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Preparation and Characterization of Polymeric Nanoparticles for Sustained Delivery of Insulin

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ABSTRACT

The present study was aimed at developing and exploring a novel sustained release formulation of PEGylated insulin encapsulated in polymeric nanoparticles that produces prolonged insulin release. The insulin was conjugated with PEG-2000 at specific amino terminus of its B chain. PEGylated insulin was encapsulated in PLGA nanoparticles made by double emulsification method. Insulin conformation and antidiabetic activity were retained after PEGylation and PLGA encapsulation. The nano-spherical particles revealed a low burst release, an important safety feature for an extended release insulin product. In PBS (pH 7.4) at 37^{o}C, formulations with drug content of approximately 14.4% showed very low initial release of insulin over one day and near zero order drug release after a lag of 2-3 days. For animal studies, PLGA nanoparticles loaded with PEGylated insulin were administered subcutaneously as a single injection and produced a release of 15% insulin in the first day but then lowered the serum glucose levels of diabetic rats to values <200 mg/dL for approximately 2 days. Based on these findings, it is suggested that the novel PEG–insulin encapsulated PLGA nanoparticles can be used as a carrier for prolonged and sustained release insulin formulation.

Keywords: Insulin, Nanoparticle, Poly(ethylene glycol), PLGA

1. INTRODUCTION

Diabetes is a worldwide disease of near epidemic proportion, with more than a 150 million people currently diagnosed with the disease and this number is expected to double by 2025 [1]. Diabetes mellitus is a metabolic disorder characterized by hyperglycemia, glycosuria, hyperlipemia, negative nitrogen balance and sometimes ketonemia. It is a group of diseases in which the regulatory activity of insulin may be defective in different ways. The morbidity and mortality associated with this increasing prevalence of diabetes have focused considerable effort in strategies to try to better manage the disease [2, 3]. Recent developments in insulin formulation and delivery, including ultra-fast acting and intermediate to long-acting (one day) basal injections have encouraged the development of basal-bolus insulin administration programs that better mimic the normal pattern of insulin secretion [4, 5]. By combining nanotechnology and chemical modification of insulin molecule, a long-acting insulin product can be formulated which may provide a basal insulin requirement with weekly single dose. One major reason for chemical modification is the inherent instability of insulin when it is exposed to the conditions normally encountered during nanoparticle fabrication [6]. Structural and chemical instability often results in irregular release of insulin from nanoparticles [7, 8]. Other problems encountered are low encapsulation efficiencies and high initial burst release of drug, both of which may be linked to the same underlying phenomena [9].

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Various methods for increasing the insulin stability during encapsulation process and release from polymeric matrices have been attempted, with limited success. Examples of such methods include use of traditional stabilizing excipients, hydrophobic ion-pairing, and the addition of surfactants to the protein solution prior to encapsulation [10-12].

PEGylation is known as attaching the PEG molecules with the active drug moiety, and can be utilized to enhance insulin stability as shown in published reports. PEGylation can effectively increase the stability of several therapeutic proteins (e.g., EGF, insulin, a-interferon, and Fab' fragments) encapsulated within PLGA microparticles [13-16]. Unfortunately, those attempts have not produced products with low drug burst and ideal release profiles. A new method of insulin entrapment is described here is PEGylation of insulin at a specific site and encapsulation in PLGA nanoparticles prepared by double-emulsification technique. Nanoparticles formulated by this method show a low burst release with a zero order release profile, and complete release of encapsulated insulin. Prominently, this method allows for high encapsulation efficiency and the insulin content of the nanoparticles is such that a low dose volume is achievable [17].

2. MATERIALS AND METHODS

2.1 Materials

Crystalline human Zinc-insulin was procured from Cadila Pharma. Ltd. (Ahmedabad, India) and used without further purification. Methoxy polyethylene glycol-succinimidyl propionate (mPEG-SPA, 2000 Da) and Poly lactic-co-glycolic acid (PLGA, molar ratio, 45:55, MW 6500Da), were purchased from Sigma (St. Louis, MO). The Accucheck-Advantage blood glucose monitor was a product of Roche Diagnostics Corp. (Mumbai). The human insulin ELISA kit (Active Insulin ELISA) was from Genei Pvt. Ltd., Bangalore. Streptozotocin sodium citrate, citric acid, and Tween 20 were purchased from Sigma (St. Louis, MO). The water used in these studies was of MilliQ quality and all other reagents and solvents used were reagent grade or analytical grade.

