 582 μU/mL) with a progressive and controlled release profile up to 24 h when compared to simple microparticles (without nanoparticles inside) and nanoparticles. These experiments demonstrate that a desirable insulin injection formulation with negligible burst effect *in vivo* has been developed. Such effect was attributed to the double polymer barrier that the drug has to diffuse through before reaching the external medium either *in vitro* or *in vivo*.

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1. Introduction

Bioactive proteins and peptides are a rapidly growing class of therapeutic agents. Injections of peptides/proteins have to be renewed frequently because their *in vivo* half-life is generally rather short. Therefore, sustained delivery of proteins and peptides is still of major interest for parenteral administrations [1].

However, parenteral peptides or proteins controlled release products based on biodegradable polymers (nano/microparticles and hydrogels) or lipid-based systems (liposome and solid lipid nano/microparticles) often present an inconsistent release profile characterized by a high initial burst effect defined as the initial release of a large bolus of drugs [2–4]. This initial burst phase is then followed by a plateau frequently associated with an incomplete release [4]. In most cases, the burst release corresponds to a significant loss of drug from both therapeutic and economic standpoints. Indeed, high initial drug release is not suitable for parenteral peptides and proteins products because drug released in this period is no longer available for prolonged release and may lead to a high risk of side effects (due to elevated serum levels) especially for drugs with narrow therapeutic index and short half-

life. For this reason, one of the goals of protein microencapsulation is to reduce the initial burst and achieve a constant release rate thereafter.

For many parenteral therapeutic proteins, achieving acceptable high drug content into particles while maintaining satisfactory release kinetics (*i.e.* minimal burst together with acceptable duration), represents a formulation challenge.

Protein release from biodegradable polymeric particles during the initial release stage depends on diffusion escape of the protein through channels existing in the polymer matrix. Given that the time required until the onset of polymer degradation ranges from weeks to months, drug release during the first few days (or hours) depends on how successfully the diffusion is controlled. In most cases, the burst release is due to poor control over the diffusion based release in this stage. The degree of initial burst from the nano/microparticles depends on the ability of the polymer matrix to encapsulate the protein, thereby making it unavailable for immediate diffusion [4,5]. For this reason, efforts to reduce the initial burst have followed in the same track as those to increase drug encapsulation efficiency.

Initial burst release of peptides and proteins from polymeric matrix may depend on their molecular weight. Peptides and proteins with small molecular weights (for example triptorelin, calcitonin) may escape from the polymeric matrices through the pores and cracks that form during the particles manufacturing process [3,6,7]. On the other hand, burst release may also occur with high

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molecular weight proteins (immunoglobulin G [8], Glial cell line-derived neurotrophic factor (GDNF) [9], α-interferon [10]) due to i) their heterogeneous distribution in the matrix ii) their loosely association with the surface or iii) their embedding in the surface layer [3,4].

In a first report [11], we have prepared composite microparticles corresponding to drug loaded poly(ε-caprolactone) (PCL) nanoparticles encapsulated into Eudragit® RS or ethyl cellulose microparticles. This study showed high encapsulation efficiency and low initial burst effect of triptorelin (hydrophilic drug model peptide). However, the concept of controlling the burst effect by encapsulating nanoparticles into microparticles was only demonstrated *in vitro*. In addition, the tested microparticle polymers (Eudragit® RS and ethyl cellulose) are not acceptable for parenteral administration (subcutaneous or intramuscular). Thus, we decided to use a biodegradable polymer such as poly (lactic-co-glycolic) acid (PLGA) to replace Eudragit® RS or ethyl cellulose in the microparticles matrix. We first tested the influence of this new composition, *in vivo*, on the burst release of a lipophilic drug, i.e. ibuprofen [12]: as a matter of fact, an important reduction of the burst release was demonstrated. In the present work, we aimed to further extend this concept to the field of peptides/proteins delivery. Indeed, most multiparticulate-marketed systems concern peptides or proteins material. Contrary to ibuprofen, peptides/proteins are highly water-soluble molecules whose burst effect is more difficult to control. Due to its broad interest and its easy serum assay, not mentioning its relatively low cost, insulin was chosen as the peptide drug model. Indeed, patients with type 1 diabetes mellitus depend on external insulin (most commonly subcutaneously injected) for their treatment. Patients with type 2 diabetes mellitus have insulin resistance, which is progressively associated with a reduced production of insulin; thus, some type 2 diabetic patients require insulin when oral antidiabetic drugs become insufficient in controlling blood glucose levels. It is also well known that insulin-dependent patients need a relatively constant basal insulin supply to attain a near-normal physiological pattern of insulin.

In order to achieve blood glucose control in diabetes, intensive insulin treatment is used. This treatment involves the daily injection of one or more doses of intermediate- or long-acting insulin to satisfy basal insulin requirement, as well as injection before each meal. The multiple daily injections regimen leads to poor patients' compliance, pain and even mental stress. Theoretically, injectable polymeric or lipidic delivery systems (microspheres, microcapsules, microemulsion, liposomes, and gel) could be used for controlled release of insulin continuously for a desired period of time [13,14]. Such a release pattern would satisfy continuous low-level of basal insulin requirement. Basal insulin delivery from these novel systems could partly relieve patients from multiple daily injections. Such formulations would contribute not only to an improvement in the patient's compliance, but also to a reduction of developing additional diabetes complications [15]. In the literature, there are a lot of attempts to prepare satisfactory parenteral sustained insulin formulation without burst release [14,16–19].

