Preparation, In Vitro Release, In Vivo Absorption and Biocompatibility Studies of Insulin-loaded Microspheres in Rabbits

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ABSTRACT

The purpose of this study was to develop a single-dose insulin delivery system based on poly (lactide-co-glycolide) (PLGA) microspheres to provide basal insulin level for a prolonged period. Insulin-loaded PLGA microspheres were prepared by water-in-oil-in-water double emulsion (batch A) and solid-in-oil-in-water emulsion (batch B) methods. Microspheres were characterized for physical characteristics and in vitro release. In vivo absorption of insulin and biocompatibility of insulin-loaded PLGA microspheres were performed in diabetic New Zealand white rabbits. Light and transmission electron microscopy were performed on the skin tissues excised from microspheres injected sites in order to study the biocompatibility. The burst release of insulin was high (47%) from batch B and low (5%) from batch A. Therefore, we mixed microspheres of batch A and B in ratio of 3:1 w/w, which produced desirable in vitro release profile. In vivo absorption study showed that insulin-loaded microspheres provided a serum insulin level of 20–40 μU/ml up to 40 days. Biocompatibility study provided evidence of normal inflammatory and foreign body reactions, which were characterized by the presence of macrophages, fibroblasts and foreign body giant cells. Neither necrosis nor tissue damage was identified. At the end of 12 weeks, no distinct histological differences were observed in comparison to the control tissue samples. In conclusion, insulin-loaded PLGA microspheres controlled the in vivo absorption of insulin to maintain the basal insulin level for longer period and the delivery system was biocompatible.

KEYWORDS: PLGA, microspheres, insulin, in vivo absorption, biocompatibility.

INTRODUCTION

Diabetes mellitus is a group of metabolic disorders characterized by persistent hyperglycemia, an excess of glucose in the blood. It affects ~17 million people, or 6.2% of the population in the United States. There are two major types of diabetes, type I and type II. Type I diabetes, also called insulin-dependent diabetes mellitus (IDDM), and is characterized by an absolute insulin deficiency due to autoimmune destruction of the beta cells in the pancreas. It typically occurs in children and adolescents. Approximately 5%-10% of patients with diabetes have type I disease. The majority (90%-95%) of patients with diabetes have type II disease, also called noninsulin-dependent diabetes mellitus (NIDDM), which is characterized by insulin resistance (ie, reduced sensitivity of cells to the action of insulin) and a relatively deficiency of insulin. Complications of hyperglycemia include the macrovascular complications (eg, coronary artery disease, myocardial infarction, stroke etc.) and microvascular complications (eg, nephropathy, end-stage renal disease, blindness etc.).[1]

In non-diabetic persons, insulin secretion can be divided into two basic components, basal and stimulated. Stimulated insulin secretion occurs in response to a meal and results in insulin concentrations of 60–80 μU/ml within 30-minute after the meal. Concentrations return to basal levels in 2 to 4 h.[2] Basal insulin is secreted continuously between meals and throughout the night at a rate of 0.5–1 Unit/h in adults.[3] Basal insulin secretion provides serum concentrations of 5–15 μU/ml.[2] The continuous low-level basal insulin secretion into portal circulation modulates the rate of overnight hepatic glucose and glucose output during prolonged periods between meals. It reduces hepatic glucose production but allows for glucose levels sufficient for cerebral energy production at bedtime. In this way, basal glucose levels are maintained constant and within a narrow concentration range.[2] It has been suggested that basal insulin supplement should be used earlier in the course of type 2 diabetes.[4,5]

Insulin therapy by frequent injection is required for the treatment of all patients with type I diabetes and many patients with Type II diabetes. At present, commercial insulin products are solutions and need to be injected subcutaneously. In order to achieve target blood glucose control, intensive insulin treatment is often used. This treatment involves daily one or more doses of intermediate- or long-acting insulin injection 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. Injectable polymeric delivery systems

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can be used for controlled release of insulin continuously for a desired period of time.[6] This 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.[4]

It is worthy to note that the biocompatibility of polymeric delivery system is important for its clinical acceptance. A desirable response of an implanted biomedical polymer would show a short-lived inflammatory response with minimum fibrosis resulting from the normal healing response of wounds.[7] The biodegradable polyesters such as PLGA and PLA have been extensively evaluated for their biodegradation and biocompatibility.[8-10] No adverse reactions either locally or systemically were observed when PLA and PLGA microspheres were used in vivo.[9] Another important thing in tissue biocompatibility study is the effect of the incorporated active agent on the tissue response. A slight increase in inflammatory and foreign body responses were observed for cisplatin-loaded microspheres compared with cisplatin-free microspheres.[11] These studies pointed out the importance of carrying out control studies using delivery system without active agent.[9]

Therefore, insulin-loaded PLGA microspheres were investigated for in vitro insulin release, in vivo basal insulin delivery and biocompatibility in rabbits.