2.2 PEGylation of insulin molecules with mPEG

PEGylation of insulin molecules was achieved by conjugating a single mPEG-2000 polymer chain specifically to the amino terminus of B chain (PheB1) of insulin via a hydrolytically stable amide bond. It is already reported that PEGylation of insulin with PEG of different molecular weights, PEG-2000 possess optimal properties as desired in the present study. Zink-insulin was reacted with mPEG-2000 SPA according to reported methods [18-19]. Resulted PEGylated insulin was isolated from other reaction species by ion-exchange chromatography, desalted and lyophilized samples were stored at -20^{o}C prior to use.

2.3 Characterization of the PEGylated insulin

2.3.1 Ion-exchange chromatography

The purity of the PEGylated insulin was analyzed using an FPLC (NIPER, Mohali) system fitted with an analytical ion-exchange column. The column was equilibrated with 1 M AcOH/7 M urea containing 0.01 M NaCl at a flow rate of 1.0 mL/min, and the bound insulin was eluted using a NaCl-gradient (0.01–0.3 M) over 30 min. Detection was at 278 nm. This gradient separates the major PEGylated insulin (PheB1) peak from later eluting peaks for other PEGylated insulin (GlyA1, LysB29).

2.3.2 RP-HPLC

Purity of PEGylated insulin was further evaluated by reversed phase HPLC (Shimadzu, Japan) instrument with detection of eluent at 278 nm. A column C18 (4.6mm X 25cm, 5 μm particle diameter) of reversed phase HPLC was equilibrated with 0.1% TFA (solvent A) in 30% acetonitrile at a flow rate of 1.0 mL/min. Following sample injection, a linear gradient of 2.0%/ min solvent B (0.1% TFA in 95% acetonitrile) was applied for 20 min.

2.3.3 MALDI-TOF mass spectrometry

Mass spectroscopy was used to determine the number of PEG chains attached to insulin molecules. All samples were run on model Micromass MALDI/TOF-Spec 2E instrument, mass spectrometer (Punjab University, Chandigarh) operated in the linear mode, and positive ions were monitored.

2.4 Nanoparticle preparation

PLGA nanoparticles containing the PEGylated insulin were prepared using a modified version of repeated double emulsification method [20]. 1% PLGA solution was prepared by dissolving 30 mg PLGA in 3 ml DCM. This solution was injected in solution of PEGylated insulin with continuous stirring for 1.5 hr at 4000 rpm. The emulsion so prepared was subjected to ultrasonication for 5 min using probe sonicator (Soniweld, Mumbai, India) the contents were immediately dispersed into distilled water, followed by vigorous mechanical stirring to remove traces of DCM. After complete removal of DCM, the formulation was filtered through 0.45 μm filter (Gelmen Filter, Mumbai, India) to remove impurities.

2.5 Characterization of nanoparticles

2.5.1 Drug loading

PEGylated insulin loaded PLGA nanoparticles were ruptured by dissolving the nanoparticles in acetonitrile, and then precipitating the polymer by diluting with water. The supernatant containing the PEG-insulin conjugate was analyzed by RP-HPLC.

2.5.2 Particle size distribution

Particle size of the prepared nanoparticles was measured using Malvern UK zetasizer and transmission electron microscope (Philips Morgani268, Eindhoven, Netherland) by negative staining of grid containing nanoparticles with aqueous solution of phosphotungstic acid at 44000X magnification.

2.5.3 SEM analysis

Samples were sputtered with gold using an Anatech Hummer 6.2. Images were made with a LEO 435 VP, Eindhoven Netherlands 30 KV for scanning electron microscope at an accelerating voltage of 15 kV and 7.80 KX magnifications.

2.5.4 In vitro drug release

PEGylated insulin loaded PLGA nanoparticles (~20 mg) were dispersed in 1.5 mL of PBS (pH 7.4) containing 0.02% Tween 20 and 0.05% sodium azide and incubated at 37^{o}C with horizontal shaking at 150 rpm. At pre decided time intervals the samples were withdrawn, centrifuged and supernatant was analyzed by RP-HPLC to determine the amount of conjugate released from the nanoparticles.