The purpose of the present study was to develop and test *in vivo* composite microparticles based on biodegradable polymers only, thus adapted to the subcutaneous injection and *in vivo* administration of insulin.

2. Materials

Regular and fast human insulin (Actrapid®) was from Novo Nordisk (Bagsvaerd, Denmark): concentration of the solution was 100 IU/mL of human insulin. Excipients of Actrapid® are metacresol, zinc chloride, glycerol, sodium hydroxide and/or hydrochloric acid and water for injectable preparation. Poly

(ε-caprolactone) (Mw 40,000 Da) and D-L poly(lactic-co-glycolic) acid 50:50 (m/m) Resomer RG 504S end-capped (M_W 48,000; viscosity: 0.47 dL/g) were purchased from Aldrich, (Saint Quentin Fallavier, France) and Boehringer Ingelheim (Ingelheim, Germany), respectively.

Polyvinylalcohol (PVA, Mw 30,000, 88% hydrolyzed) was supplied by Sigma (Saint Quentin Fallavier, France). Ethyl acetate (water solubility = 8.3 g/100 mL at 20 °C) was purchased from Fluka Chemie GmbH (Buchs, Switzerland). Methylene chloride (water solubility = 1.3 g/100 mL at 20 °C) was supplied by Prolabo (Paris, France). Acetonitrile and orthophosphoric acid were obtained from Carlo-Erba (Val de Reuil, France) and Prolabo (Paris, France), respectively. All other chemicals were of analytical grade and used without further purification.

3. Methods

3.1. Preparation of particles

3.1.1. Nanoparticles

Insulin loaded PCL nanoparticles were prepared by the water-in-oil-in water (W/O/W) solvent evaporation method [20]. Briefly, 1 mL of Actrapid® aqueous solution was emulsified for 15 s in 5 mL of methylene chloride (containing 125 mg of PCL) with the help of an ultrasound probe (Vibra cell 72 434, BioBlock Scientific, Strasbourg, France) at 80 W output. This primary emulsion was poured into 40 mL of a 0.1% PVA aqueous solution and sonicated again with the same ultrasound probe for 1 min in the same conditions in order to create the water-in-oil-in-water (W/O/W) emulsion. Three mL (±1 mL) of nanoparticles suspension were obtained after solvent evaporation under reduced pressure.

For incorporation determination, nanoparticles were separated from the bulk suspension by centrifugation (Biofuge Stratos; Heraeus Instruments. GmbH&Co., Hanau, Germany) at 42,000 × g for 20 min. The supernatant was kept for drug assay according to the methods described later and the sedimented nanoparticles were then redispersed in 3 mL of purified water before freeze-drying. After lyophilization, a dry powder of nanoparticles was obtained: the freeze-dried nanoparticles powder was used for dissolution purposes only. The nanoparticles preparation method was slightly modified for manufacturing of composite microparticles (nanoparticles in microparticles). Indeed, the only difference was that the solvent evaporation process was continued till 1.5 mL (±0.5 mL) of nanoparticles were obtained: this suspension was used directly (without freeze-drying) as the internal aqueous phase in the preparation of the composite microparticles in order to prevent any issue with regards to particle size change between manufacturing and freeze-drying process. Blank nanoparticles were prepared under the same conditions but without drug.

3.1.2. Microparticles

Microparticles containing insulin PCL nanoparticles (so-called composite microparticles) or not were prepared by the W/O/W solvent extraction method [21]. In the first step (W/O emulsion), the PCL nanoparticles suspension (1.5 ± 0.5 mL as mentioned before) was used as the internal aqueous phase, which was emulsified (ultrasound probe at 80 W output for 15 s) in the organic solution of ethyl acetate (10 mL) containing PLGA (150 mg).

This primary emulsion was poured into 30 mL of 0.1% PVA aqueous solution in order to obtain a W/O/W pre-emulsion. After magnetically stirring for 20 s (600 rpm) at room temperature, this pre-emulsion was added to 1 L of purified water and stirred mechanically (three-bladed propeller, 600 rpm) for 10 min to form the final W/O/W emulsion.

Upon solvent extraction, the polymers precipitated and the

microparticles cores solidified. Microparticles were collected by filtration (Millipore® Type: 0.45 μm acetate cellulose (low protein adsorption affinity) Macherey−Nagel, Hoerdt, France) and freeze-dried.

Blank PLGA composite microparticles (with blank PCL nanoparticles inside) and PLGA simple microparticles without nanoparticles inside *i.e.* simple microparticles (with or without insulin) were prepared under the same conditions. For insulin loaded simple microparticles, a solution of insulin (Actrapid® 100 IU/mL) was used as the internal aqueous phase.

3.2. Mean diameter and zeta potential

Mean diameter and size distribution of microparticles were analyzed by laser diffraction in a particle size analyzer (Mastersizer S, Malvern Instruments, Orsay, France) after dispersion in purified water. Each sample was measured in triplicate.

The mean diameter of nanoparticles and their surface potential were evaluated with a Malvern Zetasizer 3000 HSA (Malvern Instruments, Orsay, France) using respectively photon correlation spectroscopy and electrophoretic mobility. Nanoparticles were diluted in NaCl 0.001 M prior to zeta potential measurements. Each sample was measured in triplicate.

3.3. Determination of insulin content in the particles

The amount of insulin entrapped within polymeric particles was determined according to an established but slightly modified HPLC method [22] by measuring the amount of non-entrapped insulin in the external aqueous solution (indirect method), which was recovered after filtration of microparticles. In the case of nanoparticles, the external aqueous solution was obtained after centrifugation of the colloidal suspension for 20 min at 42,000 × g.

