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Preparation, characterization, and in vivo evaluation of insulin-loaded PLA–PEG microspheres for controlled parenteral drug delivery

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Abstract

Aim: The aim of this study was to prepare insulin-loaded poly(lactic acid)–polyethylene glycol microspheres that could control insulin release at least for 1 week and evaluate their in vivo performance in a streptozotocin-induced diabetic rat model. *Methods*: The microspheres were prepared using a water-in-oil-in-water double emulsion solvent evaporation technique. Different formulation variables influencing the yield, particle size, entrapment efficiency, and in vitro release profiles were investigated. The pharmacokinetic study of optimized formulation was performed with single dose in comparison with multiple dose of Humulin® 30/70 as a reference product in streptozotocin-induced diabetic rats. *Results*: The optimized formulation of insulin microspheres was nonporous, smooth-surfaced, and spherical in structure under scanning electron microscope with a mean particle size of 3.07 μm and entrapment efficiency of 42.74% of the theoretical amount incorporated. The in vitro insulin release profiles was characterized by a bimodal behavior with an initial burst release because of the insulin adsorbed on the microsphere surface, followed by slower and continuous release corresponding to the insulin entrapped in polymer matrix. *Conclusions*: The optimized formulation and reference were comparable in the extent of absorption. Consequently, these microspheres can be proposed as new controlled parenteral delivery system.

Key words: *In vitro release; insulin; microspheres; pharmacokinetic study; w/o/w double emulsion*

Introduction

Over the past few decades, there has been a considerable interest in the development of effective drug delivery systems for proteins and investigation of a large number of recombinant proteins for therapeutic applications[1]. Insulin, a 51-amino acid protein, is the most important regulatory hormone in the control of glucose homeostasis. There are more than 150 million people suffering from diabetes mellitus worldwide, and this number will double by the year of 2025[2]. The diabetic patients need to take one or more doses of intermediate or long-acting insulin injections per day to stay healthy and live normally.

In an attempt to avoid injection therapy, various noninvasive routes have been explored for insulin dosing such as oral[3–6], pulmonary[7–9], buccal[10,11], and nasal[2,12–14] but so far researchers have not yet developed a successful formulation for clinical application.

Insulin is poorly suited to oral administration because of extensive proteolytic degradation by intestinal enzymes, insufficient membrane permeability because of its high molecular weight with complex structure, and low lipophilicity[15]. Transdermal bioavailability of insulin is very low because of the large molecular size. The pulmonary delivery was limited by proteases in the lung that causes the poor pulmonary bioavailability of insulin[16]. The poor bioavailability of insulin administered via buccal route is largely due to low permeability across mucosal epithelium and easily washed out by saliva. Yet, as large molecule, insulin might require the help of excipients such as penetration enhancers for improving bioavailability via this route[17,18]. Ocular and nasal deliveries are also unfavorable because of degradation by

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enzymes present in eye tissues and nasal mucosa[19]. Thus, parenteral delivery of insulin is still the most suitable and preferred route compared with all other routes.

In most of the cases, the half-lives of proteins injected parenterally can only last for a few hours. Hence, multiple injections are needed daily to maintain the therapeutic effectiveness. It causes problems of inconvenience such as pain, tenderness, local tissue necrosis, and nerve damage with poor patient compliance[20]. To obviate the need for painful injection given a number of times to the diabetic patients, investigators have focused their attention on the development of controlled release insulin delivery systems based on microparticle technology using various types of biodegradable polymers. The biodegradable polymers used in microspheres and nanospheres preparations include poly(lactic-co-glycolic acid) (PLGA)[9,21–26], poly(lactic acid) (PLA)[27–29], polyphosphazene[30], poly(ε-caprolactone)[21,31], poly(fumaric-co-sebacic anhydride)[5,20], and poly(acryloyl-L-proline methyl ester)[32]. Much recent research has also focused on the use of polymer blends as drug carriers for controlled release applications. The blends of PLGA/PLA with PEG have been utilized to improve the delivery capacity and modulate the release profiles of the formulation[33–36].

In general, protein release from PLGA/PLA microspheres is characterized by bimodal behavior, an initial burst release phase within the first day, followed by slow and incomplete release. Wang et al.[37] reported that after incubation of microspheres in release medium, numerous small pores were observed on the surface of the microspheres at zero time. These pores increased in number and were present after 5 hours of incubation. Furthermore, the interconnecting channels between pores on the surface and those inside microspheres are also observed throughout the microspheres. These pores cause an initial burst release at an initial time. However, after 24 hours, the microsphere surface appeared smooth as the pores on the surface had disappeared and decreased the release rate of protein.

The preparation of stable protein pharmaceuticals, including insulin, represents a significant formulation challenge because of their inherent physical and chemical instability. Many authors reported the protein instability in PLGA/PLA polymers caused by the method of preparation, adsorption of protein onto polymer surface, moisture effect and generation of an acidic pH within the microspheres, and/or the release medium during release[1,28,38–42]. In the preparation of w/o/w emulsion, insulin acts as a surface active molecule and tends to adsorb at the water/oil interfaces. This adsorption causes insulin unfolding, inactivation, and irreversible aggregation[40,43]. Furthermore, the high-shear stress during the preparation may affect the three-dimensional structure of insulin, which is important for its biological activity. A large portion of insulin may not be released from the microspheres after a period of time because of either aggregation of insulin molecules or adsorption of insulin onto the strong hydrophobic surface of the polymer[43]. Further inactivation of insulin may also result from exposure to increasing moisture inside the microspheres. The inactivation of insulin from the prospective polymer may be because of the decrease in the pH within the microspheres and/or the release medium because of the polymer degradation into soluble acidic products, which can diffuse into the release medium. This decrease in pH may cause unfolding and irreversible inactivation of insulin[28,40,42].