EXPERIMENTAL

Materials

Human recombinant insulin was supplied by Serologicals Corporation (Norcross, GA). PLGA (50:50, intrinsic viscosity 0.63 dl/g) was purchased from Birmingham Polymer, Inc (Birmingham, AL). Poly vinyl alcohol (PVA, Mw 3000–7000) was obtained from Sigma Chemical Co (St Louis, MO). Methylene chloride and MicroBCA Kit were procured from Fisher Chemical Co (Fair Lawn, NJ) and Pierce (Rockford, IL), respectively. All the other reagents were of analytical grade.

Preparation of Insulin-loaded PLGA Microspheres

Water-in-oil-in-water (w/o/w) Double Emulsion Method

Fifteen milligram of insulin was dissolved in 200 μl of glycine-HCl buffer (pH 2.5, 100 mM). Insulin solution was then emulsified with 2 ml of methylene chloride solution containing 400 mg of PLGA. The emulsification was performed by an ultrasonicator with a microtip (Sonifier® cell disruptor, Model W185, Heat System-Ultrasonics, Inc., Plainview, NY) for 30 seconds at 40 W. The primary emulsion was added into 100 ml of aqueous solution containing 2% w/v PVA as a surfactant and stirred using a Silverson L4RT Homogenizer (Silverson Machines, East Longmeadow, MA) for 2-minute at 3000 rpm. The resultant double emulsion was then transferred into 800 ml of distilled water and magnetically stirred for 3 h at room temperature at 500 rpm to evaporate methylene chloride. The hardened microspheres were collected by filtration (Aspirator Pump, Cole-Parmer, Chicago, IL) and washed three times with deionized water and finally freeze-dried using a lyophilizer (VIRTis, VIRTIS Co., Gardiner, NY) to obtain free-flowing powder.

Solid-in-oil-in-water (s/o/w) Emulsion Method

Insulin (40 mg) was accurately weighed and suspended in 2 ml of methylene chloride solution containing 400 mg of PLGA under homogenization at 8000 rpm for 2-minute. The homogenous suspension of insulin in polymer solution was then added to 30 ml of aqueous solution containing 2% w/v PVA as a surfactant and stirred using a Silverson L4RT Homogenizer (Silverson Machines, East Longmeadow, MA) for 2-minute at 3000 rpm to form an o/w emulsion. This emulsion was then stirred continuously at reduced speed (500 rpm) for 3 h to evaporate the methylene chloride. The hardened microspheres were collected and freeze-dried to obtain free-flowing powder.

Determination of Insulin Content in Microspheres

Insulin content was determined by dissolving accurately weighed microspheres into 0.5 ml of 1 M sodium hydroxide solution overnight at 37°C. The solution was then neutralized by adding 0.5 ml of 1 M HCl. The resultant solution was analyzed for total insulin content by MicroBCA protein assay.[12] Samples were assayed in triplicate. Encapsulation efficiency of insulin in microspheres (ie, percentage of insulin entrapped in the microspheres with respect to the total amount of insulin added during the preparation of microspheres) was determined.

In Vitro Release

In vitro release studies were performed by suspending 20 mg of microspheres into 1 ml phosphate-buffered saline solution (10 mM, pH 7.4, 0.05% w/v NaN_3) in a centrifuge tube, incubating at 37°C in a water bath (Model 50 Reciprocal Shaking Water Bath, Precision Scientific, Winchester, VA), and shaking at 40 rpm. At predetermined time intervals, the samples were centrifuged (Sorvall® RC-5 Super-speed refrigerated centrifuge, DuPont Co, Newtown, CT), and the supernatant was withdrawn completely and replaced with the fresh buffer. The amount of the released insulin was determined by MicroBCA protein assay.[12] In vitro release

Table 1. Characterization of insulin-loaded microspheres

ID	Fabrication Method	Theoretical insulin loading (%, w/w)	Encapsulation efficiency (%)*,†	Mean size(μm)
A	w/o/w	3.3	85.9 (± 3.2)	55
B	s/o/w	9.0	84.2 (± 0.6)	20

* Encapsulation efficiency (%) = mass of the encapsulated drug/mass of the total drug used in preparation.
†$N = 3 \pm$ s.d.

studies were performed in triplicate for each batch of microspheres. A mixture of microspheres prepared by w/o/w and s/o/w methods in a ratio of 3:1 w/w was also studied for in vitro release. The results were expressed as mean ± s.d. ($n = 3$). Statistical comparisons were made using Student's *t*-test. The probability value of less than 0.05 was considered significant.

Diabetic Animal Model

New Zealand White rabbits with initial body weight of 2–3.5 kg and maintained on standard laboratory chow and water ad libitum were injected with10% w/v alloxan (125 mg/kg body weight) freshly prepared in sterile saline via a marginal ear vein. To counteract the hypoglycemia caused by insulin release from necrotic β-cells due to the acute action of alloxan, the rabbits were provided with 5% sucrose instead of water for the first 24 h after injection. The rabbits were considered diabetic if the blood glucose level, as determined by the glucose oxidase method using a glucometer (Glucometer Elite®, Bayer Corporation, Elkhart, IN), was 300 mg/dl or more one week after alloxan injection. These diabetic rabbits were used in the in vivo insulin absorption study. Institutional Animal Care and Use Committee of North Dakota State University approved the animal experimental protocol.