Briefly, 50 μL of the external aqueous solution were injected into the HPLC system (Shimadzu HPLC 10A vp, Shimadzu, Champs sur Marne, France) with UV detection (SPD-10 A VP, Shimadzu, Champs sur Marne, France) and a data processing software (model Class VP). The separation was achieved by using a C-18 reversed phase column (250 × 4.6 mm, 5 μm, 300 Å − Vydac, Interchim, Montluçon, France) at 40 °C. The detection wavelength was set at 214 nm. The flow rate of the mobile phase was 1.2 mL/min [(A) water acidified with 0.1% trifluoroacetic acid; (B) acetonitrile acidified with 0.1% trifluoroacetic acid] changed by gradient way to separate metacresol peak from insulin one. A standard calibration curve was performed with the Actrapid® solution in the 0.1% PVA aqueous solution. The established linearity range was 0.05−2 IU/mL (r > 0.99).

3.4. In vitro drug release from both nanoparticles and microparticles

Fifty mg of freeze-dried loaded particles were suspended in 20 mL of saline phosphate buffer (KH_2PO_4 4.4 mM, Na_2HPO_4 45.1 mM, NaCl 0.1 M, pH 7.4 adjusted by orthophosphoric acid). Dissolution studies were carried out under sink conditions. The particles suspension was gently stirred (200 rpm) at 37 °C into a water bath. One minute before sampling, stirring was stopped in order to prevent microparticles or nanoparticles elimination from the medium. One milliliter of suspension was withdrawn at appropriate intervals (5, 15, 30, 45 min, 1, 2, 3, 4, 5, 6, 8, 24 h) and centrifuged at 42,000 × g for 10 min (due to established insulin filter adsorption during the preliminary trials). Withdrawn volume was replaced by the same volume of fresh buffer. The amount of released insulin was determined by HPLC coupled with UV detection at 214 nm as previously described. A standard calibration curve

was performed with the Actrapid® solution in the aqueous solution of saline phosphate buffer. Each particle batch was analyzed in triplicate.

3.5. In vivo studies of insulin-loaded particles in diabetic rats

3.5.1. Animals

Adult male Wistar rats (300 ± 20 g) (Charles River laboratories, France) were housed in air-conditioned quarters under a photo-period schedule of 12 h light/12 h dark. They received standard laboratory chow diet (UAR, Villemoisson-sur-Orge, France) and tap water, available ad libitum. All experiments were carried out in accordance with the European Community Council Directive of November 24, 1986 (86/609/EEC) (authorization 54−68 from Direction Départementale des Services Veterinaires, Malzeville, 54220, France).

3.5.2. Induction of diabetes

Diabetes was induced in male Wistar rats by an intravenous injection of streptozotocin (65 mg/kg) in a 10 mM citrate buffer at pH 4.5 as previously described [23]. Rats were considered diabetic when glycemia was higher than 300 mg/dL (about three weeks after streptozotocin treatment). Type 1 diabetic rats provide the best *in vivo* model available for our study as these rats produce very little endogenous insulin; therefore, the observed serum insulin concentrations are purely a result of the insulin delivery system [18].

3.5.3. Treatment and serum collection

Unloaded composite microparticles (control) and insulin-loaded particles (PCL nanoparticles, PLGA simple microparticles and PLGA composite microparticles) were injected subcutaneously (20 IU/kg) as a single administration (aqueous suspension) to overnight fasted (water at libitum) diabetic rats. As a reference, an insulin aqueous solution (Actrapid® Novo-Nordisk, 100 IU/mL) was administered intravenously in control animals at 1 IU/kg.

The administered volume was 100 μL/100 g of rat in a 2.25% carboxymethylcellulose aqueous solution (m/v) for microparticles. Rats were anesthetized (slight anesthesia prior and sequentially from 30 min to 24 h after administration) by intraperitoneal injection of pentobarbital sodium (15 mg/kg). Animal groups (5 groups of 3 rats each) were randomly done. Blood (400 μL) was collected by cardiac puncture 15, 30, 45 min and 1, 2, 4, 6, 8, 10, 24, 48, 72 and 96 h after administration into 1.5 mL polypropylene vials. After 30 min storage at 4 °C and centrifugation at 3000 × g for 10 min at 4 °C, the obtained serum was immediately stored at −20 °C. Due to the 5 animal groups' randomization, only 2 blood samples per animal were collected during the first 24 h for each formulation. For further times, blood was sampled from two groups leading to 6 samples at each time.

3.5.4. Insulinemia and absolute bioavailability after sub-cutaneous administration of insulin-loaded nanoparticles

Insulin (loaded or unloaded) particles (20 IU/kg) were administered subcutaneously to overnight fasted diabetic rats according to the scheme shown before. Aqueous free insulin (1 IU/kg) was intravenously administered in a second group of rats. Blood serum insulin concentrations were measured by radioimmunoassay (Insulin-CT kit from CIS Bio International, Gif-sur-Yvette, France). The areas under the curves (AUC) of the concentration−time profiles were calculated with the linear trapezoidal method. The absolute bioavailability was calculated by the ratio of the respective AUCs corrected by the administered doses.

3.5.5. Statistical analyses

The results were expressed as mean values ± standard deviation

(SD). For the pair-wise comparison, the Mann–Whitney U test was used to investigate statistical differences. The statistical treatment with more than two groups was performed with Kruskal–Wallis test followed by Dunn's test, except when normality and equal variance were passed, it was followed by the Tukey test. In all cases, $p < 0.05$ was considered to be statistically significant.

