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Sustained prolonged topical delivery of bioactive human insulin for potential treatment of cutaneous wounds

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ABSTRACT

Skin damaged by heat, radiation, or chemical exposure is difficult to treat and slow to heal. Indeed full restoration of the tissue is difficult to obtain. Sub-dermal insulin injection was recently shown to stimulate wound healing of the skin by accelerating wound closure, stimulating angiogenesis and inducing a regenerative process of healing. We have developed a topical delivery vehicle that is capable of releasing therapeutic levels of bioactive insulin for several weeks with the potential to stimulate and sustain healing. By encapsulating the crystalline form of insulin within poly(D,L-lactide-co-glycolide) microspheres, we succeeded in stabilizing and then releasing bioactive insulin for up to 25 days. To measure bioactivity we used Rat L6 myofibroblasts, stimulated them with this slow release insulin and determined activation of the receptors on the cell surface by quantifying AKT phosphorylation. There was only a minor and gradual decrease in AKT phosphorylation over time. To determine whether the slow release insulin could stimulate keratinocyte migration, wounding was simulated by scratching confluent cultures of human keratinocytes (HaCaT). Coverage of the scratch "wounds" was significantly faster in the presence of insulin released from microspheres than in the insulin-free control. Extended and sustained topical delivery of active insulin from a stable protein crystal-based reservoir shows promise in promoting tissue healing.

1. Introduction

Recombinant peptides are an increasingly important therapeutic intervention against a variety of medical conditions including diabetic, oncologic, cardiovascular, immunosuppressive and gastroenterological diseases (Liu et al., 2009; Stevenson, 2009; Thomas et al., 2009; Parker et al., 2004; Dudley-Brown et al., 2009). Therapeutic peptides are part of a larger class of biopharmaceuticals that also encompasses monoclonal antibodies and nucleic acid-based medicinal products (Walsh, 2005; Aberra and Lichtenstein, 2006; Golzio et al., 2005). Approximately 150 recombinant biopharmaceuticals have been approved by the Food and Drug Administration (FDA) and the list of submissions and approvals continues to grow (Ferrer-Miralles et al., 2009).

While growth is rapid, two major challenges remain. Instability and a need for sustained delivery mechanisms both influence the therapeutic potential of peptide-based biopharmaceuticals. Unlike small molecular weight drugs less than 1 kDa, polypeptides are complex molecules with molecular weights ranging from 5 to 200 kDa (Rathore, 2009). Maintaining amino acid sequence and unique three-dimensional structure is essential for preserving polypeptide bioactivity and avoiding undesirable immunological reactions (Wang, 2005; Maas et al., 2007). Furthermore, polypeptide delivery has often been accomplished via intravenous infusion, or injection as is practiced with insulin (Degim and Çelebi, 2007; Chen and Singh, 2008). Unfortunately, these routes of delivery offer little to no protection from enzymatic degradation, and require frequent dosing to maintain therapeutic levels within systemic circulation.

Although insulin has been studied for the treatment of Type 1 diabetes, it has also been investigated for wound healing since the 1920s (Joseph, 1930; King et al., 1928). Rosenthal (1968) demonstrated that topically applied insulin increased wound tensile strength and decreased healing times in Wistar rats. Similarly, Udupa and Chansouria (1971) applied a linear musculoperitoneal wound in rats and administered 2 units of insulin per 100 g subcutaneously. Faster wound healing was observed in insulin-treated rats versus the non-treated group. Histological analysis of wound tissue

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revealed an earlier appearance of collagen fibers with more compact, dense and well-oriented morphology versus control animals (Udupa and Chansouria, 1971). Recently, Zhang et al. (2007) tested local insulin–zinc injections on skin donor site healing in rabbits. Subcutaneous injections of 0.25 U long acting insulin–zinc suspensions were directly administered to the backs of dermatomed adult rabbits. Injections were delivered every other day and compared to controls consisting of placebo and zinc suspension alone. With localized injection, wound healing was accomplished in 9 days, significantly faster than any of the control groups and without signs of major systemic side effects. However, until very recently, little was known about the molecular mechanisms involved in the effects of insulin on speeding healing. Liu et al. (2008) have shown that insulin stimulates keratinocyte migration and proliferation, that this effect is independent of EGF and that the epidermis assumes a more mature morphology when the wounds are treated with insulin. Moreover, these investigators also showed that insulin stimulates angiogenesis and that the blood vessels in the presence of insulin are more stable. Taken together, these findings indicate that local insulin delivery is a viable treatment option, as long as improvements in the formulation and delivery are developed.

Biodegradable, poly(D,L-lactide-co-glycolide) (PLGA) microspheres are one delivery vehicle capable of releasing peptides in a controlled manner for up to several weeks (van de Weert et al., 2000; Taluja et al., 2007; Crotts and Park, 1998). The use of PLGA in FDA approved medical devices such as Lupron Depot®, has underscored the polymer's superior efficacy as a drug delivery vehicle for long term, controlled release applications (Mitwally et al., 2005; Mittal et al., 2007). Although PLGA microspheres have been applied as oral or injectable peptide delivery vehicles, they have also been explored as peptide carriers for topical application in wound healing (Değim, 2008). PLGA microspheres are favorable for topical delivery applications because of their hydrophilic, lubricious, smooth, permeable, biodegradable, and biocompatible qualities (Değim, 2008; Boateng et al., 2008). Unfortunately, conventional PLGA encapsulation techniques such as the water-in-oil-in-water (W/O/W) emulsion, solvent evaporation method have proven detrimental to peptide bioactivity (Manoharan and Singh, 2009). Factors such as heat and shear forces associated with mixing, and interfacial tension can lead to unfolding and deactivation of polypeptides (Krishnamurthy and Manning, 2002; Chi et al., 2003; Chang et al., 2009; Daniel, 1996). Likewise, polypeptides such as insulin can be exposed to highly acidic microenvironments within PLGA microspheres, causing peptide denaturation during the bulk erosion process (Shao and Bailey, 1999; Ding et al., 2006; Rosa et al., 2000; Yeh, 2000). As a result, crystalline forms of enzymes and peptides have been used to overcome formulation stresses and maintain bioactivity during release *in vitro* (Wang et al., 2004; Castellanos et al., 2001). As a consequence of these findings, better methods of release need to be developed using FDA approved materials.