In Vivo Insulin Absorption

The calculated amount of insulin-loaded microspheres was suspended in 0.5 ml of an aqueous injection vehicle containing 2% carboxymethylcellulose, 2% mannitol, 0.1% Tween 80. Microsphere suspension was injected subcutaneously at neck region of diabetic rabbits at a dose of 30 U insulin/kg body weight. The control group consists of rabbits without insulin treatment (blank microspheres). At predetermined time points (0 h, 6 h, 1 day, 1, 2, 4, 6, 8, and 12 weeks), blood samples (~1ml) were withdrawn from ear vein to measure plasma insulin and blood glucose level. All blood samples were withdrawn in the morning after overnight fasting, except the sample taken 6 h after dosing.

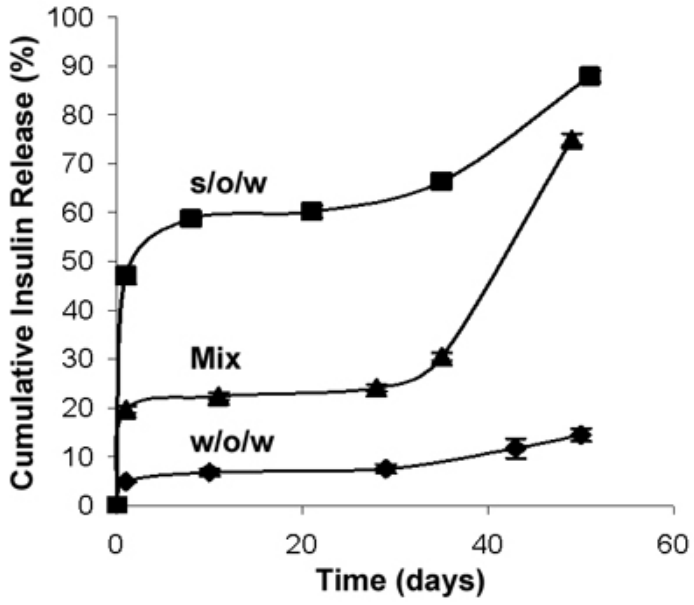

Figure 1. In vitro release profiles of insulin from PLGA microspheres prepared by different techniques. Key: (♦) w/o/w emulsion method, (■) s/o/w emulsion method, and (▲) the mixture of microspheres prepared by the w/o/w and s/o/w methods.

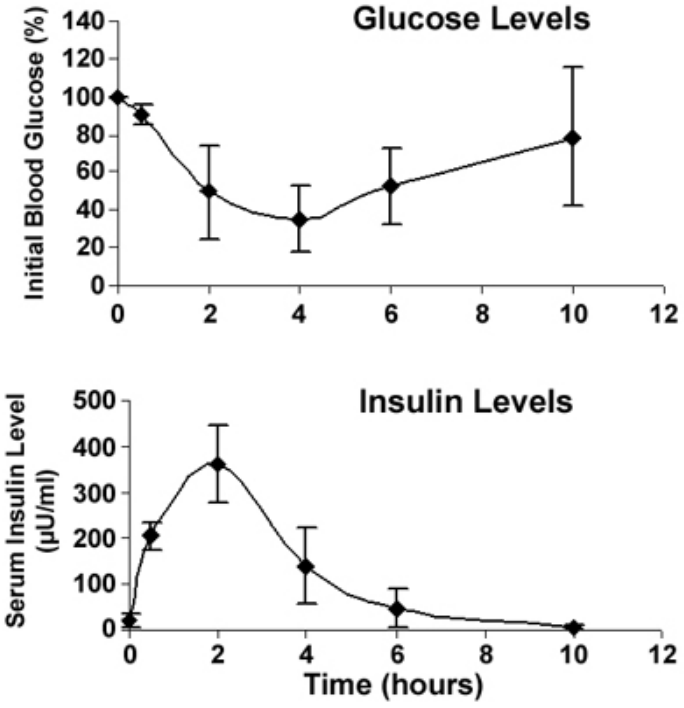

Figure 2. Blood glucose levels and serum insulin levels following subcutaneous injection of insulin (2U) solution.

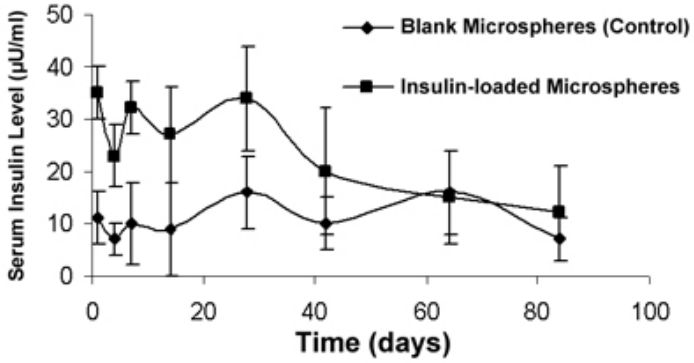

Figure 3. Serum insulin levels in diabetic rabbits following a single subcutaneous injection of insulin-loaded PLGA microspheres at a dose of 30U/kg body weight.