The aim of this study was to prepare and characterize controlled release insulin-loaded PLA–PEG microspheres that could maintain optimum plasma glucose levels for at least a period of 1 week following a single subcutaneous injection of the preparation. In addition, the microspheres that produced desired in vitro performance were evaluated for its in vivo performance in streptozotocin-induced diabetic rat model.

Materials and methods

Materials

Actrapid® HM human insulin injection (100 IU/mL) was purchased from Novo Nordisk (Bagsvaerd, Denmark). Humulin® 30/70 was purchased from Eli Lilly (Fegersheim, France). PLA polymer, R 203 H (M_W 21,400 g/mol, i.v. 0.29 dL/g), was purchased from Boehringer Ingelheim (Ingelheim, Germany). Polyethylene glycol (PEG) 2000 was purchased from Merck (Darmstadt, Germany). Polyvinyl alcohol (PVA) (M_W 140,000 Da) and mannitol were purchased from BDH (Poole, UK). Streptozotocin, sodium citrate, glycerol, and sodium azide were purchased from Sigma (St. Louis, MO, USA). Sodium carboxymethylcellulose (Cekol 10,000) was purchased from Metsa-Serla (Skoghal, Sweden). Sodium chloride was obtained from Hamburg Chemicals (Hamburg, Germany). Tween 80, sodium sulfate anhydrous, dichloromethane, and zinc oxide were purchased from R&M Chemicals (Essex, UK). High-performance liquid chromatography (HPLC) grade of acetonitrile was purchased from J.T. Baker (Ecatepec Estado de Mexico, Mexico).

Preparation of insulin-loaded PLA–PEG microspheres

Insulin-loaded PLA–PEG microspheres were prepared using a water-in-oil-in-water (w/o/w) double emulsion solvent evaporation technique described by Okochi and Nakano[44] with slight modification. Briefly, 3.6 mL (12.5 mg) of Actrapid® HM insulin injection solution was emulsified with 10 mL of dichloromethane solution, prepared by dissolving polymer blend of PLA and PEG to produce primary emulsion (w/o). The primary emulsification was performed using a probe sonicator (model XL-2020; Heat Systems, New York, NY, USA) equipped with a 3.2-mm probe in an ice bath for 2 minutes followed by homogenizer (Ultraturrax T-18; IKA Works Inc., Wilmington, DE, USA) at a speed of 18,000 rpm in an ice bath for 3 minutes. The resulting primary emulsion was added to 25 mL of aqueous solution containing 3% (w/v) of PVA as an emulsifier and homogenized at the same speed in an ice bath for 5 minutes to produce a w/o/w double emulsion. The resultant double emulsion was magnetically stirred for 6 hours at room temperature at 250 rpm to allow solvent evaporation and microsphere formation. The formed microspheres were isolated by centrifugation at 2185 × *g* for 15 minutes and washed thrice using distilled water. Mannitol (1%, w/v) was added to prevent aggregation of the microspheres, which were then lyophilized for 24 hours. The resultant free flow microspheres were stored at 4°C until further use.

Investigation of various formulation parameters

Yield, particle size, entrapment efficiency, loading, and in vitro release profile of microspheres are important parameters in microspheres formulation. Thus, the following factors that could affect these parameters were investigated.

Different ratios of PLA and PEG 2000

The formulations (F1–F6) were prepared with the blend of PLA and PEG 2000 at the ratios of 8:2, 6:4, 5:5, 3:7, 2:8, and 1.5:8.5 (Table 1).

Insulin to polymer (PLA–PEG 2000) ratio

The PLA–PEG 2000 polymer at the ratio of 3:7 (F4) was selected to study the effect of insulin to PLA–PEG 2000 polymer ratios at 1:30 (F7), 1:45 (F8), and 1:60 (F4) (Table 1).

Concentration of PVA in external aqueous phase

The ratio of insulin to PLA–PEG 2000 (3:7) polymer at 1:60 (F4) was selected to study PVA concentration effect in external aqueous phase. The PVA concentrations of 1%, 2%, and 3% (w/v) (Table 2) were investigated while maintaining a constant volume of the external aqueous phase (25 mL).

Effect of glycerol

The effect of different amounts of hydrophilic additive, glycerol in oil phase of the primary emulsion, was investigated with the amounts of 25, 50, 100, and 200 mg (Table 2). The formulation F4, which was prepared using PLA–PEG 2000 (3:7) with insulin to polymer at ratio of 1:60, was used as a control.

Effect of zinc oxide

The formulation F13 prepared with glycerol at 100 mg was selected for the investigation of the effect of zinc oxide in the oil phase of the primary emulsion. The amount of zinc oxide examined was 5, 10, 15, and 20 mg (Table 2).

Characterization of insulin-loaded PLA–PEG microspheres

Yield

The microspheres recovered at the end of preparation were weighed, and the yield was calculated as a percentage of

Table 1. Composition of insulin-loaded PLA–PEG microsphere formulations, F1–F8.

Ingredients (per batch)	F1	F2	F3	F4	F5	F6	F7	F8
Insulin (mg)	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
PLA (mg)	600.0	450.0	375.0	225.0	150.0	112.5	112.5	168.8
PEG 2000 (mg)	150.0	300.0	375.0	525.0	600.0	637.5	262.5	393.8
PVA (%, w/v)	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Dichloromethane (mL)	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
PLA–PEG 2000 ratio	8:2	6:4	5:5	3:7	2:8	1.5:8.5	3:7	3:7
Insulin to PLA–PEG 2000 ratio	1:60	1:60	1:60	1:60	1:60	1:60	1:30	1:45

Table 2. Composition of insulin-loaded PLA–PEG microsphere formulations, F9–F18.