Therefore in this study, we present the encapsulation of human recombinant crystalline insulin into PLGA microspheres by a solid-in-oil-in-water (S/O/W) suspension technique. Various insulin loadings were tested to determine the encapsulation efficiency, microsphere morphology, sphere diameter and pore size. Differential scanning calorimetry (DSC) analysis, insulin release kinetics and bioactivity were assessed *in vitro* and in cultured cells. The results demonstrate the viability of this encapsulation technique for implementation in topical wound healing devices.

2. Materials and methods

2.1. Materials

PLGA (D,L;50:50, 5–15 kDa), crystalline human recombinant insulin, poly(vinyl alcohol) (PVA), LR white embedding kit, NaOH, sodium dodecyl sulphate (SDS) and sodium phosphate dibasic anhydrous were supplied by Sigma Aldrich (Oakville, Canada). Bovine serum albumin (BSA) and micro-BCA protein assay kit were obtained through ThermoFisher (Ottawa, Canada). Dichloromethane (DCM) was supplied by Caledon Labs (Georgetown, Canada). Rat L6 myoblasts were supplied by ATCC (Manassas, USA). Human keratinocytes (HaCaT) were provided by Cell Line Services, Eppelheim, Germany (Boukamp et al., 1988). Dulbecco's modified Eagle's medium (DMEM), Dulbeco's phosphate buffered saline (DPBS), and 0.25% trypsin with EDTA were obtained through Gibco (Carlsbad, USA). Fetal bovine serum (FBS) and penicillin–streptomycin were purchased from Hyclone (Fair Lawn, USA). FACE AKT ELISA assay kit was obtained through Active Motif (Carlsbad, USA).

2.2. Microparticle preparation

Human recombinant crystalline insulin (hRcI) was microencapsulated using a solvent extraction method from a solid-in-oil-in-water (S/O/W) suspension method (Fig. 1). Microspheres were prepared with 0 (placebo), 2.5, 5 and 10% w/w hRcI by dissolving 90–100 mg of PLGA in 1 mL of dichloromethane (DCM) and combining with 0, 2.5, 5 or 10 mg of hRcI. The suspension was aspirated several times with glass Pasteur pipette to disperse the crystals, then transferred drop-wise into 30 mL of a chilled aqueous solution of 5% PVA. The resulting S/O/W emulsion was stirred for 1 min at 430 rpm with a high shear impeller mixer (Caframo; Wiarton, Canada), then the mixture was poured into a beaker filled with 400 mL distilled water chilled to 5–7 °C, and stirred overnight in an ice bath at 200 rpm with a propeller mixer. The microspheres were collected by vacuum filtration, rinsed 3 times with ice cold distilled water, then transferred to a −20 °C freezer overnight. The microspheres were then refrigerated at 4 °C for 12 h prior to being placed under vacuum for another 12 h to remove residual DCM. Microspheres were stored at −20 °C when not in use.

2.3. Encapsulation efficiency (E.E.%)

Microspheres (8–10 mg) were dissolved in 3 mL DCM. DCM was evaporated off, then the residue was dissolved in 10 mL aqueous 5% SDS, 0.1 M NaOH with stirring at 80 rpm for 24 h at 37 °C. Protein concentration was determined by micro-BCA protein assay and read spectrophotometrically at 562 nm. Encapsulation efficiencies were calculated by taking the ratio of residual insulin versus the initial insulin added during microsphere formulation. All samples were measured in triplicate.

2.4. Particle size and morphology

Mean sphere and pore sizes were determined from SEM images (JEOL 840, USA). To examine the internal morphology, microspheres were embedded into LR white resin, sectioned and mounted onto copper grids. TEM images (JEOL 1200EX, USA) were later digitally captured and then analyzed for presence and location of insulin crystals.

2.5. DSC analysis

Approximately 4 mg of microspheres were placed into aluminum pans that were hermetically sealed. Both empty and sample pans were placed into a DSC (TA Instruments, USA), cooled to −30 °C and equilibrated for 1 min. Samples were then heated to 110 °C and cooled back to −30 °C at rate of 10 °C per min.