4. Results

Tables 1 and 2 summarize the main physico-chemical parameters (mean diameter, zeta potential and encapsulation efficiency) of nanoparticles and microparticles, respectively. Both unloaded PCL nanoparticles and insulin loaded PCL nanoparticles showed a diameter around 380 nm and beared almost no charge. The encapsulation efficiency was high ($80 \pm 4\%$) (Table 1).

Insulin loaded simple microparticles (71 ± 7 μm) were smaller than blank ones (92 ± 4 μm), but insulin loaded composite microparticles had the same size as blank ones (Table 2). Although it is very difficult to see a general trend, it can be noticed that simple microparticles (either unloaded or loaded with insulin) are somehow larger than composite microparticles. For both types of microparticles, the insulin encapsulation efficiency was high (composite microparticles; 99%, simple microparticles; 90%) (Table 2). Therefore it is obvious that nanoparticles (blank and insulin loaded) are very well incorporated in the matrix of composite microparticles.

Fig. 1 displays the *in vitro* release profiles of insulin from the three types of formulations (PCL nanoparticles, PLGA simple microparticles and PLGA composite microparticles). Insulin loaded PCL nanoparticles and PLGA simple microparticles display an important drug release or burst (around 41% and 36%, respectively) in the first 15 min followed by a plateau up to 24 h for PCL nanoparticles (around 50% at 24 h). The same type of profile is observed for PLGA simple microparticles with a maximum release of around 56% (Table 3). A somehow different release profile was obtained with PLGA composite microparticle formulations. Indeed, the initial insulin burst release was the lowest (around 19% after 15 min) with a progressive and controlled release profile up to 24 h for PLGA composite microparticles (around 39% after 24 h). Moreover, a common trend for all tested particles was the non-complete release of insulin in 24 h.

An *in vivo* study was conducted by injecting subcutaneously the 3 types of polymer particles into diabetic rats. The serum insulin profiles (Fig. 2) show the insulin level in the 3 treated diabetic groups as well as the control group (diabetic rats treated with blank PLGA composite microparticles). In Fig. 2A, the intravenous administration of insulin solution (Actrapid®) (1 IU/kg) gave rise to an immediate peak at the first sampling time. In contrast, serum insulin levels of the control group (diabetic rats treated with blank PLGA composite microparticles) showed a low insulin level (around 20 μU/mL) throughout the study, which is the same as insulin serum level of rats without any treatment. Treatment with insulin PCL nanoparticles led to a high initial insulin serum level of about 2400 μU/mL within 30 min after particles injection (Fig. 2B) which can be related to the 40% (at 15 min.) *in vitro* insulin burst release (Tables 3 and 4); this initial peak is followed by a very fast decrease
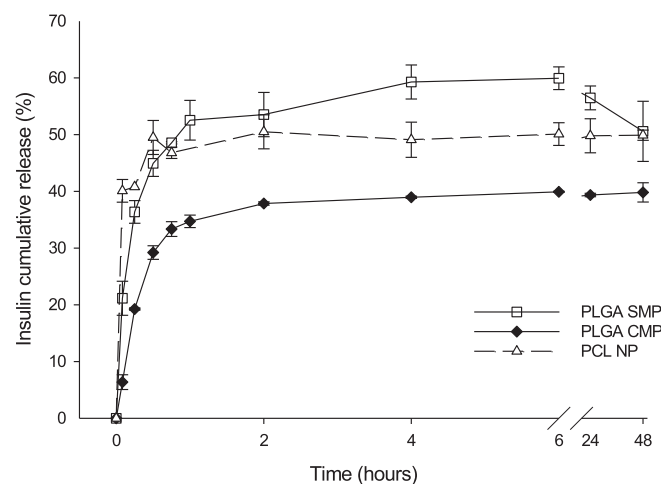
Table 1
Mean diameter, drug encapsulation efficiency and zeta potential of blank or insulin loaded PCL nanoparticles (NP) (n = 3 ± SD).

	Blank NP	Insulin NP
Mean diameter (nm)	375 ± 6	390 ± 17
Zeta potential (mV)	−1.3 ± 1.6	0.2 ± 0.6
Encapsulation efficiency (%)	−	80 ± 4

Table 2
Mean diameter and drug encapsulation efficiency of blank, insulin simple microparticles (SMP) and composite microparticles (CMP) (n = 3 ± SD).

	Mean diameter (μm)		Encapsulation efficiency (%)	
	Blank	Insulin	Blank	Insulin
SMP	92 ± 4	71 ± 7	−	90 ± 3
CMP	113 ± 2	111 ± 4	−	99 ± 0.3