Ingredients (per batch)	F9	F10	F11	F12	F13	F14	F15	F16	F17	F18
Insulin (mg)	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
PLA (mg)	225.0	225.0	225.0	225.0	225.0	225.0	225.0	225.0	225.0	225.0
PEG 2000 (mg)	525.0	525.0	525.0	525.0	525.0	525.0	525.0	525.0	525.0	525.0
PVA (%, w/v)	1.0	2.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Glycerol (mg)	—	—	25.0	50.0	100.0	200.0	100.0	100.0	100.0	100.0
Zinc oxide (mg)	—	—	—	—	—	—	5.0	10.0	15.0	20.0
Dichloromethane (mL)	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0

the total amounts of polymer and drug added during the preparation of microspheres.

Particle size determination

The particle size of insulin-loaded microspheres after lyophilization was examined using a Mastersizer S (Malvern Instruments Ltd., Malvern, UK) fitted with MS1 small volume sample dispersion unit connected to a dispersion unit controller. The microspheres were dispersed by bath sonication for 2 minutes in the saline medium (0.9% sodium chloride) containing a surfactant (0.1% Tween 80) to prevent aggregation before examination. Samples were analyzed in triplicate.

Determination of insulin content in microspheres

The content of insulin in microspheres was estimated by the method described by Carino et al.[3] with slight modification. Briefly, 10 mg of dried microspheres were dissolved in 5 mL of dichloromethane and then 10 mL of phosphate-buffered saline (PBS; pH 7.4) was added. The mixture was stirred vigorously using a magnetic stirrer at a speed of 500 rpm for 1 hour to extract insulin into PBS from the organic solution. After centrifuging at 4500 rpm for 15 minutes, the aqueous solution was withdrawn and insulin was quantitated using chromatographic conditions of our previously reported HPLC–UV method[45]. The HPLC apparatus was comprised of a Waters 510 delivery pump (Milford, MA, USA) equipped with a six-valve sample injector port fitted with a 20 μL sample loop, a UV spectrophotometric detector (Shimadzu SPD-6A; Shimadzu, Kyoto, Japan), and a chromato-integrator (D-2500; Hitachi, Tokyo, Japan). A reverse phase C18 column (150×4.6 mm^2, 5 μm) was used as stationary phase and 0.2 M sodium sulfate (pH 2.3) adjusted with orthophosphoric acid and acetonitrile (74:26, v/v) at the flow rate of 1.2 mL/min as mobile phase. The detection was set at a wavelength of 214 nm. The calibration plot was linear over the concentration range 0.35–5.25 μg/mL with a correlation coefficient value of 0.9999.

The encapsulation efficiency was expressed as the ratio of actual to theoretical insulin content. The samples were analyzed in triplicate for each formulation studied. The entrapment efficiency and insulin loading were calculated using the following equations:

$$\text{entrapment efficiency (\%)} = \frac{\text{weight of insulin in microspheres}}{\text{weight of insulin fed initially}} \times 100\%$$

$$\text{insulin loading (\%)} = \frac{\text{weight of insulin in microspheres}}{\text{weight of microspheres}} \times 100\%.$$

In vitro insulin release study

Lyophilized microspheres containing insulin at a concentration of 1 mg/mL were accurately weighed and suspended in PBS buffer (pH 7.4) containing 0.02% sodium azide as a bacteriostatic agent and 0.01% Tween 80 to prevent the microspheres from agglomeration in the dissolution medium in stoppered flasks. The flasks were placed in a reciprocal shaker water bath maintained at 37 ± 0.5°C with a continuous agitation of about 60 cycles per minute. At predetermined time intervals of 2, 12, 24, 72, 120, and 168 hours, the supernatant was collected after centrifugation at 4500 rpm for 15 minutes. The collected amount of supernatant was replaced with fresh PBS. The amount of insulin released was quantitated using HPLC–UV method[45]. The experiments were run in triplicate for each batch of microspheres. The burst release and $T_{25\%}/T_{50\%}$ (the time required for the 25% and 50% of insulin release) were used for comparison of release profiles.

Scanning electron microscopy

The morphology of microspheres containing insulin was examined using scanning electron microscopy (SEM; Leica Cambridge S360; Leica Cambridge, Cambridge, UK). For the shape and surface analysis, the freeze-dried microspheres were mounted onto aluminum stub using double-sided adhesive tape and then sputter coated with a thin layer of gold under argon atmosphere (Emitech K750; Emitech, Kent, UK) before examination. The coated specimen was then examined under the microscope at an acceleration voltage of 2.0 kV and photographed.

Pharmacokinetic study

Healthy female Sprague–Dawley rats, weighing between 220 and 260 g, were fasted for 16 hours prior to the experiment. The rats were made diabetic by injecting streptozotocin intraperitoneally at a dose of 60 mg/kg body weight, freshly dissolved in 10 mM cold citrate–sodium citrate buffer (pH 4.5). Standard rat pellets and water were given ad libitum. The study was conducted in accordance with Animal Ethical Guidelines for Investigations in Laboratory Animals and was approved by the Animal Ethics Committee of the Universiti Sains Malaysia.

Animals showing blood glucose of 16.7 mmol/L or higher 3 days post injection were used for the study. Diabetic rats were divided into two groups containing six rats in each group. First group received 25 U/kg/day (0.875 mg/kg/day) of Humulin® 30/70 commercial insulin injection as reference, once daily for 5 days. The second group received the test formulation of a single dose of 125 U/kg (4.375 mg/kg) of insulin-loaded microspheres. Before administration, the microspheres were suspended in a suitable vehicle consisting 20% (w/v) of 0.5% (w/v) sodium carboxymethyl cellulose, 5% (w/v) mannitol, and 0.1% (v/v) Tween 80 in aqueous solution. The injections were administered subcutaneously using a 21-gauge needle[21]. Food was supplied after 2 hours of dose administration. Blood (0.5 mL) was withdrawn periodically from rat tail vein into heparinized Eppendorf tubes. Blood samples were kept on ice until centrifugation. Plasma was separated by centrifugation at 11,269 × *g* for 10 minutes.