For comparison purpose, insulin solution was also injected at a dose of 2U/rabbit. Blood samples were taken at time 0 and 0.5, 2, 4, 6, and 10h after injection. Blood glucose level was determined by the glucose oxidase method[13] using a glucometer (Glucometer Elite®, Bayer Corporation, Elkhart, IN). Serum insulin was measured by radioimmunoassay, using a RIA kit. In this assay, the concentrations of the insulin in serum samples were measured by an indirect method based on the competition between radiolabeled ^{125}I-insulin and unlabeled insulin in an antigen-antibody reaction. A second antibody goat anti-guinea pig IgG directed against the first antibody, guinea pig anti-human IgG, is used that leads to the formation of large complexes which upon centrifugation are counted with a gamma-counter.[14] Three animals were used in each group.

In Vivo Biocompatibility of Microspheres

The biocompatibility of microspheres was studied in New Zealand white rabbits. For each rabbit, two doses of microspheres (with and without insulin) were injected at separate sites of the back of rabbits. At 1, 4, 8 and 12 weeks after injection, rabbits were euthanized by injecting an overdose of Nembutal (100mg/kg body weight). Tissue samples from the injection sites were removed using a scalpel for microscopic evaluations.

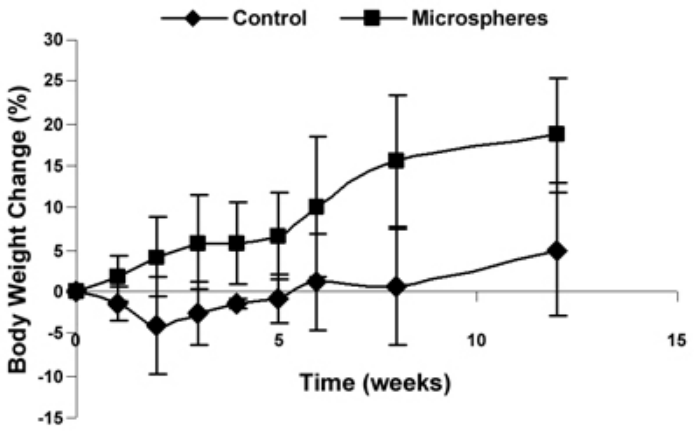

Figure 4. Body weight of diabetic rabbits following a single subcutaneous injection of insulin-loaded PLGA microspheres at a dose of 30U/kg body weight.

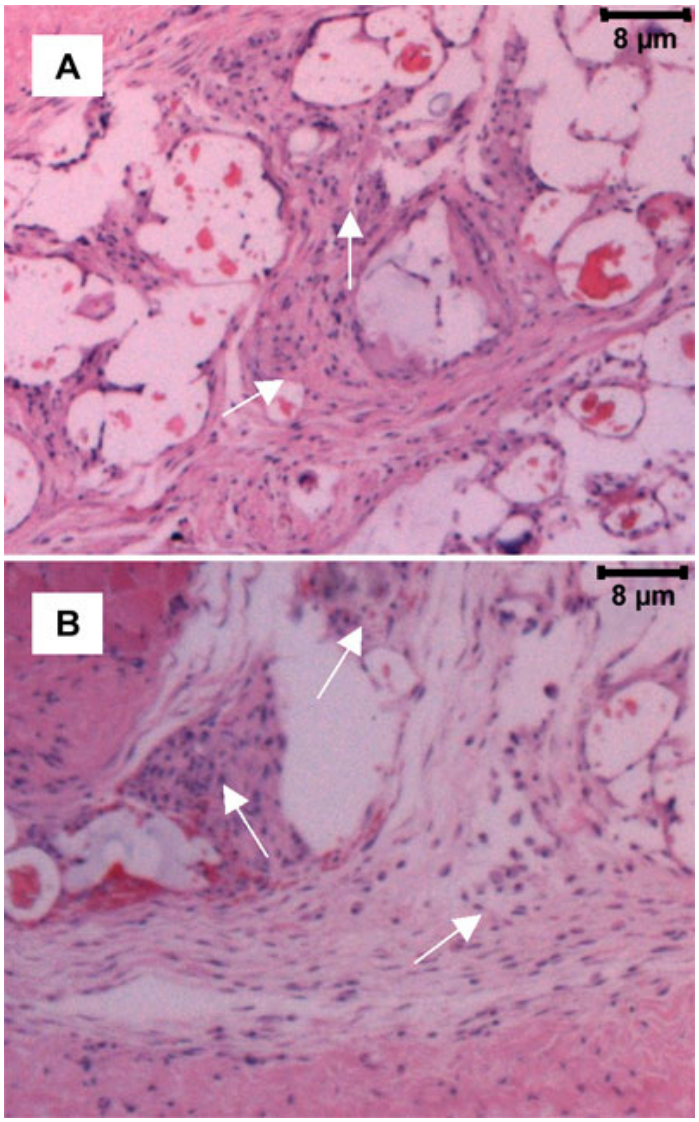

Figure 5. Light photomicrograph of subcutaneous tissue 1 week after subcutaneous injection of blank (A) and insulin-loaded (B) microspheres showing inflammatory responses to the implants, represented by the inflammatory cells (indicated by arrows) surrounding the tissues.