Fig. 1. Release profiles of insulin [mean cumulative release (%) versus time (hours)] from PCL nanoparticles (NP), PLGA simple microparticles (SMP) and PLGA composite microparticles (CMP) in pH 7.4 phosphate buffer under sink conditions (n = 3 ± SD).
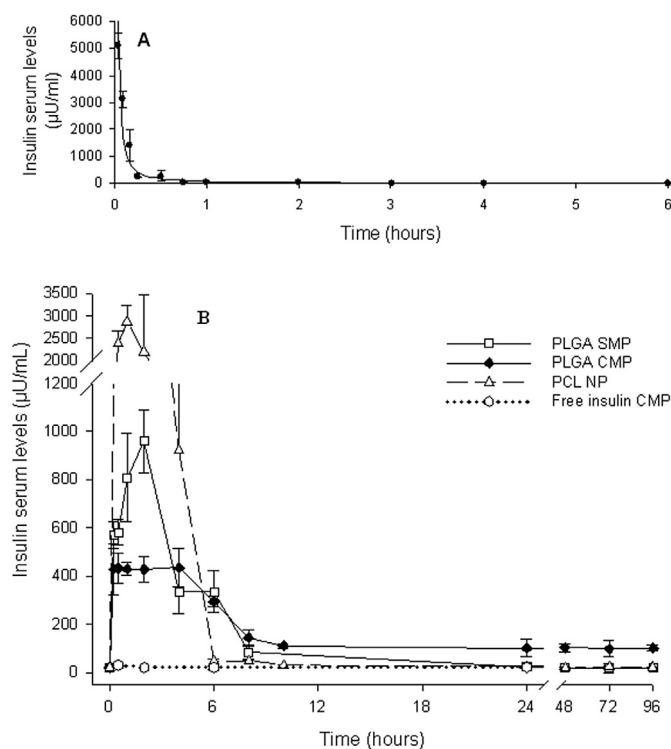
in serum insulin which reach the initial basal level after 6 h (Fig. 2B). Relatively lower serum insulin levels were initially detected (around 582 μU/mL in 30 min) when PLGA simple microparticles were administered (Fig. 2B). Then insulin serum levels increased progressively to a peak value of 959 μU/mL that was reached after 2 h. With later times, a gradual decrease back to initial serum insulin levels was observed after 24 h. On the other hand, in the case of composite microparticles, the increase and decline of insulin serum level was more gradual and there was a steady insulin plateau up to 72 h (Fig. 2B). Indeed, during the first 24 h the insulin serum levels following the composite microparticles subcutaneous administration displayed fairly stable concentrations without any remarkable modification. Moreover, insulin serum level decreased slowly and the initial serum basal level was reached after a much longer time (up to 96 h) than with PLGA simple microparticles. Such a profile means a continuous insulin release leading to stable blood concentrations for longer times with remarkably low burst release as already noticed *in vitro*.

The pharmacokinetic parameters derived from the serum concentrations are summarized in Table 5. The highest serum concentration (Cmax 2872 μU/mL) was observed with the PCL nanoparticles after 1 h but only at 2 h for the PLGA simple

Table 3
Mean percentage (n = 3 ± SD) of insulin released after 15 min and 24 h from PCL NP, PLGA simple (SMP) and PLGA composite (CMP) microparticles *in vitro*.

Formulations	Polymers	Insulin released (%)	
		15 min	24 h
PCL NP	PCL	40.8 ± 0.1	49.8 ± 3.0
PLGA SMP	PLGA	36.4 ± 2.0	56.5 ± 2.0
PLGA CMP	PLGA/PCL	19.3 ± 0.2	39.4 ± 1.7

Fig. 2. Mean insulin serum concentration versus time (hours) after intravenous injection of 1 IU/kg of insulin as a solution (A) or after subcutaneous injection of 20 IU/kg of insulin [PCL nanoparticles (NP), PLGA simple microparticles (SMP) and PLGA composite microparticles (CMP) (B)] to rats (n ≥ 3 ± SD).

microparticles (Cmax 959 μU/mL). Finally, the lowest insulin Cmax (435 μU/mL) was obtained at 4 h after PLGA composite microparticles administration. Based on the concentration−time profiles, areas under the insulin concentration curves (AUCs from 0 to 24 h) were calculated and ranked according to the following order: PCL nanoparticles >PLGA simple microparticles = PLGA composite microparticles. However, in the case of PLGA composite microparticles, the AUC still increased up to 96 h. The *in vivo* results show that the highest absolute bioavailability (with regards to the insulin solution) was obtained with the nanoparticles suspension [65% (0−10 h)] whereas simple microparticles and composite microparticles exhibit only around 35% (0−24 h). It has to be noted that these results have been calculated between 0 and 24 h although insulin was still detected much later in serum for composite microparticles leading, for instance, to a 48% absolute bioavailability after 48 h.

Table 4
Mean insulin serum concentrations (μU/ml) in diabetic rats 30 min and 8 h after an intravenous injection of insulin solution (1 IU/kg) or a subcutaneous injection of polymeric insulin loaded particles (20 IU/kg) (n = 4 ± SD). *Significantly different from PCL nanoparticles (p < 0.05).

Formulations	Polymers	Insulin serum concentration (μU/ml)	
		30 min	8 h
No injection	−	22 ± 2.2	22 ± 2.2
Insulin solution (IV)	−	262 ± 190	16 ± 2
Free insulin loaded CMP (SC)	PLGA (PCL)	30 ± 5	21 ± 6
PCL NP (SC)	PCL	2389 ± 280	47 ± 7
PLGA SMP (SC)	PLGA	582 ± 54	83 ± 24
PLGA CMP (SC)	PLGA (PCL)	433 ± 71*	145 ± 20

Results in Table 5 also indicate that the insulin burst release is significantly (in terms of Tmax, Cmax, and AUC 0-10) more reduced with composite microparticles than with both insulin nanoparticles and simple microparticles confirming the results observed *in vitro*.

5. Discussion

Recent improvements in insulin formulations and delivery, including ultra-fast acting and intermediate to "long-acting" (once a day) injections have allowed the development of basal-bolus insulin administration regimens that better mimic the normal pattern of insulin secretion [24,25]. By combining existing delivery technologies, a simple method was developed to manufacture a unique long-acting insulin formulation that shows promise of providing prolonged basal serum insulin levels.