To 100-μL aliquot of plasma, 20 μL of 15 μg/mL methyl paraben (internal standard), 50 μL of PBS (pH 7.4), and 3 mL of mixture of dichloromethane and *n*-hexane (1:1, v/v) extraction solvent were added. The mixture was vortexed for 2 minutes and centrifuged at 1700 × *g* for 10 minutes. The supernatant was transferred to reacti-vial and dried up to approximately 200 μL under nitrogen gas before the addition with 200 μL of 0.05 M HCl for back extraction. The balance of 200 μL of organic layer was evaporated, and the residual aqueous phase was analyzed using HPLC–UV method[45]. The calibration curve exhibited an excellent linearity over the concentration range of 0.7–8.4 μg/mL of insulin with a correlation coefficient of 0.9994. The pharmacokinetic parameter, namely, area under the plasma concentration–time curve from 0 to the last measurable concentration (AUC_{0-t}), was calculated using trapezoidal rule from the obtained data for reference and test.

Statistical analysis

The results are presented as mean ± SD. The particle size, entrapment efficiency, insulin loading, $T_{25\%}$, and $T_{50\%}$ values of in vitro release profiles of insulin microspheres were treated statistically using one-way analysis of variance (ANOVA). When there was a statistically significant difference, a post hoc Tukey's HSD (honestly significant difference) test was performed. The pharmacokinetic parameter, AUC_{0-t}, was compared statistically using independent samples *t*-test. A statistically significant difference was considered at $P < 0.05$.

Results and discussion

The selection of a particular encapsulation method is usually determined by solubility of the protein and the coating polymer; it also has utmost importance for the efficient entrapment of active substance. In this study, double emulsion solvent evaporation technique was adopted for the efficient incorporation of insulin in PLA–PEG polymeric microspheres because of the solubility of protein in aqueous phase, and the organic phase or oil phase acts as a barrier between the two aqueous compartments, preventing the diffusion of the active material toward the external aqueous phase. It is also known to be superior to other encapsulation methods in terms of stability of proteins because of minimal exposure to organic solvent during preparation of microspheres[46,47].

Biodegradable polymers are synthetic or natural polymers, which are degraded into biocompatible or nontoxic products in the body. The biodegradable polyester polymer, PLA, was chosen because of its biocompatibility, predictable biodegradation kinetics, commercial availability, and nontoxicity[19]. PEG could also be blended with PLA to improve the release rate of insulin microspheres because PEG is considered as a nontoxic hydrophilic polymer and approved by US FDA.

Yield

The yield of formulations F1–F18 is shown in Table 3. In the formulations F1–F6, the product yield was decreased from 70.57% to 17.96% when PEG amount was increased from 20% to 85%. The decrease in yield associated with an increase in amount of PEG might be attributed to a higher rate of leaching of PEG from the internal phase of primary emulsion to the external phase of the secondary emulsion during the preparation of microspheres. Cleek et al.[33] in nuclear magnetic resonance studies reported that a substantial amount of PEG originally dissolved in the dichloromethane of the emulsion was extracted into the aqueous phase and did

Table 3. Results of yield, particle size, entrapment efficiency, and insulin loading of insulin-loaded PLA–PEG microspheres.

Formulation	Yield (%)	Particle size (μm)	Entrapment efficiency (%)	Insulin loading (%)
F1	70.57	4.81 ± 0.08	53.16 ± 3.80	0.87 ± 0.06
F2	65.04	3.50 ± 0.44	46.42 ± 4.87	0.76 ± 0.08
F3	48.55	3.21 ± 0.51	46.72 ± 2.94	0.76 ± 0.04
F4	32.50	2.82 ± 0.03	43.35 ± 0.90	0.70 ± 0.02
F5	19.58	2.76 ± 0.03	41.81 ± 1.01	0.68 ± 0.02
F6	17.96	2.64 ± 0.06	42.11 ± 1.80	0.68 ± 0.08
F7	31.48	2.97 ± 0.04	17.89 ± 3.98	0.58 ± 0.06
F8	30.37	2.77 ± 0.15	21.40 ± 0.97	0.46 ± 0.02
F9	30.91	6.84 ± 0.13	41.09 ± 2.07	0.67 ± 0.03
F10	30.81	4.96 ± 0.04	41.61 ± 0.57	0.68 ± 0.01
F11	27.54	2.82 ± 0.02	44.09 ± 0.59	0.72 ± 0.01
F12	30.42	2.91 ± 0.10	43.28 ± 1.06	0.70 ± 0.02
F13	32.52	3.20 ± 0.04	42.67 ± 2.47	0.69 ± 0.03
F14	30.42	3.81 ± 0.10	40.36 ± 2.35	0.66 ± 0.04
F15	31.86	2.55 ± 0.01	40.18 ± 2.31	0.65 ± 0.04
F16	33.18	2.48 ± 0.01	42.66 ± 0.82	0.69 ± 0.01
F17	32.55	3.07 ± 0.01	42.74 ± 1.69	0.70 ± 0.03
F18	33.29	3.02 ± 0.10	41.82 ± 2.61	0.68 ± 0.04

Mean ± SD, *n* = 3.

not incorporate into the final microspheres. Thus, the higher the initial PEG content, the lower the percentage incorporated in the microspheres. This phenomenon led to a decrease in the yield of microspheres with an increase in PEG content. Other parameters, insulin to polymer ratio, PVA concentration in external aqueous phase, and amount of glycerol and zinc oxide incorporated in primary emulsion, did not show any effect on the yield of microspheres.