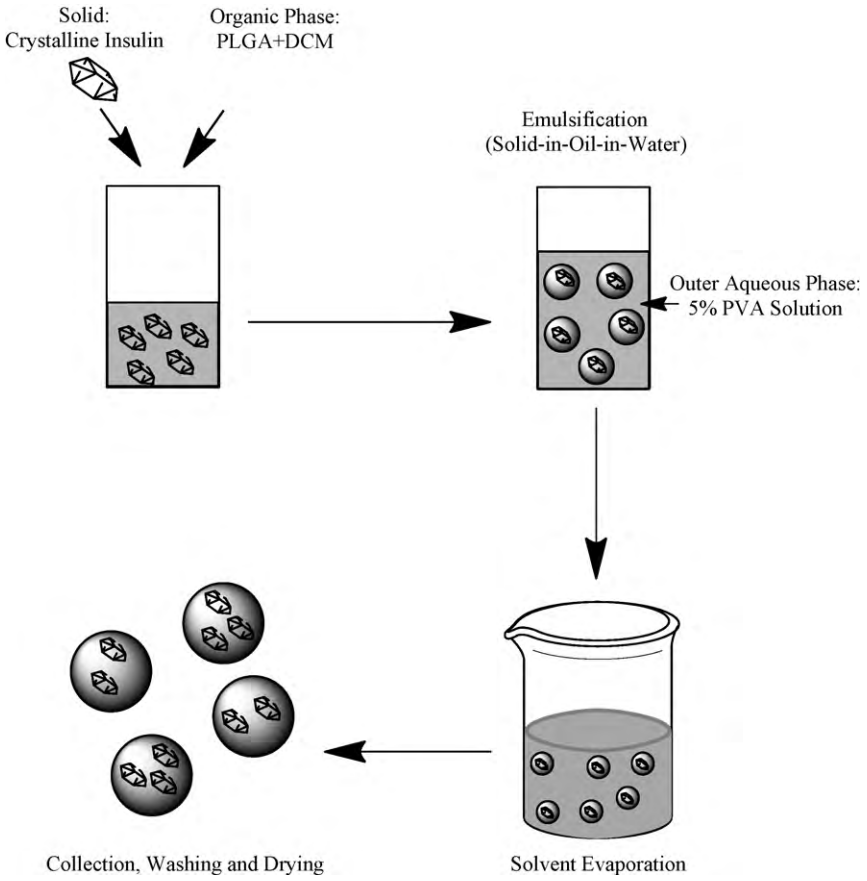

Fig. 1. An illustration indicating the preparation of crystalline insulin loaded PLGA microspheres using a S/O/W technique.

2.6. In vitro kinetics

Microspheres (4–6 mg) were added to 2 mL of 20 mM phosphate buffer at pH 7.4 and mixed on an orbital shaker at 60 rpm and 37 °C. Insulin in supernatants was measured by a micro-BCA assay compared to insulin standards. Supernatants were replaced with fresh buffer after each sampling. All samples were tested in triplicate.

2.7. Cell culture of Rat L6 myoblasts

Rat L6 myoblasts were cultured in T75 flasks containing 15 mL complete DMEM media supplemented with 20% FBS and 1% pen-strep. Cells were incubated at 37 °C and maintained in an atmosphere of 5% CO_2. When cells reached 80–90% confluence, the media was aspirated and cells were washed with 15 mL PBS. Cells were then detached with 5 mL of a 0.25% trypsin and EDTA solution and incubated for 5 min. The trypsin was neutralized with complete media, and cells centrifuged at 200 × *g* for 5 min, then resuspended in 10 mL of fresh complete media. Cells were counted by removing 10 μL of cell suspension and combining it with an equal volume of trypan blue solution. This mixture was vortexed and a sample loaded into a hemacytometer chamber (Fisher; Pittsburgh, USA) and cells counted under an inverted microscope (Nikon; Melville, USA).

2.8. Cell culture of HaCaT Cells

HaCaT cells were cultured in T75 flasks containing 15 mL complete DMEM media supplemented with 10% FBS and 1% pen-strep. Cells were incubated at 37 °C and maintained in an atmosphere of 5% CO_2. Cell passaging and counting were performed as described above for the L6 myoblasts.

2.9. Insulin bioactivity: FACE AKT ELISA

L6 myoblast cell suspensions were diluted to 112,500 cells/mL in complete media and seeded onto 96 well plates (Costar; Lowell, USA) to a final well volume of 200 μL. Cells reached 80% confluency in 24 h, at which time the cells were washed twice in the wells with PBS. Then, 200 μL of starvation media consisting of 99% DMEM and 1% pen-strep were placed into the wells 6 h prior to stimulation. Cells were then stimulated with starvation medium used to dilute (i) fresh insulin dissolved in starvation medium to 150 nM; (ii) supernatant containing 150 nM insulin released from microspheres; or (iii) supernatant from placebo microspheres. Supernatant samples from release experiments were taken from days 1, 7, 14 and 23. After 20 min stimulation, the medium was aspirated off and 100 μL of 4% formaldehyde in PBS was added. The wells were then processed according to the Fast Activated Cell-based ELISA (FACE) AKT kit protocol. The level of AKT phosphorylation was normalized for cell density using crystal violet. Six replicates were used for each sample.