Optimal sustained release insulin dosage forms for parenteral formulation should have high insulin loading together with a significant burst reduction. As mentioned before, drug burst release may occur due to high drug water solubility, non homogeneous drug distribution in matrix systems and/or drug adsorption on surface of the different delivery systems. Insulin formulations (nanoparticles, microparticles, liposomes and hydrogels) frequently display an initial burst release. For instance, Reis et al. [26] prepared insulin-loaded alginate-dextran nanospheres which showed a high *in vitro* burst effect at physiological pH (80% of insulin release after 30 min) but almost zero at pH 1.2. Such a burst effect (high at pH 7.5 and nil at pH 1.2) may be advantageous in the case of formulations intended for oral or pulmonary administration. At the opposite when insulin formulations are intended for parenteral administration, burst release is a major drawback especially when sustained-release is needed.

Among the different attempts to reduce the insulin burst effect, composite structures were already prepared by combination of different techniques: particles coating [27], nanoparticles micro-encapsulation [28], gel or insulin/cyclodextrin complex in microparticles [29–31] and gel containing particles (nanoparticles or liposome) [32,33]. Due to a homogenous distribution of drug-loaded particles (gel, nanoparticles, liposomes) throughout the polymer matrix, microparticles seem to be the most effective to reduce the initial burst. Except for Jiang et al. [31] who showed successful 10-days glucose suppression in diabetic rats after subcutaneous administration of gel in PLGA composite microspheres, there is very little information on the *in vivo* behavior of such composite systems.

On the other hand, heterogeneous composite systems based on hydrophilic polymers cannot effectively control the burst effect of insulin [11]. In addition, some composite systems were prepared by combining hydrophobic polymers (PLGA, ethyl cellulose) with hydrophilic inner polymers (acryloyl hydroxyethyl starch, cyclodextrin complex, gelatin). Such systems were shown to stabilize the entrapped insulin and to incorporate insulin very efficiently (≥80%) but they always displayed, *in vitro*, a rapid and initial burst release of insulin (up to 40%) followed by a slow release.

This is the reason why we have proposed composite microparticles made with hydrophobic polymers. However, the encapsulation of a lipophilic polymer (as nanoparticles) in another lipophilic polymer (as microparticles) is really challenging with classical microparticles techniques.

So, the use of insulin in our composite microparticles would certainly have the potential to eliminate or at least decrease the burst effect. Furthermore, it could be interesting to develop a new injectable and possibly once a day dosage form of insulin (drug with a narrow therapeutic index so with serious risk of side effects from high serum levels), a short elimination half-life drug [34]. Nevertheless, it should be noticed that developing a once a day

Table 5
Main insulin pharmacokinetic parameters after subcutaneous administration to rats (n $\geq$ 3 $\pm$ SD) of different formulations: insulin solution, PCL nanoparticles (NP), PLGA simple microparticles (SMP) and PLGA composite microparticles (CMP). *Significantly different from PCL nanoparticles (p < 0.05).

	Insulin solution IV	PCL NP SC	PLGA SMP SC	PLGA CMP SC
Tmax (hours)	0.033	1	2	4
Cmax (μU/mL)	5109 ± 467	2872 ± 366	959 ± 128*	435 ± 21*
Mean AUC				
AUC 0 → 1 h	662	–	–	–
AUC 0 → 10 h	662	8704	3832	3096*
AUC 0 → 24 h	662	8704	4680	4463
Rel. F (%)				
(0–10 h)	100	65.8	28.9	23.4
(0–24 h)	100	65.8	35.4	33.7
(0–48 h)	100	65.8	35.4	48

dosage form would not be possible with the tested PLGA because of its molecular weight (40,000 Da) allowing drug release *in vivo* for much longer times. In addition to insulin, such composite dosage forms may be an alternative delivery system for those peptides/proteins microparticles suffering from initial burst release.

However, with a view to injecting the composite microparticles subcutaneously, it was mandatory to use two biodegradable polymers. This is why PLGA was selected as the polymer of microparticles.

In our already mentioned previous work [11], the original idea was to use a solvent (ethyl acetate) in which one of the polymers (PCL) would not be soluble during the double emulsion process. Replacing the previous non-biodegradable polymers (ethylcellulose, Eudragit® RS) by PLGA did not change anything since the latter is also soluble in ethyl acetate. Indeed, microparticles were prepared by dissolving PLGA in ethyl acetate, which is a poor solvent for PCL. Therefore, it was possible to use the PCL nanoparticles suspension as the internal aqueous phase in the preparation of the composite microparticles since this polymer is mostly not dissolved in ethyl acetate.

PCL nanoparticles were found in the classical particles size range i.e. under 500 nm [35]. It has to be noticed that loaded and unloaded nanoparticles bear almost no charge.

Insulin was encapsulated with a high efficiency both in nanoparticles and microparticles (simple microparticles or composite microparticles). This high encapsulation efficiency of insulin especially with microparticles may be explained by i) the use of a pre-emulsification step into a smaller volume of external aqueous phase before the final organic phase extraction (necessary in order to avoid premature polymer precipitation and to obtain microparticles) and ii) the overall fast PLGA precipitation when ethyl acetate was extracted in water [4,21]. However, the encapsulation efficiency of insulin in PLGA composite microparticles (99%) was higher than in both PLGA simple microparticles and PCL nanoparticles (90 and 80% respectively). This might be due to the use of the insulin solid suspension as internal phase (S/W/O/W). Thus, it would consequently be more difficult for insulin to diffuse towards the outer water phase than in the case of the two others particles types.