Particle size

The particle size of microspheres results are shown in Table 3. The particle size of microspheres was slightly decreased with increasing percentage of PEG 2000 from 20% to 85% (F1–F6) and found to be in the range of 2.64–4.81 μm. A statistically significant difference ($P < 0.05$) in the size of microspheres prepared with the different ratios of PLA and PEG 2000 between F1 and F2 to F6 as well as between F2 and F6. The microspheres prepared with different ratios of insulin to PLA–PEG 2000 polymer (F4, F7, and F8) did not show any statistical significant difference ($P > 0.05$) on the particle size of microspheres. The size of microspheres and PVA concentration was inversely related with a decrease in microsphere size from 6.84 to 2.82 μm as the concentration of PVA in external aqueous phase was increased from 1% to 3% (w/v). It might be due to the presence of PVA in the external aqueous phase that could stabilize the emulsion droplets against coalescence, resulting in the formation of small-size emulsion droplets. Yang et al.[48] reported that the stabilization effect was more dominant at higher PVA concentrations. There was a statistically significant difference ($P < 0.05$) in the particle size of microspheres (F9, F10, and F4) at the different PVA concentrations in the external aqueous phase. The size of microspheres varied slightly from 2.82 to 3.81 μm (F11–F14) with the addition of glycerol in oil phase of the primary emulsion. When compared to control formulation, F4 (0 mg glycerol), formulations prepared with different amount of glycerol, F13 (100 mg) and F14 (200 mg), have shown a statistically significant difference ($P < 0.05$) in the size of microspheres. There was a slight change in size of microspheres (2.48–3.07 μm) with the addition of zinc oxide in oil phase of the primary emulsion. Formulation (F13) prepared without zinc oxide has shown a statistically significant difference between the particle size of microspheres prepared with the varying concentrations of zinc oxide, F15 (5 mg), F16 (10 mg), and F18 (20 mg).

Entrapment efficiency and insulin loading

The entrapment efficiency and insulin loading results are shown in Table 3. Among the formulations studied, microspheres prepared with PLA–PEG 2000 ratio of 8:2 (F1) produced the highest entrapment efficiency of 53.16%. A statistically significant difference ($P < 0.05$) in the entrapment efficiency and insulin loading of formulations prepared with different ratios of PLA–PEG 2000 were observed between F1 and F4 to F6 with varying ratios of PLA and PEG in microspheres. The percentage of insulin entrapment efficiency was increased from 17.89% to 43.35%, while increasing the ratio of insulin to PLA–PEG 2000 ratio from 1:30 to 1:60. A statistically significant difference ($P < 0.05$) in the entrapment efficiency and insulin loading of formulations prepared with different ratios of insulin to PLA–PEG 2000 were observed between F4 and F7, F8 and insulin loading between F7 and F8. The formulations prepared with the changes in PVA concentration (F9, F10, and F4), incorporation of glycerol (F11–F14), and zinc oxide (F15–F18) did not show any statistically significant effect on the entrapment efficiency and insulin loading ($P > 0.05$).

In vitro release profiles

The in vitro release profiles of insulin formulations prepared with various ratios of PLA–PEG 2000 (F1–F6) are shown in Figure 1. There was no effect on the percentage release of insulin during 1 week when the PEG content increased from 20% to 50% (F1–F3). For these three formulations, about 60% of insulin was released in 1-week period. The higher release rate of insulin (76.96%) was observed for microspheres prepared with the ratio of 3:7 (F4), but about 64% of insulin was released within 1 day. The formation of large pores favored diffusion of insulin from microspheres with high content of PEG. Where, the leaching of PEG from internal aqueous phase to external aqueous phase during microsphere manufacturing process caused the formation of pores. Hence, the porosity of microspheres increased with an increasing amount of PEG[49]. The percentage of insulin released was surprisingly decreased to 49.75% and 41.03%

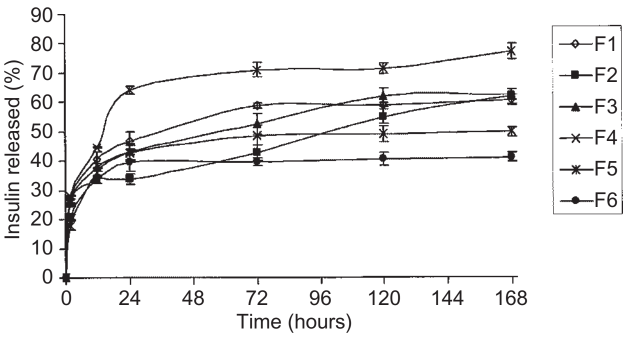

Figure 1. In vitro release profiles of microspheres prepared with various ratios of PLA and PEG 2000, 8:2 (F1), 6:4 (F2), 5:5 (F3), 3:7 (F4), 2:8 (F5), and 1.5:8.5 (F6). Mean ± SD, $n = 3$.

with PLA–PEG 2000 at ratios of 2:8 and 1.5:8.5 (F5 and F6). The increase in PEG content at 80% and above (F5 and F6) could have been associated with the lower amount of PEG incorporated in the microspheres. As a result, the amount of PEG present might not be sufficient to induce the insulin release. The produced results were similar to the findings of Cleek et al.[33] Formulation F4 at PLA–PEG 2000 ratio of 3:7 was selected for further experiments because of the higher release rate of insulin compared with all other ratios over a period of 1 week. Further experiment was conducted to modify the release profile of insulin to achieve a low burst with a slow and continuous release.

The in vitro insulin release profiles of formulations prepared with different ratios of insulin to PLA–PEG 2000 (F4, F7, and F8) are depicted in Figure 2. The release profiles of microspheres prepared with the ratio of 1:30 (F7) showed about 93.84% of insulin released within 1 day and for the ratio of 1:45 (F8), 100% insulin was released within 3 days. The formulation prepared with the ratio of 1:60 (F4) released about 76.96% of insulin during 1 week. The mean $T_{50\%}$ values for the formulations with increasing insulin to polymer (PLA–PEG 2000) ratios 1:30, 1:45, and 1:60 were 3.16 ± 0.54, 6.28 ± 0.76, and 15.29 ± 0.09 hours, respectively. It could be noted that increase in insulin to polymer ratio decreased the rate of insulin released, and the $T_{50\%}$ values were significantly different statistically ($P < 0.05$). Hence, formulation F4 at insulin to PLA–PEG 2000 ratio of 1:60 was selected for further experiment with the aim of producing once-a-week controlled release insulin microspheres.