2.5.5 Far-UV circular dichroism

Insulin, PEGylated insulin, and PEGylated insulin released from PLGA nanoparticles in a dissolution experiment were tested by circular dichroism spectroscopy (Jasco J-720 Spectropolarimeter, Japan). Sample concentrations (~20 μM) were determined using UV-spectroscopy employing an extinction coefficient of $6.2X10^3$ M^{-1} cm^{-1} at 278 nm, with corrections being made to account for the difference in conjugate molecular weight.

2.6 Bioanalytical methods

2.6.1 Glucose assay

Blood glucose levels were measured using a commercial glucose meter (Accu-Checkk AdvantageR). Results are presented in units of mg/dL (range 10–600 mg/dL).

2.6.2 Insulin assay

PEGylated insulin levels in rat serum were determined using a commercially available human insulin ELISA kit (Active Insulin ELISA; Genei Pvt. Ltd., Bangalore) whose output was given in micro-international units per milliliter (μIU/mL).

2.7 In vivo studies

PEGylated insulin loaded PLGA nanoparticles were injected intravenously (IV) into the diabetic albino rats (Sprague Dawley strain) and blood glucose levels were compared with a marketed product regular human insulin injection (40 IU/mL, Sc; Cadila Pharma. Ltd. Ahmedabad, India). The environmental conditions for relative humidity and the temperature in the housing area were monitored in accordance with the study site SOP. The rats were fed a laboratory animal feed (Purina Rodent Chow 5001) and were provided tap water ad libitum. Lighting was on a standard 12 h on /12 h off cycle.

On Day 1, all animals were treated with streptozotocin at 40 mg/kg SC followed by 5 mL of normal saline injected at a different SC location. Glucose levels on Day 0 prior to treatment ranged from 560–724 mg/dL. Six animals were treated once on Day 0 by SC injection of the nanoparticulate formulation containing PEGylated insulin at a dose level of 75 IU/kg and one group of six animals was left untreated to serve as a negative control. Prior to treatment on Day 0 and at post treatment Hours 1, 3, 6, 12, and 24 and Days 3, 5, 7, 9, and 11, blood was collected via the tail vein for analysis of insulin and glucose concentrations.

3. RESULTS AND DISCUSSION

3.1 PEGylation of insulin molecule

3.1.1 Conjugate analysis

Chromatographic analysis by RP-HPLC and ion-exchange chromatography showed a purity of >95% for the isolated PEGylated insulin. Mass spectrometry showed a characteristic peak of PEG-linked protein at mass 7,830. No signals for plain insulin or multiply conjugated insulin were observed. As the molecular weight of insulin is 5808 Da, and PEG molecule is 2018 Da the result of mass spectra is reliable for the attachment of a single PEG chain with insulin. Figure 1 shows the effects of mPEG conjugation and encapsulation in PLGA on the far UV circular dichroism spectrum of insulin. Negligible effects on secondary structure are seen for insulin after conjugation with PEG-2000 at PheB1 and after encapsulation in, followed by release into buffer from, PLGA nanoparticles.

3.1.2 In vivo activity of PEGylated insulin

Blood glucose level reduction after treatment with 0.3 IU/kg of PEGylated insulin in rats was similar as seen with the same dose of HumulinR (Figure 2). Serum glucose levels run closely for both PEGylated and unconjugated insulin as there were no significant differences in any of the parameters (C_{nadir}, T_{nadir} and AUC) indicating that the biological activity of this site-specific PEGylated insulin construct is retained. This result is consistent with earlier reported work for the PEGylation of insulin at PheB1 with PEG of molecular weights of 750 or 2000 Da.[21]

3.2 PEGylated insulin entrapment in PLGA nanoparticles

3.2.1 In vitro characterization

3.2.1.1 Drug content and encapsulation efficiency

Drug content of PEGylated insulin loaded PLGA nanoparticles was found to be 14.4% (w/w) with an encapsulation efficiency of 65%.