There are two ways to verify that the burst is controlled i.e. either by an *in vitro* dissolution test or by an *in vivo* approach for instance after subcutaneous or intramuscular administration. We have used both approaches to verify the potential burst reduction with composite microparticles.

In vitro (Fig. 1 and Table 3), it is obvious that the lowest burst was achieved with the composite microparticles but the same higher burst effect was observed with PCL nanoparticles and PLGA simple microparticles. Indeed, the burst at 15 min was almost 2 times more with the simple microparticles or with the PCL nanoparticles than with composite microparticles.

Several hypotheses can be taken into account to explain the insulin burst. Among them, the heterogeneous distribution of insulin including surface distribution, physico-chemical nature of the polymeric matrix, morphology of particles and insulin/matrix interactions are likely to happen. In the case of PCL nanoparticles, the main factor driving insulin release is the large exchange surface area developed with the outer medium due to the very small diameter of nanoparticles. Although PLGA simple microparticles have a larger size (300 times more) than PCL nanoparticles, insulin burst effect is almost the same for two formulations. The size effect can be balanced by the relative hydrophobicity of tested polymers. Indeed, PCL is more hydrophobic than PLGA and water uptake is faster for PLGA leading to faster insulin diffusion to the PBS external buffer in the case of PLGA simple microparticles.

Since simple and composite microparticles have been prepared according to the same conditions, differences in process and formulation parameters affecting insulin burst release can be ruled out. However, for composite microparticles it is not the drug but the PCL nanosuspension, which is distributed in the PLGA polymer matrix; thus drug distribution could be different between simple and composite microparticles. Therefore, composite microparticles present mixed characteristics of both PCL nanoparticles and PLGA simple microparticles.

As for the polymer matrix, it is also obvious that, in composite microparticles, there are two barriers for the drug to diffuse through before reaching the outside release medium. The first barrier is due to the PCL polymer of nanoparticles and the second barrier is the outer PLGA matrix: so we can assume that there is no insulin on the particles surface in the case of PLGA composite microparticles. It is reasonable to make the assumption that the double layer of polymers is the main parameter explaining the marked *in vitro* burst reduction.

As observed in Fig. 1, composite microparticles release insulin very slowly since only around 39% of insulin is found in the outer phase after 24 h. This is another interesting feature of the composite microparticles whose very slow release pattern could also be of interest in the development of a long lasting injectable dosage form for any type of peptide or protein.

Although the entire encapsulated insulin was not released within 24 h, the dissolution test was limited to this time since the goal of this research work was to demonstrate the influence of the encapsulation of nanoparticles into microparticles on the initial burst release.

Nevertheless, it has to be noted that prolonging the time of the release study does not always lead to a 100% insulin release. Indeed, Furtado et al. [18], carried out an *in vitro* release study with insulin microparticles made of poly (fumaric-co-sebacic anhydride) and achieved only 40% of insulin release after 12 days. In addition, Jiang et al. [30], prepared insulin composite microparticles made of an inner hydrogel [poly (acryloyl hydroxyethyl starch)] in a PLGA

matrix and they only found 5% of insulin released after 8 days in a glycine buffer (pH 2.8). In the case of nanoparticles, the non total release may also be explained by a partial loss of nanoparticles during sampling but this has no impact on the comparison between simple and composite microparticles.

Due to their small average diameter, the suspension of nanoparticles was easily injected subcutaneously. As for the simple microparticles and composite microparticles, it was first necessary to disperse them in a relatively viscous aqueous solution prior to injection. The carboxymethylcellulose aqueous suspension of simple microparticles and composite microparticles also allowed an easy subcutaneous injection and was characterized by i) a good physical stability (no sedimentation observed during preparation and injection), ii, good syringability (21 G) and iii) a physiological pH of 7.4.

It has also to be mentioned that the different experimental models of diabetes and the different dosages of insulin reported in the literature make results comparison very difficult. For instance, Furtado et al. [18], used rats, which were predisposed to spontaneously develop type I diabetes via cell-mediated autoimmune destruction of the pancreatic β cells, whereas Takenaga et al. [13,31], used rats with streptozotocin-induced diabetes. Also administered doses of insulin were different and ranged from 45 IU/kg [17], 250 IU/kg [13] to 345 IU/kg [31]. In our case, we have chosen to administer the lowest possible dose, which was 20 IU/kg. Other differences included sampled fluids (plasma [13,18], or serum [31]), insulin dosage method (ELISA [18] or RIA [31]) and type of insulin (human [13] or bovine [30]).

As already observed, the insulin nanoparticles display a fast absorption (Tmax 1 h) whereas absorption is much slower for simple microparticles (Tmax 2 h) and even more delayed for composite microparticles (Tmax 4 h). As well known for matrix microparticles, diffusion of the drug through the microparticles is generally the rate-limiting step of absorption. The diffusion pathway is even longer for the composite microparticles due to the double layer of polymers.