Light Microscopy

Tissue samples were fixed in 10% buffered formalin solution and then embedded in paraffin. Transverse sections (5 μm) were cut using a microtome. Slides of the skin sections were prepared and stained with hematoxylin and eosin. The slides were observed under light microscope (Meiji Microscope, Osaka, Japan).

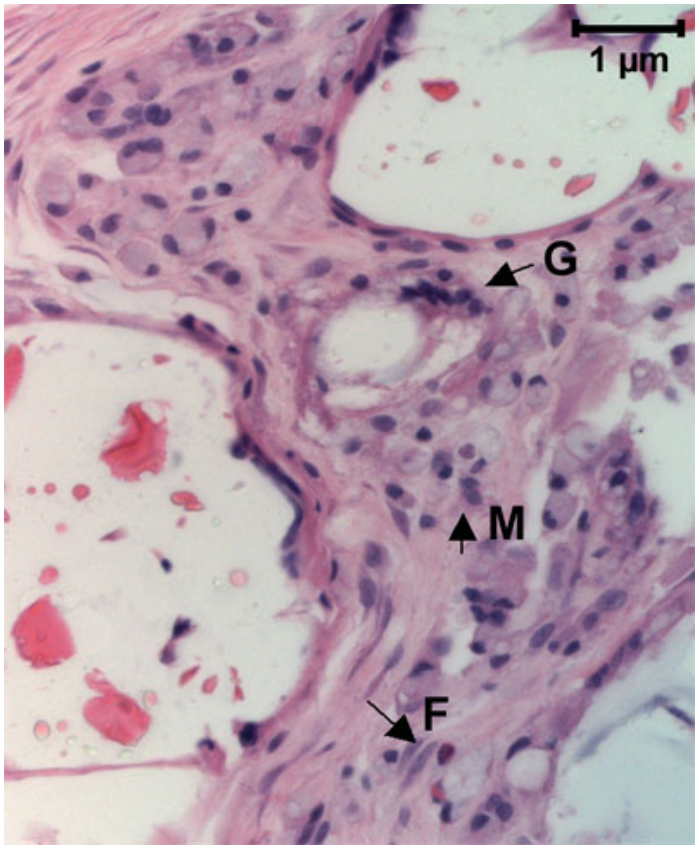

Figure 6. Light photomicrograph of subcutaneous tissue 1 week after subcutaneous injection of insulin-loaded microspheres. The inflammatory cells are mainly macrophages (M) and foreign body giant cells (G). "F" stands for fibroblast.

Transmission Electron Microscopy

Skin sections were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.35 (Tousimis Research Corporation, Rockville, MD) for 2 h. The samples were then rinsed with sodium phosphate buffer and placed in 2% osmium tetroxide in phosphate buffer (pH 7.35) for 2 h. Following dehydration in a graded acetone series, samples were embedded in Epon-Araldite-DDSA and sectioned at 60 nm thickness. Sections on grids were stained with lead citrate for 2.5-minute before observing on a JEOL JEM-100CX II electron microscope.

Results and Discussion

Physico-chemical Characterization of Microspheres

Insulin-loaded microspheres were prepared by two different techniques. Table 1 shows the characteristics of microspheres. The average size of microspheres prepared by the w/o/w and s/o/w methods was 55 µm and 20 µm, respectively. High insulin encapsulation efficiency (> 84%) was obtained for microspheres fabricated by both w/o/w and s/o/w methods.

Figure 1 shows the in vitro release of insulin from three batches of microspheres (A, B, and C) obtained from w/o/w (A), s/o/w (B), and microsphere mixture (C: A/B=3:1w/w). The insulin release from formulation A was slower. The burst release of insulin was ~5%, and total release in 2 mo was less than 20%. In contrast, the release profile from formulation B showed a different pattern. The burst release (47.1 ± 1.8%) was significantly ($P<0.05$) higher than formulation A (5.0 ± 0.1%), and the total release within 2 mo was greater than 90%. Clinically, these two formulations may not be desirable. High burst release may lead to a toxic level in patients. Low release may not provide sufficient therapeutic insulin concentration. Therefore, a better-controlled release pattern should have a proper burst followed by gradual release. In this study, the release pattern was changed by physically mixing microspheres of batches A and B (Figure 1). The mixture of microspheres has an initial release of 20%, which is between formulations A and B. A higher release rate was observed in formulation C after 30 days when the polymer erosion started. This result may be due to faster degradation product of formulation B generating acidic microenvironment and facilitating the degradation of microspheres of the batch A. Desirable release profiles have been observed by physically