3.2.1.2 Nanoparticle size and morphology

SEM and TEM analysis shown in Figure 3 for nanoparticles made from 45:55 lac:gly PLGA with a PEGylated insulin content of 14.4% (w/w). The particles were found to be spherical in shape with a relatively smooth (non-porous at the SEM resolution) surfaces (Figure 3A). The mean diameter of the nanoparticles was around 275 nm with a narrow range of 250–300 nm (Figure 3B) as demonstrated by TEM analysis.

3.2.1.3 In vitro drug release

In PBS pH 7.4 at 37^{o}C, after an initial release of <0.5% in the first day and a subsequent 4-day lag period, the nanoparticles released their contents almost completely (90%) in a nearly continuous fashion over the next 14 days (Figure 4). High initial drug release typically seen for proteins in PLGA nanoparticles is often ascribed to molecules at or near the outside surface and especially facile diffusion to the surface through internal pores and channels formed during manufacture [9]. In contrast, PEGylated insulin nanoparticles observed in the SEM are completely homogeneous within the resolution of the instrument, suggesting a lack of pores and channels. In preliminary experiments differential scanning calorimetry of a physical mixture of 65% (w/w) PLGA and 35% (w/w) PEGylated insulin was compared with the trace for PEGylated insulin nanoparticles of the same composition. Distinct phase transitions for the PLGA and PEG in the physical mixture are absent for the nanoparticle sample, which shows only a single glass transition. This result is interpreted to indicate that the PEGylated insulin and PLGA are intimately dissolved, with no separate phase. Nearly complete release of PEGylated insulin over the course of in vitro dissolution suggests that PEG conjugation helps prevent denaturation of the protein. This is in contrast to experience with unconjugated insulin in other PLGA particulate systems where degradation and covalent dimer formation prevent release of much or most of the protein as native structure [22].

3.2.2 In vivo characterization of PEGylated insulin loaded PLGA nanoparticles

After a single SC injection in STZ treated rats, a very small initial burst release (C_{max} ~10.5 μIU/mL) of PEGylated insulin was detected 1 h after nanoparticle injection. Beginning 24 h post-injection, PEGylated insulin levels rose steadily over 2 days to levels of approximately 30 μIU/mL on day 3 reaching levels of 85 μIU/mL on Day 7. PEGylated insulin levels ≥30 μIU/mL were maintained over a 7-day period from Days 3 to 10 before returning to near baseline levels by Day 13 (Figure 5A). The pharmacodynamic response mirrored the kinetic profile in that a decrease in serum glucose only became evident after serum insulin levels increased appreciably beginning after Day 1. By Day 5, serum glucose levels for PEGylated insulin loaded PLGA nanoparticles treated rats were in the euglycemic range and glucose levels remained low until Day 11 by which time glucose levels increased to near pre treatment levels (Figure 5B).

4. CONCLUSION

Polymeric nanoparticles like PLGA nanoparticles typically release high levels of proteins immediately after administration which is a major cause of hypoglycemia. They often fail to release their payloads in a complete and controlled manner, potentially resulting in harmful immune responses and glucose excursions, respectively. However, insulin release from such formulation in a controlled manner may provide a prospective benefit to diabetes patients. Data generated to date suggest the methods of insulin PEGylation and encapsulation in PLGA nanoparticles described here protect the protein from the normally deleterious effects of nanoparticle manufacture. In addition, the process results in a formulation characterized by high drug loading, encapsulation efficiency and activity, negligible initial burst release, and a relatively continuous (near zero order) drug release profile. The combination of two complementary technologies (PEGylation and nanoencapsulation) offers the potential for sustained delivery of basal insulin with a single weekly dose. Further, ongoing research indicates that this approach is useful for controlled delivery of other proteins for several therapeutic applications.