2.10. Insulin bioactivity: scratch assay

HaCaT cell suspensions were diluted to 250,000 cells/mL in complete media and seeded onto 24 well plates (Costar; Lowell, USA) to a final well volume of 1 mL. Cells formed a confluent layer 48 h later, at which time a single scratch was made using a 10 μL pipette tip. All wells were washed twice with 1 mL PBS to remove cellu-

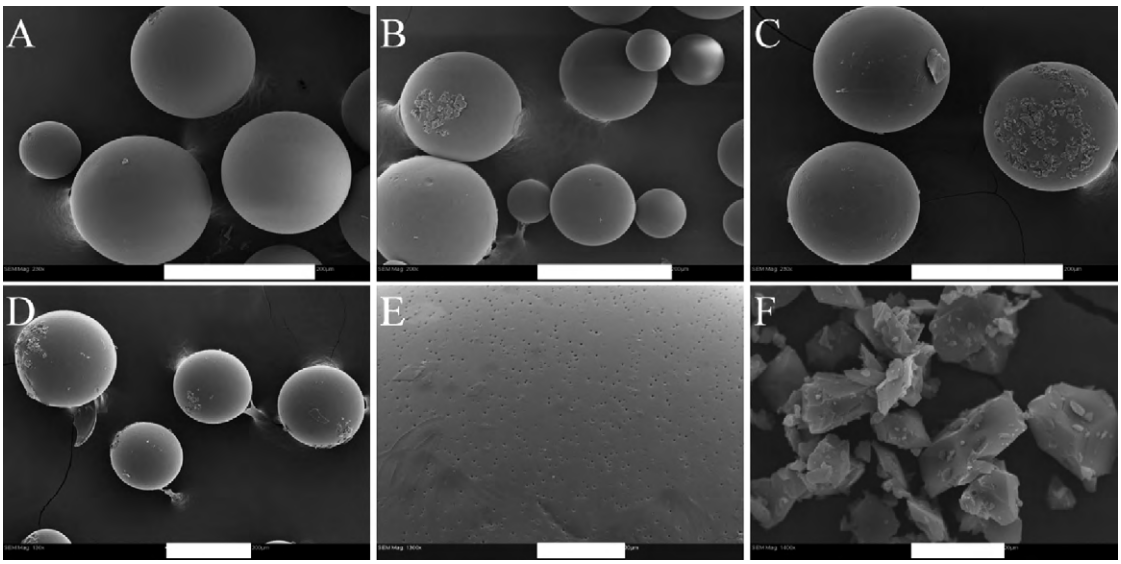

Fig. 2. Shape and surface morphology of microspheres with (A) 0%, (B) 2.5%, (C) 5%, and (D) 10% insulin crystal loadings. Magnified view of the microspheres indicating a highly porous surface structure (E). Recombinant human insulin crystals (F). White size bar found at the bottom of each image represents 200 μm in (A–D), 20 μm in (E) and (F).

lar debris. Then, 500 μL of starvation media consisting of DMEM, 2% FBS and 1% pen-strep was used to dilute (i) fresh insulin to 10^{-7} M; (ii) supernatant containing 10^{-7} M insulin released from microspheres; or (iii) supernatant from placebo microspheres. Supernatant samples from release experiments were taken from days 1, 7, 14 and 23. Scratches were photographed and measured using a bright field inverted microscope (Nikon; Melville, USA) at time points 0, 4, 24 and 48 h. All samples were performed in triplicate.

2.11. Statistical analysis

Graphing and statistical analysis were performed using JMP™. A one-way ANOVA, followed by a pairwise comparison was performed on bioactivity and scratch assay data with a $p = 0.05$.

3. Results

3.1. Insulin content in PLGA microspheres

Microencapsulation of crystalline insulin was performed by solvent evaporation from a multiple phase emulsion. At the lowest loading of 2.5%, insulin was fully accounted for in the microspheres with an encapsulation efficiency of 99 ± 10%. With 5% insulin, encapsulation efficiency decreased to 83 ± 8% and at 10%, encapsulation efficiency was 78 ± 1%.

3.2. Particle size and morphology

The mean microsphere diameters for formulations containing 0, 2.5, 5 and 10% crystalline insulin were 154 ± 57, 131 ± 92, 153 ± 53, and 137 ± 52 μm respectively. SEM micrographs shown in Fig. 2A–D revealed spherical and discrete microspheres, while the emergence of insulin crystals on the sphere surface was more apparent with increased loading. Microspheres appeared smooth, but an enlarged view in Fig. 2E revealed a highly porous surface structure, which was observed in all formulations. The mean pore diameter was 318 ± 121, 282 ± 106, 314 ± 126 and 387 ± 145 nm for 0, 2.5, 5 and 10% loading of crystals, respectively. Insulin crystals seen in Fig. 2F appeared rhombic with sharp and distinct edges and composed of various sizes. The mean crystal size was 4 ± 5 μm. TEM imaging revealed insulin crystals embedded within the polymer matrix of the microsphere, as well as at the periphery as seen in Fig. 3A. A magnified view of the outer edge of the microsphere revealed a porous structure going well into the matrix, as illustrated in Fig. 3B.

3.3. DSC analysis

DSC analysis was performed to determine the effect of increasing insulin crystal content on the glass transition temperature (T_g) of fabricated PLGA microspheres. Thermograms in Fig. 4 illustrate T_g of 13.2, 19.4, 20.8, and 24.0 °C for microspheres with 0, 2.5, 5 and 10% insulin respectively. During formulation, PLGA microspheres with highest crystalline insulin content were noticeably much more rigid when being handled at room temperature.

3.4. In vitro insulin release kinetics

In vitro release kinetics were determined for PLGA microspheres with 2.5–10% crystalline insulin entrapment. All formulations achieved nearly 100% insulin release over the 25-day period as seen in Fig. 5. A burst release of 28–46% was observed within the first 24 h followed by a period of near zero order release for approximately 10 days thereafter. Between days 12 and 14, all formulations exhibited a rapid increase in release of approximately 27–29% before the insulin release reached a plateau by day 25. All formulations exhibited similar biphasic release profiles.