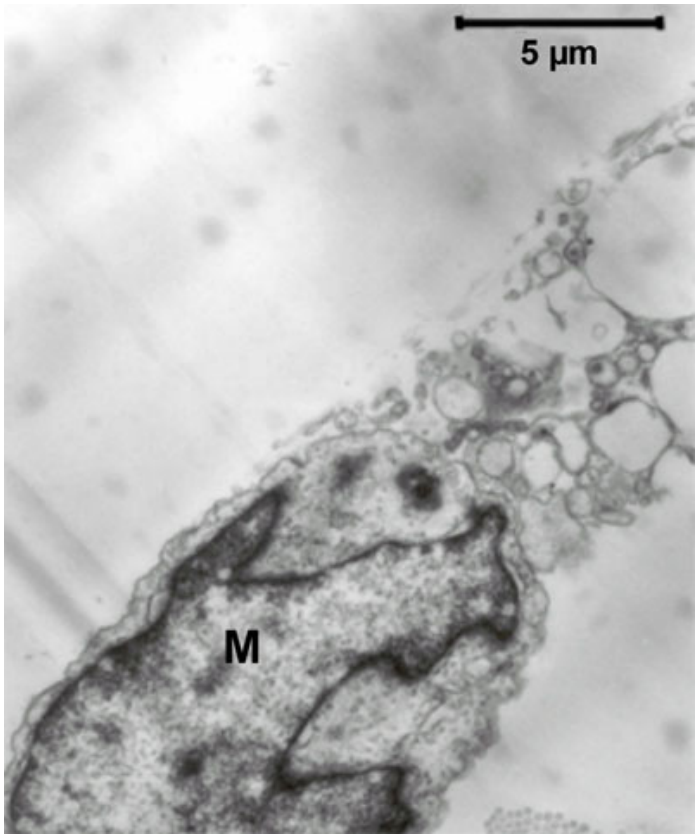

Figure 7. TEM micrograph of subcutaneous tissue 1 week after subcutaneous injection of insulin-loaded microspheres, showing parts of macrophage (M) that had invaded the material.

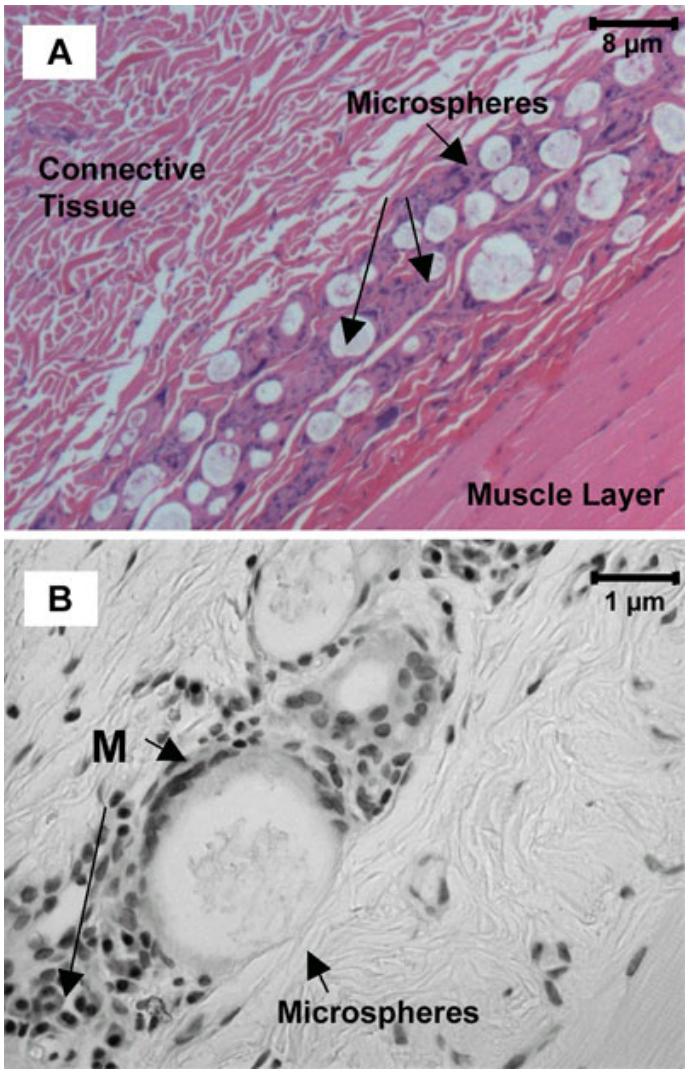

Figure 8. Light photomicrograph of subcutaneous tissue 4 weeks after injection of blank microspheres. A few isolated microspheres can be identified and surrounded by macrophages (M).

blending polymers with different molecular weights or mixing microspheres prepared with these polymers.[15] These approaches are believed to be practical alternatives to the expensive process of customizing the polymer properties to obtain desired drug release profiles.

In Vivo Absorption of Insulin

Figure 2 shows the blood glucose level (A) and serum insulin concentration (B) following the subcutaneous administration of insulin solution in phosphate-buffered saline (pH 7.4). The blood glucose level decreased to its lowest level, 35% of initial value, 4 h after administration. Blood glucose level returned to its initial level 10 h after injection. The mean peak serum insulin concentration (C_{max}) of 363 µU/ml was observed at 2 h after administration. Serum insulin level decreased rapidly and reached to 10 µU/ml after 10 h.