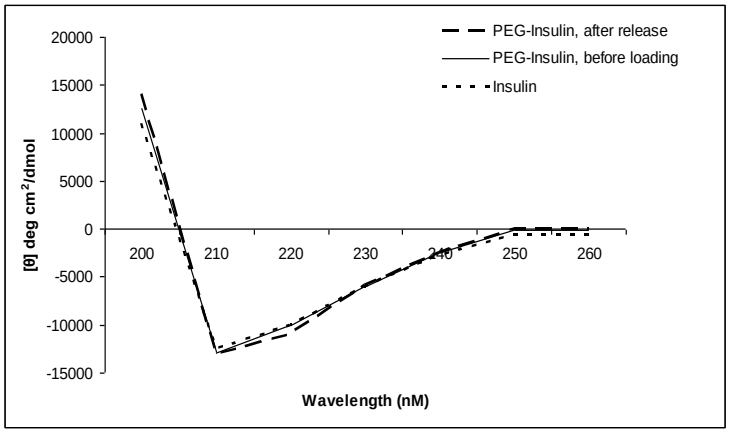


Figure 1. Far ultraviolet circular dichroism spectra of insulin and insulin following mPEG conjugation and formulation in PLGA nanoparticles

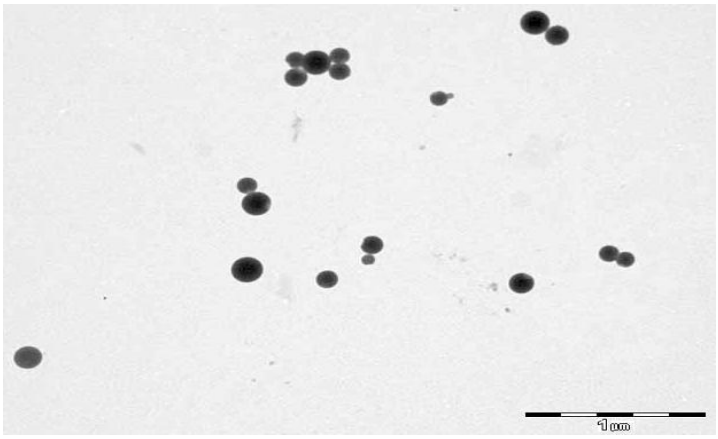


Figure 3. Scanning electron micrographs (A) and Tramission Electron micrographs (B) of PEGylated insulin nanoparticles

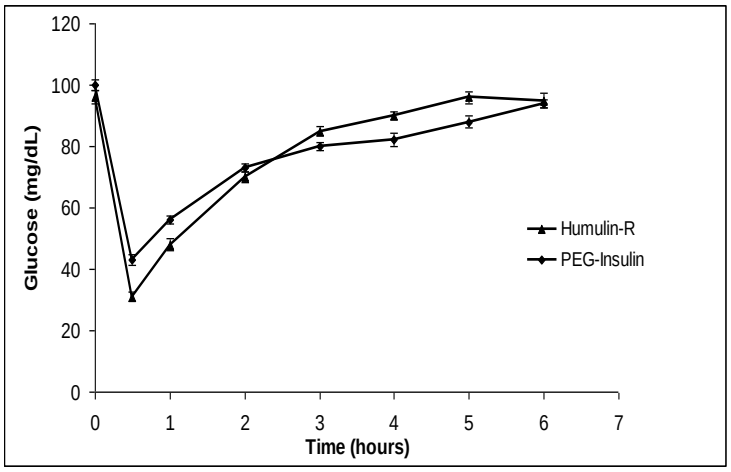


Figure 2. Mean (±SD) blood glucose levels in fasted normal rats (n =4/group) after a single IV dose of HumulinR or PEG–insulin