3.5. Insulin bioactivity: FACE AKT ELISA

Bioactivity of released insulin was determined by measuring the levels of phosphorylated AKT in Rat L6 myoblasts after stimulation with supernatants. The level of AKT phosphorylation was then compared relative to the activity of a fresh insulin control at the same protein concentration. One-way ANOVA was conducted for each day to compare treatments (2.5, 5 and 10%). As seen in Fig. 6, released insulin on day 1 was fully active and exhibited higher levels of activity for all formulations compared to placebo activity, with the activity dropping gradually over the 23 day period. In general, microspheres with the lowest load-

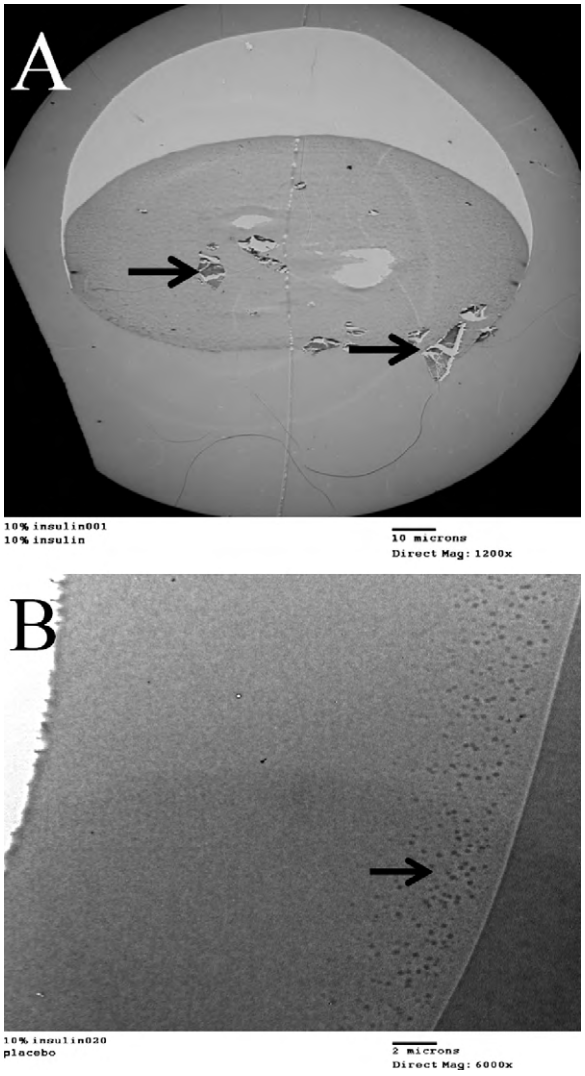

Fig. 3. TEM cross sectional images of PLGA microspheres with 10% w/w crystalline insulin entrapment. Black arrows point to insulin crystals embedded into the inside, and extending out from the surface of PLGA microspheres (A). The highly porous outer region the PLGA microsphere highlighted by a black arrow (B). Size bars represent 10 μm in (A) and 2 μm in (B).

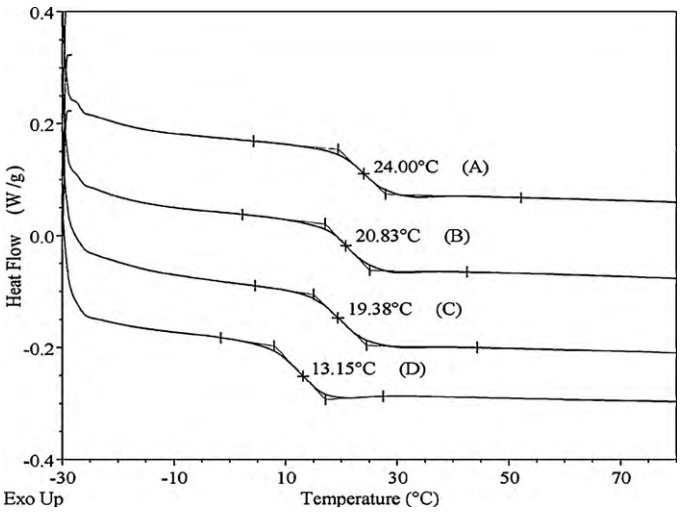

Fig. 4. DSC thermograms for PLGA microspheres loaded with (A) 10%, (B) 5%, (C) 2.5%, and (D) 0% crystalline insulin.

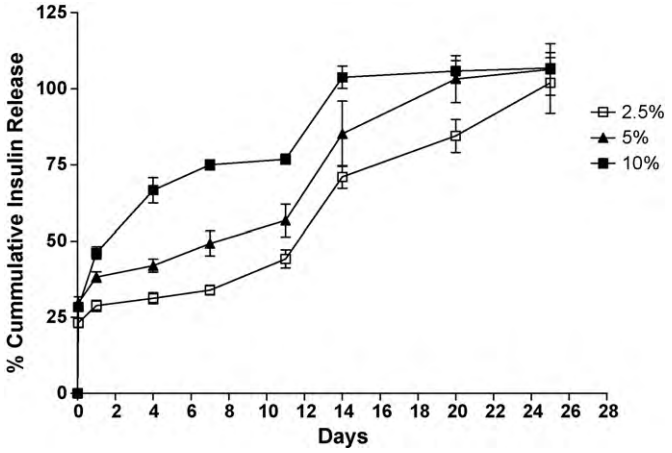

Fig. 5. Insulin release kinetics *in vitro* for PLGA microspheres containing 2.5–10% insulin.

ing of insulin showed the highest level of released activity to 14 days. In general, microspheres with the lowest loading of insulin showed the highest level of released activity to 14 days. Pairwise comparison for all bioactivities were statistically significant ($p < 0.05$) when compared to placebo microsphere supernatant on time points up to day 23. By day 23, the activity of released insulin ranged from 25 to 37%, depending on the formulation. A Welch test of means, which accounts for non-equal variances, concluded a significant difference between the sample and placebo groups.