The in vitro insulin release profiles with different PVA concentrations in external aqueous phase are shown in Figure 3. The change in the PVA concentration in external aqueous phase had a significant effect on the release profiles of microspheres. The PVA concentrations at 1%, 2%, and 3% (w/v) (F9, F10, and F4) released 50.46%, 41.38%, and 27.90% of insulin, respectively, at initial 2 hours and 88.82%, 80.19%, and 76.96%, respectively, over a period of 1 week. The results showed that the larger microspheres prepared with a low concentration of PVA showed a faster insulin release. The formation of a less stable emulsion at low PVA concentration resulted in the diffusion of insulin from the internal aqueous phase to external aqueous phase. This phenomenon led to the distribution of more insulin on the surface of microspheres and faster release of insulin. In contrast, a higher PVA concentration increases the viscosity of the external water phase and results in an increased difficulty for the insulin aqueous solution to diffuse out. Moreover, as a surfactant, it would be expected that PVA would bind to the microsphere surface and might block insulin from diffusing outward. Thus, insulin was distributed more evenly within the interior of the microspheres. The produced results were similar to the findings of Yang et al.[48] As such, the release of insulin was slower, although particle size of microspheres was smaller. The mean $T_{50\%}$ values for PVA concentration of 1%, 2%, and 3% (w/v) were 2.32 ± 0.63, 11.79 ± 1.35, and 15.29 ± 0.09 hours, respectively, and were found to be significantly different statistically ($P < 0.05$). From the results obtained, formulation F4 with PVA concentration of 3% (w/v) was chosen for further experiment because at this concentration a more stable emulsion was formed and the release was comparatively slower than those of 1% and 2% (w/v) of PVA (F9 and F10).

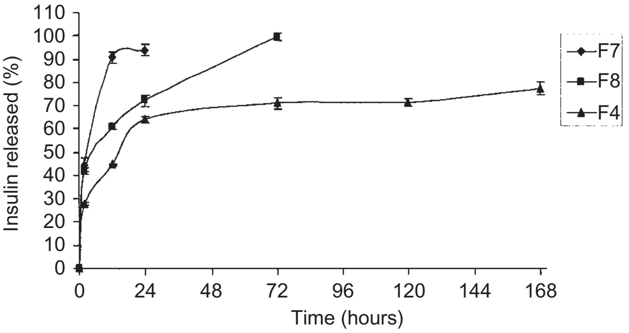

Figure 2. In vitro release profiles of microspheres prepared with different ratios of insulin to PLA-PEG 2000: 1:30 (F7), 1:45 (F8), and 1:60 (F4). Mean ± SD, $n = 3$.

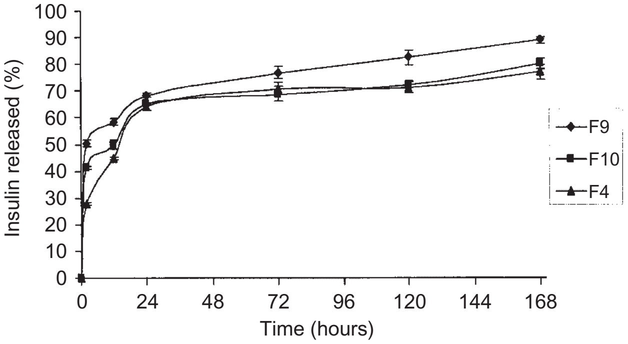

Figure 3. In vitro release profiles of insulin microspheres prepared with different concentrations of PVA in the external aqueous phase: 1% (w/v) (F9), 2% (w/v) (F10), and 3% (w/v) (F4). Mean ± SD, $n = 3$.

The insulin microspheres prepared with formulation F4 produced high initial burst release. The burst release, which could cause severe hypoglycemia with serious consequences when the microspheres are administered, had to be overcome. As reported by Tracy[50], it was important to prevent the rapid release of the microspheres when size was reduced to 2–3 µm. The smaller size of microspheres had a faster release of insulin and produced hypoglycemia.

Yamaguchi et al.[26] reported that addition of the hydrophilic polar solvents such as glycerol, water, and ethanol suppressed the initial burst release of insulin, and glycerol was found to be more effective than water and ethanol. Therefore, in this study, glycerol was incorporated into the oil phase of primary emulsion to suppress an initial burst release. The formulation F4, prepared without glycerol (0 mg), was used as control. The in vitro release profiles of formulations prepared with different amount of glycerol (F4 and F11 to F14) are depicted in Figure 4. The microspheres prepared with incorporation of 25 mg (F11) and 50 mg (F12) of glycerol did not show any effect on the suppression of burst release when compared to control formulation. This might be attributed to the smaller amount of glycerol, and the resultant formulations produced a burst release of insulin. The mean $T_{25\%}$ values for F4, F11, and F12 were 1.79 ± 0.04, 2.11 ± 0.33, and 2.76 ± 0.25 hours, respectively, and there was no statistically significant difference ($P > 0.05$) in burst release among the formulations prepared. The burst release was reduced to 18.79% with the increase of glycerol to 100 mg (F13) and also the percentage of insulin released in the period of 1 week was comparable to control formulation. Further increase in glycerol to 200 mg (F14) not only suppressed burst release but also reduced insulin release to 64.68% after 1 week. The mean $T_{25\%}$ values for the formulations prepared with 100 and 200 mg of glycerol were 6.73 ± 1.14 and 7.01 ± 1.08 hours, respectively, and there was a statistically significant difference in insulin burst release compared with control ($P < 0.05$). The mean $T_{50\%}$ values for the formulations prepared with 0 (F4), 25 (F11), 50 (F12), 100 (F13), and 200 mg (F14) of glycerol were 15.29 ± 0.09, 17.06 ± 1.01, 16.62 ± 0.55, 81.39 ± 4.97, and 135.68 ± 2.52 hours, respectively. The increase in glycerol to 50 mg did not affect the release rate of microspheres in the 1-week period, and there was no statistically significant difference in the formulations of F4, F11, and F12 ($P > 0.05$). The amount of insulin released was slower and continuous with the glycerol content of 100 mg and above compared with control and found to be significantly different statistically between the formulations of F4, F13, and F14 ($P < 0.05$). When a hydrophilic solvent like glycerin is added to oil phase (dichloromethane), the refractive index of glycerin (1.4746) approaches to that of dichloromethane (1.4244) and thereby rendering the oil phase less turbid. When another hydrophilic solvent like water was added to oil phase, there was a decrease in turbidity of the oil phase but initial burst release was not overcome to the same extent with that of glycerol. Therefore, the reason behind decrease in initial burst release may be attributed to altering the localization of insulin in microspheres rather than decrease in turbidity of oil phase. When an appropriate amount of glycerol was added to the oil phase, the insulin molecule would mainly be present on or inside of w/o emulsion droplets but not on the surface of microspheres. Moreover, the presence of glycerol may cause the microspheres to swell soon after coming into contact with the dissolution medium that might contribute to the suppression of insulin burst release from PLA–PEG microspheres[24]. On the basis of the low burst and higher percentage of insulin release, the microsphere formulation prepared with the addition of 100 mg of glycerol (F13) was selected for further experiment.