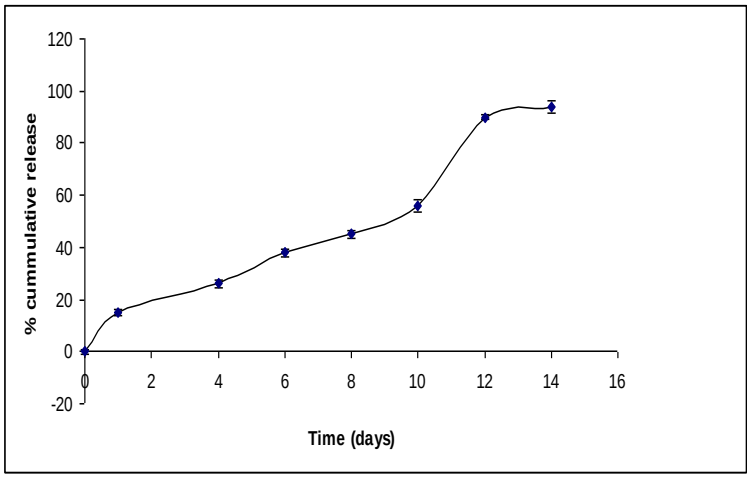
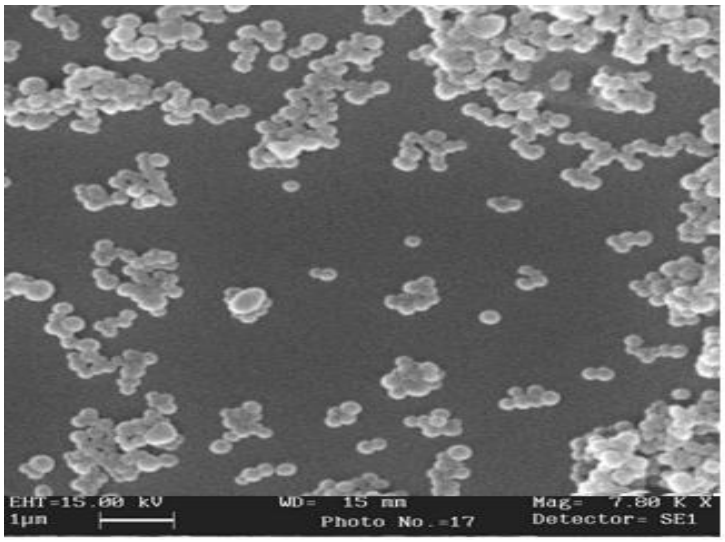


Figure 4. In vitro release of PEGylated insulin from PLGA nanoparticles (n =3)



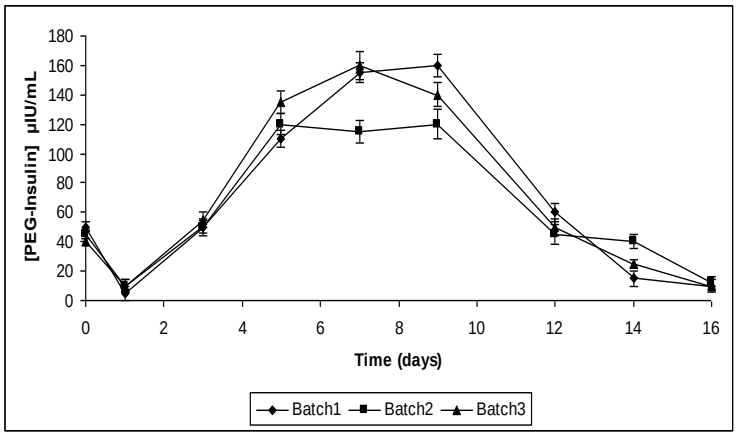


Figure 5. (A) Mean serum insulin levels in diabetic rats (n =10/group) following a single SC administration of PEGylated insulin loaded PLGA nanoparticles (n =3 batches)

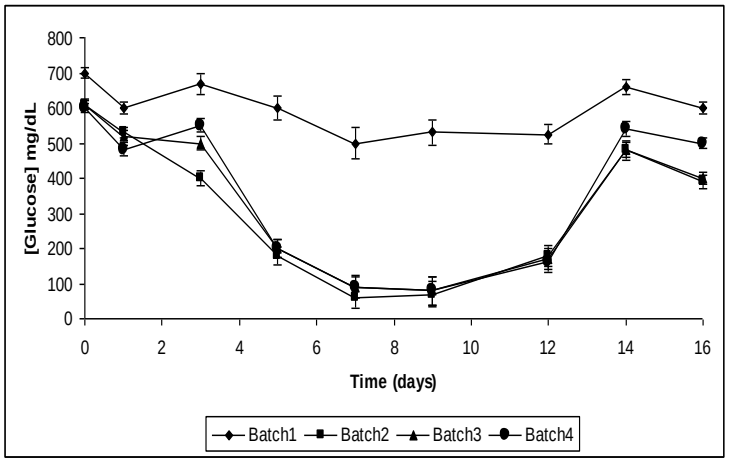


Figure 5. (B) Mean serum glucose levels in diabetic rats (n =10/group) following a single SC administration of PEGylated insulin loaded PLGA nanoparticles compared to untreated diabetic controls. (n =3 batches)

5. ACKNOWLEDGEMENT

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