3.6. Insulin bioactivity: scratch assay

Scratch repair assays were performed on HaCaT cells to further investigate the bioactivity of released insulin. The approximate initial width of the applied scratch was 0.5 mm, which filled rapidly due to cell migration, stimulated by applied insulin as may be seen in Fig. 7. The amount of insulin-protein applied to the tissue scratch at the beginning of the assay was 10^{-7} M for all conditions, with the exception of supernatant from blank microspheres (0% insulin control), in which case protein free supernatant was assayed to determine if there was a tissue response to PLGA degradation products. Plots A, B and C represent assays for supernatants from 2.5, 5 and 10% insulin loaded microspheres, respectively. Assay of supernatant from 2.5% insulin loaded microspheres was most problematic because of the very small quantities of insulin being released on individual days, which may explain the irregular response as was observed for example, with insulin sampled on day 14. Clearer and more consistent trends are evident with insulin released from 5 and 10% insulin microspheres where standardization of insulin–protein was more accurately conducted.

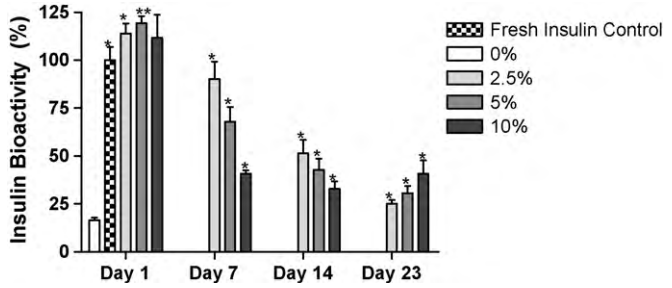

Fig. 6. Insulin bioactivity measurements for PLGA microsphere formulations with 0–10% w/w crystalline insulin. Asterisks indicate statistical significance between placebo and sample ($p = 0.05$).

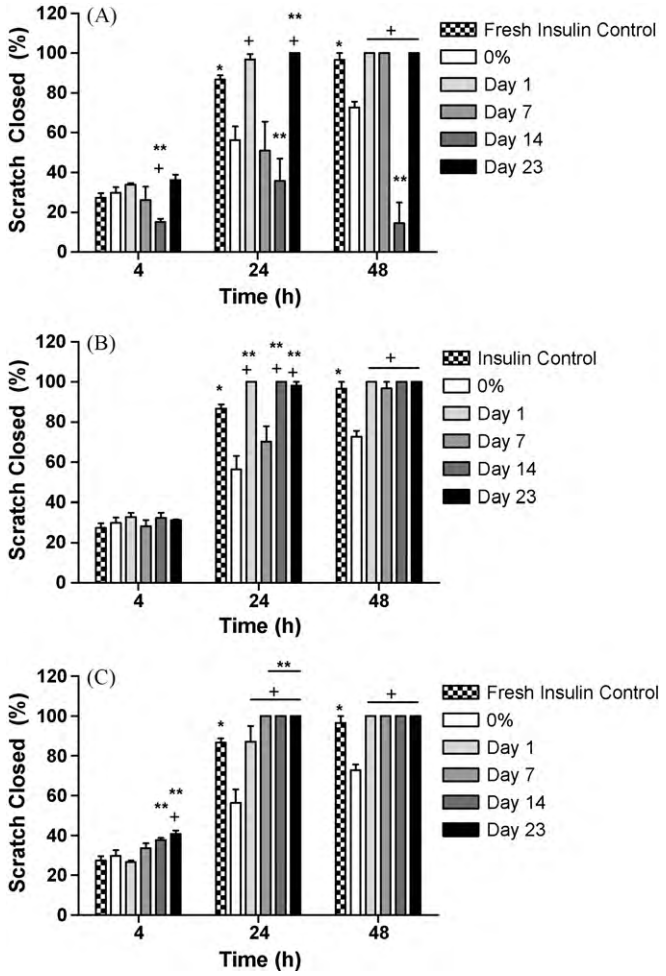

Fig. 7. HaCaT cell scratch repair assays evaluating bioactivity of insulin released from 2.5 (A), 5 (B), and 10% (C) insulin loaded PLGA microspheres into supernatants sampled on days 1, 7, 14 and 23 days of sustained release. Single asterisks indicate statistical significance between fresh insulin control and insulin free PLGA supernatant, double asterisk indicates difference between insulin released to supernatant, and fresh insulin control, and plus sign indicates difference between insulin released to supernatant and insulin free PLGA supernatant ($p = 0.05$).

The most rapid cell migration in the scratch assay took place between 4 and 24 h for cells exposed to 10^{-7} M fresh insulin, with cells approaching confluence at 24 h, and fully confluent at 48 h. Cell migration was also evident in the absence of insulin, but the differences between insulin exposed cells and cells growing in absence of insulin were large, and significantly different based on a one-way ANOVA test followed by a pairwise comparison, beyond 4 h of stimulation. For example, at 24 h, percent scratch closure was 84% for cells exposed to fresh insulin in comparison to 46% for cells migrating in absence of insulin. At 48 h, the scratch was fully closed in the presence of fresh insulin, in comparison to 67% for cells growing in the absence of insulin. Thus it is clear that insulin strongly stimulates growth and migration of HaCaT cells.