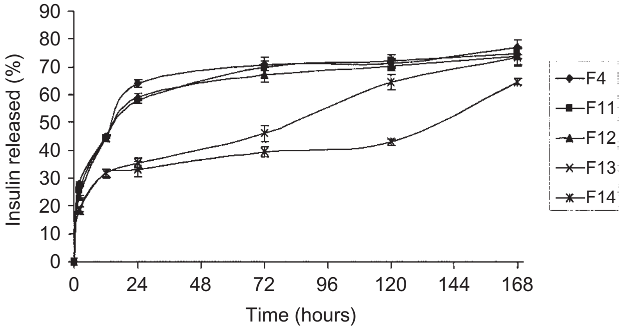

Figure 4. Effect of glycerol on release profiles of insulin microspheres: 0 mg (F4), 25 mg (F11), 50 mg (F12), 100 mg (F13), and 200 mg (F14). Mean ± SD, $n = 3$.

The amount of zinc oxide was incorporated into the oil phase of primary emulsion to further suppress an initial burst release from insulin-loaded PLA–PEG microspheres. The importance of zinc in the prevention of insulin deactivation was reported by Scott[51]. The use of zinc compounds was reported not only to stabilize the human growth hormone but also to control release from microspheres[50,52]. As described by Manoharan and Singh[43], the addition of zinc salts would be an effective solution to stabilize insulin within the microspheres. According to the authors, depending upon the concentration, insulin monomers self-associate to form dimers. In the presence of zinc ions, three such dimers associate to form hexamer, which is highly stable between pH 5 and 8, less soluble than insulin monomer, and has an increased molecular weight. The increased molecular weight and decreased solubility of insulin may effectively reduce the initial burst release. The authors also reported that increased insulin stability attributed to the superior stability of the formed insulin hexamers and neutralization of the acidic environment produced by degradation of polymer by the zinc salts. The formulation F13, prepared without zinc oxide (0 mg), was used as control. The in vitro release profiles of formulations prepared with different amounts of zinc oxide (F13 and F15 to F18) are depicted in Figure 5. The results of burst release of microspheres prepared with 0, 5, 10, 15, and 20 mg zinc oxide were 31.98%, 31.52%, 23.43%, 18.79%, and 18.42%, respectively. There was no

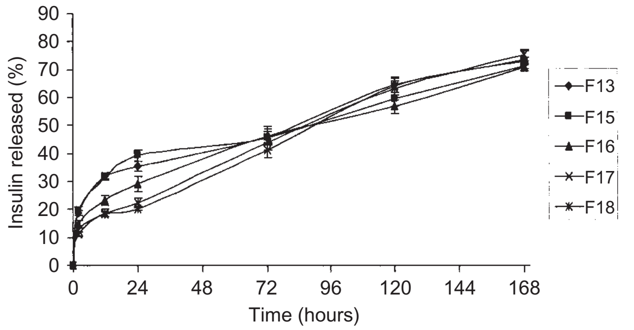

Figure 5. Effect of zinc oxide on release profiles of insulin microspheres: 0 mg (F13), 5 mg (F15), 10 mg (F16), 15 mg (F17), and 20 mg (F18). Mean ± SD, $n = 3$.

significant effect on the suppression of burst release with the addition of 5 mg zinc oxide (F15). The burst release was reduced with respect to increase in zinc oxide from 10 to 20 mg (F16–F18). The mean $T_{25\%}$ values for formulations with 0, 5, 10, 15, and 20 mg of zinc oxide were 6.73 ± 1.14, 6.52 ± 0.92, 14.32 ± 2.25, 29.63 ± 4.34, and 34.02 ± 3.71 hours, respectively. It can be noted that a statistically significant difference ($P < 0.05$) was observed in burst release of insulin for all the formulations except the formulation containing 5 mg zinc oxide ($P > 0.05$) when compared with control formulation (F13). The microspheres prepared with the addition of 15 (F17) and 20 mg (F18) of zinc oxide strongly suppressed initial burst release of insulin. This contributed to a slow and continuous release of insulin for a long period. The mean $T_{50\%}$ values for formulations with 0, 5, 10, 15, and 20 mg of zinc oxide were 81.39 ± 4.97, 86.55 ± 3.16, 88.17 ± 13.6, 87.76 ± 6.32, and 90.59 ± 5.79 hours, respectively, and found to be not significantly different statistically ($P > 0.05$) in insulin release with all the formulations prepared with and without the addition of zinc oxide. From the results observed, the formulations prepared with the addition of 15 and 20 mg of zinc oxide showed low burst release compared with the formulations prepared with 5 and 10 mg of zinc oxide. There was no difference in the burst release of insulin microspheres prepared with 15 and 20 mg of zinc oxide. The addition of zinc oxide did not show any effect on the amount of insulin released over a period of 1 week. Among the formulations studied, formulation F17 was selected as the final formulation of controlled release injectable insulin microspheres for in vivo study because of the low burst and constant release profile over a period of 1 week.