Cmax and Tmax are parameters, which reflect the *in vivo* absorption rate of a drug. Therefore the initial burst is correlated to the values of the two latter parameters. In terms of burst effect, the three dosage forms can be ranked as: PCL nanoparticles >simple microparticles >composite microparticles. There is a significant difference (six fold times) in Cmax between the PCL nanoparticles and the PLGA composite microparticles. In addition, Tmax is also very different between the two dosage forms (1 and 4 h for PCL nanoparticles and PLGA composite microparticles, respectively). The difference in Cmax between the composite microparticles and simple microparticles is less spectacular but is still about 2.2 times whereas Tmax are 2 and 4 h for simple microparticles and composite microparticles, respectively. However, despite such difference in Cmax between composite and simple microparticles, it was not statistically different. Larger groups of animals should probably be used if one wants to demonstrate a significant difference.

Thus it is confirmed that the microparticle systems, and more particularly the composite microparticles, have a dramatic influence on the initial burst release *in vivo*. The same hypotheses, as already discussed for the *in vitro* dissolution results, may explain the observed *in vivo* results. It has been definitely confirmed that the composite microparticles present a strong potential in reducing the burst effect not only *in vitro* but also *in vivo*.

The *in vivo* study was performed for 96 h in order to evaluate the potential of the 3 multiparticular dosage forms as prolonged release compositions. The insulin serum concentrations reach the quantification limit of insulin in diabetic rats after 10 h and 24 h for PCL nanoparticles and PLGA simple microparticles, respectively. It probably means that insulin release (based on diffusion

mechanism) from particles stopped and the second potential insulin release mechanism (based on polymers degradation) did not begin at least up to 96 h. For the PLGA composite microparticles, the insulin initial serum level for diabetic rats (22 μU/ml) was not reached (insulin serum concentration at 96 h is 101 μU/ml) after the 4th day of the *in vivo* test, which means that insulin was still released from particles till 96 h. Nevertheless, this definitely demonstrates that insulin is released for the longest period of time with composite microparticles and insulin release from particles is diffusion controlled till the next mechanism of release (based on degradation polymers) may eventually start. However, due to a sampling period limited to 96 h, we were not able to observe this later release phase.

Taking into account the relatively low number of animals and the normal *in vivo* variability, the figures of absolute bioavailability for simple and composite microparticles are relatively close and it could be considered that the quantitative bioavailability are the same at 24 h (around 35%); indeed, this is confirmed by the lack of statistical difference between all the dosage forms. In the case of composite microparticles, the bioavailability increased with time because the insulin quantification limit was not reached at 96 h. This shows the great potential of such dosage forms to act as long release microparticles. Our bioavailability results are lower than the ones found by Furtado et al. [18], after subcutaneous administration of insulin poly(fumaric-co-sebacic anhydride) microparticles, who obtained an absolute bioavailability of 61%. However, our composite microparticles still have a potential to release insulin after 96 h and bioavailability could still be increased.

Composite microparticles are characterized by delayed insulin Tmax and a decrease in Cmax. In control experiments, a bolus of insulin was injected; it enters into the circulation and bombards the tissues at a rate faster than these tissues can process insulin. Consequently, tissue receptors quickly become saturated and the remaining unbound insulin is rapidly degraded by virtue of its short half-life [36]. The pharmacokinetic data of our work and other groups [13,18,31], imply that unencapsulated (or fast insulin release formulation as simple nanoparticles) insulin has an extremely high rate of absorption for a very brief period of time at the start of the experiment after which, the insulin has been taken up or degraded and is no longer present in the body for the rest of the experiment. The inherent disadvantage with this type of unencapsulated insulin or fast insulin release formulation is that the short insulin duration fails to provide insulin for controlled glucose regulation throughout the entire experiment and is therefore unable to maintain basal plasma glucose levels past the initial hours of the experiment. In this respect, subcutaneous delivery of fast insulin release formulation fails to mimic the normal physiological insulin secretion. Additionally, excessive exposure of tissues and muscle to injected insulin increases insulin resistance and diabetes complications.

In contrast, the insulin composite microparticles (and to a lower degree the simple microparticles) degrade slowly over a period of several days or weeks, thereby providing controlled insulin diffusion throughout most of the experiment. The implications of such a system (average Tmax around 4 h, average insulin Cmax of only 435 μU/ml and minimum insulin concentration of 100 μU/ml at 96 h) show that insulin is absorbed at a slower rate for a longer duration. The major benefit of this system is that insulin is presented to tissue receptors at a constant rate, as opposed to pure insulin injections where an overload of insulin results in a high degree of insulin degradation. This prevents excessive tissue and muscle exposure to insulin and makes insulin available to regulate glucose levels in the body for a substantially longer time than subcutaneous unencapsulated insulin administrations. Therefore, this type of release more closely resembles the body's normal physiological processes whereby insulin is constantly secreted to

keep plasma glucose at a basal level.

6. Conclusion

Our results confirm the efficacy of composite microparticles to obtain high insulin encapsulation efficiency and limited burst effect with a satisfactory sustained release. Such insulin composite microparticles present an interesting behavior both *in vitro* and *in vivo*. One way to increase or decrease the release rate of drugs from such composite microparticles would be to play on the type of biodegradable polymers used in their manufacturing. Indeed, higher or smaller molecular weights of the 2 types of polymers could lead to tailor made release. Such an approach will be used in the continuation of this work.

It can be concluded that composite microparticles gave better *in vivo* profile than simple PLGA microparticles. Longer *in vivo* studies (>96 h) should be undertaken in order to verify whether insulin could still be recovered in serum after polymer degradation. Nevertheless, it has also to be noticed that such composite microparticles could be of tremendous interest for cutting the burst effect of other encapsulated peptides or proteins as already confirmed *in vitro* in the case of triptoreline [11].

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