A comparison may then be drawn between cell migration in the presence of insulin released from PLGA microspheres over the 23 day period, to that of fresh insulin. By 48 h, the scratch was fully closed for all but one supernatant insulin sample. In comparison to the fresh insulin control, there was no significant difference in tissue response at 4, 24 or 48 h for all remaining supernatant samples. It would then appear that supernatant insulin released to 23 days from all formulations, was fully active, in comparison to fresh insulin when evaluated in terms of tissue migration response.

4. Discussion

Wound healing is a dynamic process that functions to restore lost or damaged skin layers through the activity of growth factors, cytokines, extracellular matrix molecules and stimulation of various cell types (Fu et al., 2005). Early in the healing process, initiation of re-epithelialization is a critical step in closing a wound (Martin, 1997). One approach toward accelerating this process is through the topical application of insulin to the surface of wounds (Liu et al., 2009). In this study, we have shown that 2.5–10% human recombinant crystalline insulin can be encapsulated into PLGA microspheres using a S/O/W solvent extraction technique with encapsulation efficiencies greater than 80% to as high as full retention, depending on the insulin loading. The resulting microspheres appeared spherical and porous with a mean diameter between 137 and 154 μm. Glass transition temperatures increased as larger quantities of insulin were incorporated into the formulation and resulted in T_g between 13 and 24 °C. A biphasic release profile showed full release of insulin over 25 days for all microsphere formulations. AKT phosphorylation following stimulation of Rat L6 myoblasts indicated fully active released insulin, with a gradual decrease but sustained activity up to 23 days of release. We have therefore demonstrated an insulin delivery system capable of releasing bioactive insulin for up to 3 weeks.

In this case, PLGA 50:50 with a molecular weight of 5–15 kDa was selected for its known controlled release applications and good biocompatibility (Ferrer-Miralles et al., 2009; Değim, 2008). To preserve insulin bioactivity during the formulation of PLGA microspheres, a S/O/W technique at low temperatures was employed. Since insulin has reduced solubility at lower temperatures, there is less of a propensity for solubilization and partition along the water/organic solvent interface (Bromberg et al., 2005). In addition, crystalline polypeptides provide many advantages such as enhancing sustained delivery, having greater ease-of-handling, and improved chemical and physical stability (Shenoy et al., 2001), thereby protecting the therapeutic integrity of the polypeptide (Shenoy et al., 2001; Pechenov et al., 2004). As reported by Lee et al., 2007, peptides are often susceptible to unfolding and denaturation along hydrophilic/hydrophobic interfaces, which is minimized with crystalline insulin. As a result higher encapsulation efficiencies ranging from 80 to 100% were possible because less of the insulin was lost to the outer aqueous environment during microsphere formulation. A S/O/W encapsulation method therefore facilitates high encapsulation efficiencies while also minimizing the potential for insulin degradation.

Morphologically, microspheres appeared distinct with spherical geometries. The surface appeared highly porous and varying amounts of insulin crystals may be seen protruding from the surface (Fig. 2A–E). According to Yang et al. (2000), the high degree of porosity exhibited on the surface of the PLGA microspheres is a function of the low temperatures at which synthesis takes place. The authors reported that solvent diffusion occurs at a much slower rate from the hardening microsphere at reduced temperatures. This causes the polymer to remain semi-solid for a much longer period of time, thereby allowing water from the outer aqueous phase to interact at the particle interface. The small water droplets become entrapped within the outer layer of the microsphere and either leave a porous structure on the outer phase, or migrate inwards into the microsphere as hardening occurs. TEM imaging (Fig. 3) also illustrated the presence of large internal voids, which may be indicative of this process. Yang et al. (2000), suggested that as the PLGA continues to stiffen from the outside in, the water internalized near the surface can be driven inwards and coalesce to form large internal droplets.

DSC analysis was performed to determine the T_g of PLGA microspheres after drying. Microspheres with more crystalline insulin

content were firmer and more easily handled. The T_g for all formulations ranged from 13 to 24 °C, suggesting that firmness of the microspheres was due to an increased T_g. Bouissou et al. (2006) reported T_g of W/O/W formulated PLGA microspheres of 20–29 °C depending on the addition of surfactants in the formulation. The authors also reported that an added peptide acted like an anti-plasticizing agent, raising the T_g. Higher levels of crystalline insulin in the present study may have the same effect. Similarly, Okada et al. (1994) also reported an increase in T_g with an increase in drug content. Using leuprorelin acetate, a peptide-like analogue, the authors suggested that the ionic interaction between basic amino acids and the terminal carboxylic acid groups of the polymer result in stronger molecular interactions that elevate the T_g. The relationship between T_g and drug content provides insight into approaches toward manufacturing, handling and storage of microspheres to prevent aggregation and irreversible coalescence.