Figure 3 shows serum insulin levels following single subcutaneous injection of insulin-loaded microspheres (30U/kg body weight). Insulin levels decreased with time and reached ~30 µU/ml at 24 h. The insulin level remained between 20 - 40 µU/ml up to 40 days. The insulin level of the control group remained between 5–15 µU/ml throughout the study.

Figure 4 shows the change in the body weight of diabetic rabbits following subcutaneous injection of insulin-loaded microspheres. In a period of 12 weeks, there was 5% increase in the average body weight of rabbits of the control group. However, the body weight increase was 19% in the insulin-loaded microspheres group. In vivo insulin absorption showed consistent insulin level from day 1 to day 40 in diabetic rabbits. A continuous basal insulin supply may help to improve the condition of diabetics. A body weight gain in diabetic rats was observed as fast as normal rats after single injection of insulin-loaded microspheres in 28 days.[16] The above study also found very little body weight gain of diabetic rats by daily injection of insulin solution.

Biocompatibility

Figures 5 and 6 show the light micrographs of the tissue after 1 week of subcutaneous injection of microspheres. A

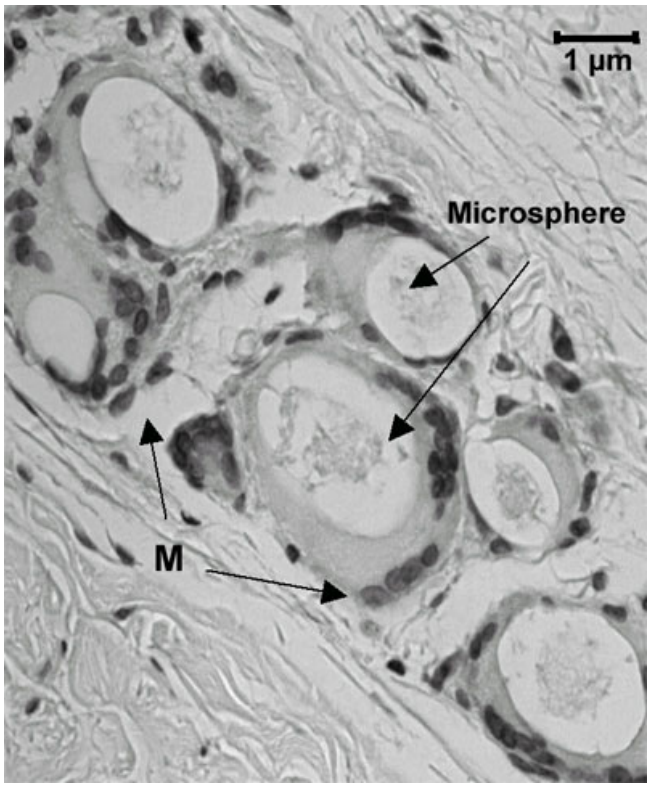

Figure 9. Light photomicrograph of subcutaneous tissue 4 weeks after subcutaneous injection of insulin-loaded microspheres. Microspheres were surrounded by macrophages (M).

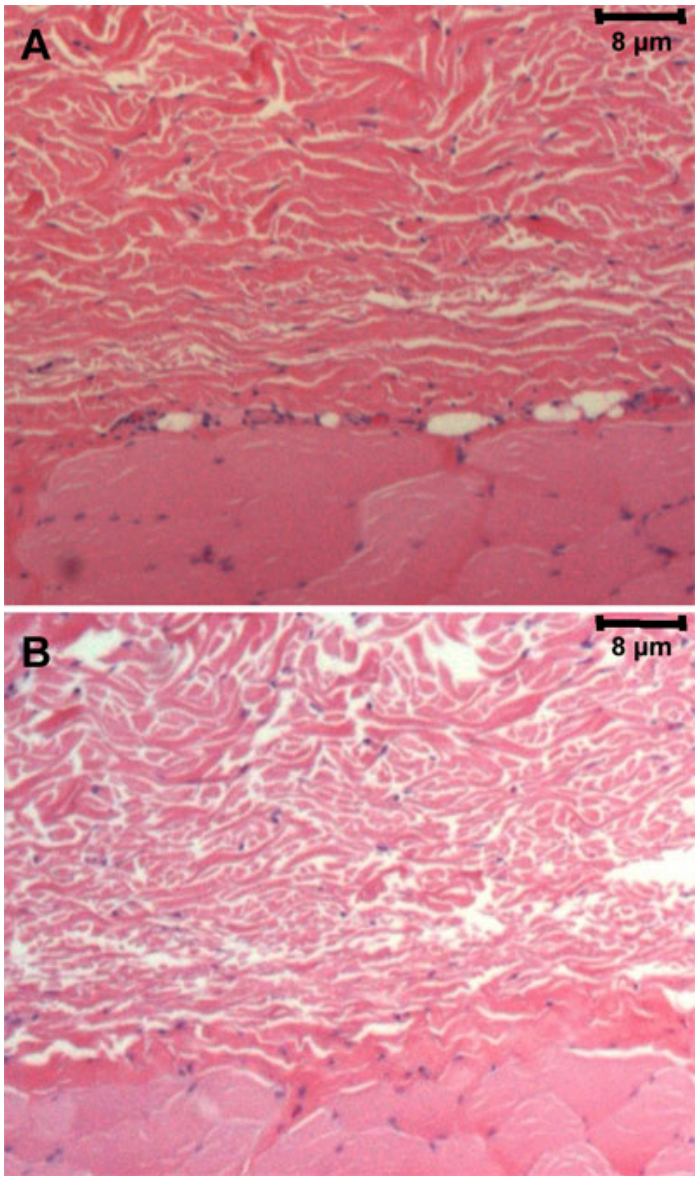

Figure 10. Light photomicrograph of subcutaneous tissue 12 weeks after subcutaneous injection of blank (A) and