SEM of optimized formulation (F17)

The optimized formulation was subjected to SEM analysis to examine the morphology of microspheres. The SEM analysis demonstrated that microspheres were spherical in shape and had a smooth and nonporous surface. The SEM images are shown in Figure 6.

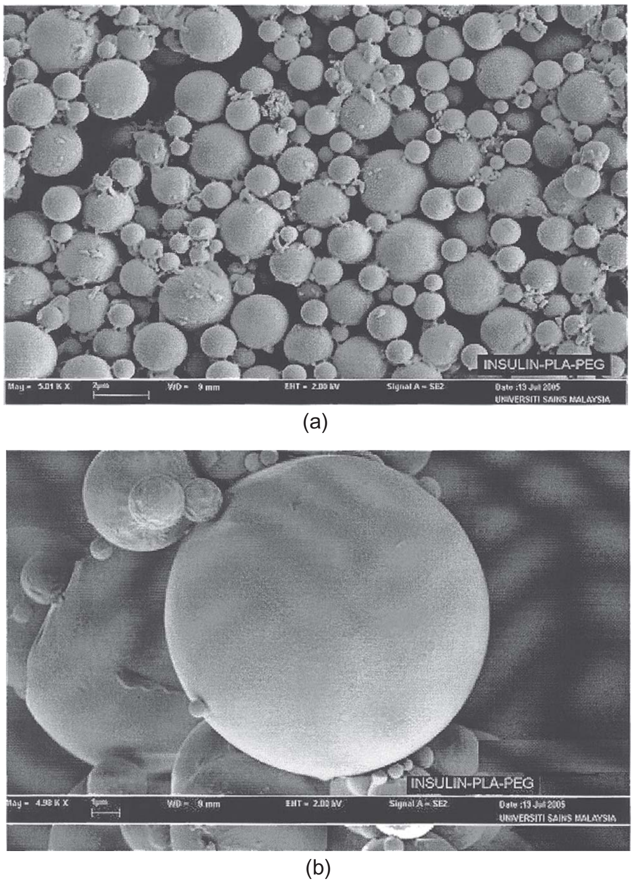

Figure 6. Scanning electron photomicrographs of optimized insulin formulation (F17). (a) Microspheres and (b) surface morphology of microspheres.

Pharmacokinetic study

The pharmacokinetics of reference and test formulations was compared to the extent of absorption (AUC_{0-t}) in diabetic rats. The mean AUC_{0-t} values for reference and test were 434.38 ± 35.13 and 483.33 ± 42.20 μgh/mL, respectively, and it was found that there was no statistically significant difference ($P > 0.05$) in the AUC_{0-t} between the two formulations. The peak plasma concentration ($C_{\max}$) for the reference and test formulations were 6.02 ± 0.46 and 4.02 ± 0.68 μg/mL, respectively. The $C_{\max}$ was obtained at 4 hours after each administration of reference and at 6 hours for the test formulation. The steady-state plasma concentrations were maintained between 24 and 168 hours after administration of single dose of test formulation. The mean plasma insulin concentration versus time profiles of reference and test are shown in Figure 7. It can

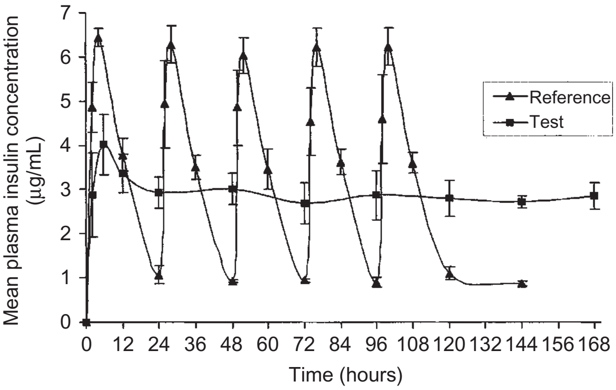

Figure 7. Mean plasma insulin concentration profiles after administration of multiple dose of Humulin® 30/70 (reference) and single dose of insulin-loaded microspheres (test) to streptozotocin-induced hyperglycemic rats. Mean ± SD, $n = 6$.

be seen from Figure 7 that test formulation did not reach the elimination phase. The period of the study was not sufficiently long to allow the collection of samples at the elimination phase for the calculation of other pharmacokinetic parameters. The data indicate that reference and test formulations were comparable to the extent of absorption.

Conclusions

In conclusion, insulin-loaded PLA–PEG microspheres were successfully formulated at laboratory scale using a w/o/w double emulsion solvent evaporation technique with good batch-to-batch reproducibility with respect to yield, particle size, and entrapment efficiency of microspheres. Formulation F17, consisting of PLA–PEG 2000 at 3:7, insulin to PLA–PEG 2000 at 1:60, 3% (w/v) of PVA, 100 mg of glycerol, and 15 mg of zinc oxide, demonstrated the desired in vitro characteristics with a yield of 32%, insulin entrapment efficiency of approximately 42.74%, and a particle size of 3.07 µm. The microspheres were spherical in shape with a smooth and nonporous surface. The release of insulin was approximately 75% with initial low burst release followed by a constant release over a period of 1 week. Insulin-loaded microspheres produced plasma insulin level in a controlled manner over a period of 1 week upon a single subcutaneous administration. The test formulation was comparable with the reference formulation in the extent of absorption. The in vitro and in vivo evaluation of insulin microspheres proved that the present microspheres had ideal properties of controlled release formulation and are also attractive for parenteral application because of their smaller size and biodegradability.

Declaration of interest: The authors report no conflicts of interest.

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