Release of insulin from the various microsphere formulations (Fig. 6) was monitored over 25 days. This time period was selected to observe the release profile beyond the 3-week life expectancy of the delivery vehicle. An initial burst effect was noted within the first 24 h time period ranging from 28 to 46% of the total encapsulated insulin. Similar burst effects have been reported due to the presence of the protein near, or on the surface of the microsphere (Schoubben et al., 2009; Cui et al., 2006). SEM images (Fig. 2) confirm the presence of large insulin crystals embedded on the surface of the microspheres, suggesting that insulin can readily dissolve into the surrounding environment without being impeded by PLGA polymer matrix. After the burst phase, insulin release rates remained relatively constant and near-zero order for about 10 days. This process is governed by insulin diffusion through pores and channels in the microsphere and erosion controlled release by polymer hydrolysis (Cho et al., 2000). Between days 11 and 14, a second phase of insulin release was observed, followed by a short period of release and eventual plateau. Thus the overall release behavior appears to be biphasic. Li et al. (2008), also reported a similar secondary burst effect of methotrexate loaded PLGA microspheres and attribute the secondary burst phase to changes in the physicochemical properties of the PLGA matrix. By days 10–15, rapid peptide release was correlated with bulk PLGA polymer weight loss due to polymer hydrolysis (Shah et al., 1992; Sandor et al., 2001). This fragmentation is what led to the biphasic release. Faisant et al. (2002) have reported similar biphasic drug release from PLGA microspheres whereby the kinetics had been modeled. The authors conclude that the initial burst followed by a zero order drug release phase is highly governed by drug diffusion. In this study, insulin protruding from the surface of the microspheres and immediately beneath the surface would have contributed to the large initial burst and zero order release. As the PLGA polymer degrades the porosity and formation of internal channels increases, thereby enhancing the drug diffusion coefficient and allowing for a secondary burst. However, since insulin is in a crystalline form, the dissolution of the peptide may also add another level of complexity to the release kinetics. Further studies are needed to elucidate how crystalline insulin dissolution can influence the release kinetics from PLGA microspheres. All microspheres in the present study eventually fully released all of the entrapped insulin.

The bioactivity of insulin was determined using an *in vitro* cell culture based phosphorylated AKT ELISA. Although *in vitro* methods such as HPLC–MS, UV circular dichromism and insulin antigenicity ELISA assays may be used to measure bioactivity, they only provide evidence of primary/secondary structural integrity, or the presence of degradation products (Emami et al., 2009; Reis et al., 2007; Naha et al., 2009). These methods however, do not determine if insulin is capable of inducing a biological response in a functional biological system. The bioactivity of insulin was assessed by measuring the induction of the insulin receptor signal transduction pathway. When insulin binds to insulin-receptor 1, the receptor becomes autophosphorylated and dimerizes (Van Obberghen, 1984). The insulin receptor is then capable of activating other messenger molecules such as AKT through phosphorylation, which induces both cell migration, proliferation and survival responses (Liu et al., 2009; Goren et al., 2008; Chen et al., 1998). These responses not only indicate the direct bioactivity of insulin, but also shed light on the potential for wound healing applications. Fig. 6 illustrates the bioactivity as measured by the detection of phosphorylated AKT relative to the signal response from freshly prepared insulin at the same concentration. On day 1, all formulations produced responses that were approximately the same as compared to the fresh insulin control. The use of a novel encapsulation technique for insulin seems to suggest that the conditions are much milder than traditional W/O/W preparation techniques and could be beneficial for other protein therapeutic formulations. Between days 7 and 23, bioactivity continues to drop yet significant levels of bioactive insulin continue to be released over the 23-day period. Loss of insulin activity may be attributed to the acidic microenvironment within the particle due to PLGA hydrolysis (Shao and Bailey, 1999). Overall, active insulin continues to be released beyond a 3-week period, indicating that a long term PLGA mediated insulin delivery is a viable option.

Insulin bioactivity was further assessed by measuring migration of HaCaT cells in response to an applied scratch. Unlike the FACE AKT ELISA that provides information about the ability to induce an insulin stimulated signal cascade, the scratch assay provides a measure of cell migration, a process critical for wound healing. It was demonstrated that cell migration was strongly stimulated by insulin, applied at 10^{-7} M. Following scratch injury and stimulation with insulin, the scratch was fully closed between 24 and 48 h. In the absence of insulin, cell migration was evident, but at significantly lower levels in comparison to cells stimulated by insulin. Thus, the scratch assay provides a model by which cellular responses to applied insulin may be assessed. Using this model, it was possible to observe whether insulin bioactivity changes, or remains stable over time of release by comparing microsphere supernatants collected from days 1, 7, 14 and 23. Overall, most insulin containing supernatants resulted in full scratch closure within 48 h, and at a much faster rate than in the absence of insulin, as was observed with supernatants from blank microspheres. This indicates that released insulin was bioactive, and PLGA degradation products did not have a stimulatory effect. Therefore, we conclude that the cellular response was due to the addition of insulin alone. As early as 24 h into the assay, many of the supernatants resulted in nearly full closure of the scratch, and at a faster rate than that of the supernatant from blank microspheres (Fig. 7A–C). When comparing the cell migration response of PLGA supernatant insulin to the response to fresh insulin, there were virtually no significant differences in the comparisons. Thus these results show that insulin released over a 23-day period stimulates the same cell migration response as fresh insulin, indicating a high level of sustained bioactivity.

5. Conclusion

Crystalline insulin can be encapsulated into PLGA microspheres by a solid-in-oil-in-water (S/O/W) suspension solvent evaporation technique with high recovery efficiency and reproducibility. Release studies demonstrated that insulin can be delivered for 25 days in a controlled release manner. High level of insulin bioactivity over a 23-day release period was demonstrated by stimulating cell signaling responses in Rat L6 myoblasts. Fur-

thermore, an *in vitro* scratch assay established that insulin released from PLGA microspheres over 23 days stimulates rapid cell migration following an induced scratch. These data suggest that crystalline insulin encapsulated within PLGA microspheres offers potential for long-term delivery of bioactive insulin for topical delivery devices and could have significant clinical implications.

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