large number of macrophages and a few polymorphonuclear (PMN) cells were observed infiltrating into the connective tissue. The macrophages were further identified in TEM micrograph (Figure 7). The presence of large number of macrophages indicates a chronic inflammatory reaction in response to the foreign body invasion and injury.[9] Similar inflammatory reactions were observed with the blank and insulin-loaded microspheres.

Figures 8 and 9 show the tissue responses after 4 weeks of subcutaneous injection of microspheres. Compared with the tissue samples after 1 week, less inflammatory cells were identified. A cluster of microspheres was seen at the injection sites. Microspheres were surrounded by macrophages, foreign body giant cells and fibroblasts.

Figure 10 shows the tissue responses after 12 weeks of subcutaneous injection of microspheres. No signs of cell damage such as rough endoplasmic reticulum or swollen mitochondria were observed in any tissue samples. Normal connective and muscular tissues were seen in all samples, which were highly comparable to the control tissue sample (Figure 11).

The primary concern in this study is the local tissue responses instead of systemic toxicity to the polymer formulations. Histological evaluation of tissue samples retrieved from injection sites at different time points within three months showed a typical foreign body and wound healing responses. There is trauma, a wound at the implant site, since microspheres injected. An inflammatory response to tissue injury, even in the absence of microspheres, would start and proceed through the steps of wound healing. This response is considered as components of the tissue or cellular host reaction to injury.[9] The first phase of inflammation is acute inflammation characterized by presence of polymorphoneclear lymphocyte. It occurs within 24–48 h. Chronic inflammatory response is the next phase of reaction. Monocytes migrate into the site of injury and differentiate into macrophages. The acute and chronic inflammatory reactions are generally similar regardless of the degradation

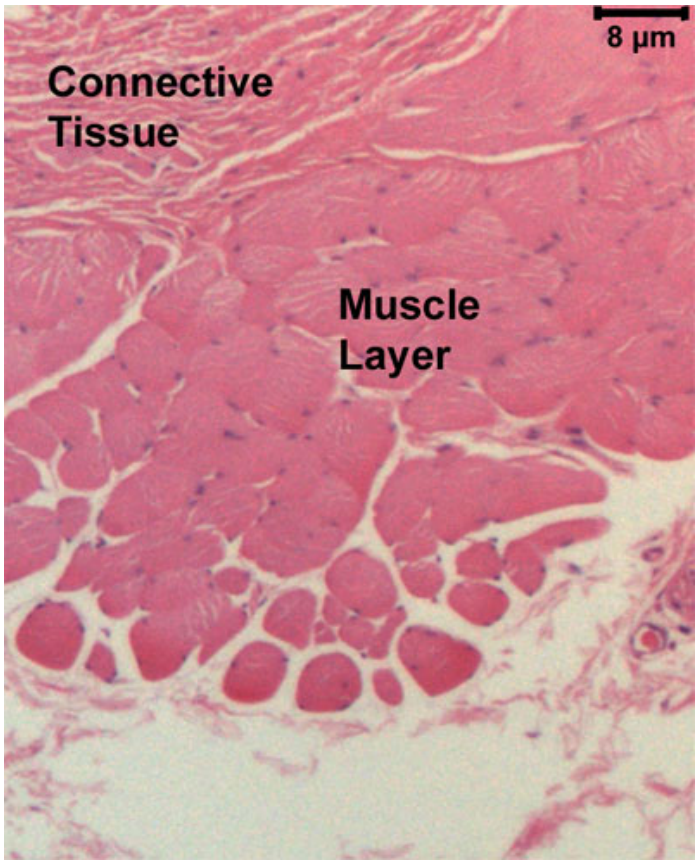

Figure 11. Light photomicrograph of subcutaneous tissue from control tissue sample. No inflammatory cells were identified.

rate of polymer and may contain components of injury caused by injection itself.[9] Macrophage is the predominant cell type in the tissue reaction to the polymer devices. Since macrophages initiate a cascade of responses, infiltration of macrophages towards the implantation site is important. Macrophages can secrete polypeptide hormones and enzymes, which are important for the sequence of events during wound healing process.[7] Macrophages also play an essential role in the phagocytosis of the polymer and its degradation.

CONCLUSIONS

In vitro release of insulin from microspheres was improved by physically mixing microspheres prepared by w/o/w double emulsion and s/o/w emulsion methods. Insulin-loaded microspheres maintained basal insulin level in diabetic rabbits for 40 days. Histological evaluations suggested that the insulin-loaded microspheres were biocompatible.

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