O5-1 **Sustained and prolonged topical delivery of bioactive human insulin for potential treatment of cutaneous wounds**

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INTRODUCTION AND OBJECTIVES

Burns are painful and morbid forms of thermal injury that usually result when the skin is overexposed to extreme heat, caustic chemicals, or radiation (Herdon 2007). Depending on the size and the extent of the burn, recovery is often slow, prone to infection and resource intensive.

Insulin has been shown to improve healing in a variety of situations. Recent studies both *in vitro* and *in vivo,* have indicated that insulin accelerates wound closure by stimulating keratinocyte migration, and minimizes scar formation (Liu 2009). However, an effective topical insulin delivery system for wound healing has yet to be discovered.

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. Insulin release kinetics and bioactivity were assessed in vitro. The results demonstrate the viability of this encapsulation technique for implementation in topical wound healing devices.

MATERIALS AND METHODS

PLGA (*L*,*G* 50:50, 5-15kDa), crystalline human recombinant insulin, poly(vinyl alcohol) (PVA), LR white embedding kit and sodium phosphate dibasic anhydrous (Sigma Aldrich, Oakville). Micro BCA Protein Assay Kit (ThermoFisher, Ottawa) and FACE AKT ELISA kit (Mercodia, USA).

Preparation of PLGA Microparticles – Four different 100 mg batches of PLGA microspheres were prepared with 0, 2.5, 5 and 10% w/w crystalline insulin. PLGA was dissolved into 1 mL of DCM along with crystalline insulin to form the primary S/O suspension. The suspension was the added drop-wise into 30 mL 10% PVA solution pre-chilled to approximately 5-10°C. An impeller mixer (Caframo, Wiarton), stirred the resulting S/O/W emulsion under constant mixing at 430 rpm for 1 min. After mixing, the emulsion was transferred to 500 mL pre-chilled distilled water stirring at 180 rpm for 10 h on ice. The microspheres were filtered, washed and stored in the freezer until use.

Encapsulation Efficiency (E.E.%) – Approximately 8-10 mg microsphere samples were dissolved in 3 mL DCM. After the DCM had evaporated, 20 mL of 5% SDS, 0.1 M NaOH solution was added (VWR, Mississauga). Supernatants were removed and analyzed spectrophotometrically 24 hours later using a micro BCA protein assay kit. E.E. % was defined as actual loading versus theoretical loading.

Sizing and Morphology Analysis– Particle and pore size distributions were determined by SEM (JEOL 840, USA). TEM (JEOL 1200EX, USA) imaging of the internal morphology was performed on thin sections of microspheres embedded in LR white resin.

Insulin In Vitro Release Study – Approximately 5 mg of insulin loaded microspheres were placed into 2.5 mL plastic centrifuge tubes (Diamed, Mississauga) filled with 2 mL 20 mM phosphate buffer, pH 7.4. All samples were incubated at 37°C on a flat orbital shaker rotating at 60 rpm.

Insulin bioactivity: FACE AKT ELISA– L6 myoblasts were seeded onto 96 well plates (Costar; Lowell, USA). Cells were then stimulated with (i) fresh insulin dissolved in starvation medium; (ii) supernatant containing insulin released from microspheres; or (iii) supernatant from placebo microspheres. After 20 min stimulation the cells were treated according to the Fast Activated Cell-based ELISA (FACE) AKT kit.

Insulin bioactivity: scratch assay – HaCaT cell suspensions were seeded onto 24 well plates (Costar; Lowell, USA). Cells formed a confluent layer 48 h later, at which time a single scratch was made using a 10 µL pipette tip. Cells were then washed, starved and treated with (i) fresh insulin; (ii) supernatant containing insulin released from microparticles; or (iii) supernatant from placebo microparticles in triplicate. Scratches were measured using a bright field inverted microscope (Nikon; Melville, USA) at time points 0, 4, 24 and 48 h.

RESULTS AND DISCUSSION

E.E.% was measured to determine loading concentrations for PLGA microspheres. Table 1 indicates that the highest encapsulation efficiencies were achieved when insulin loading was low.

Sizing analysis (Table 1) performed on the microspheres and their pores reveals that the mean diameters remained relatively consistent as insulin loading increased.

Table 1. Microsphere E.E.% and Physical Properties

Loading	0%	2.5%	5%	10%
E.E.%	0	99±10	83±8	78±1
Mean Diam. µm	154 ±57	131±92	153±53	137±52
Mean Pore Size nm	318±121	282±106	314±126	387±145

Scanning and transmission electron microscopy revealed porous surfaces with insulin crystals appearing within and protruding from the surface (Fig. 1A-B).

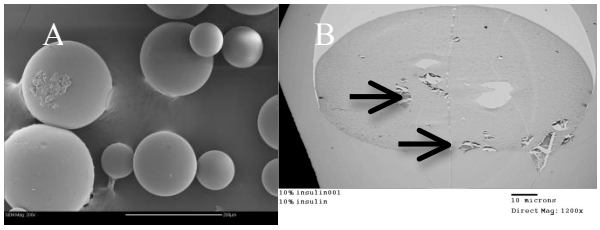

Figure 1. A) Insulin loaded microspheres, B) TEM cross section of a 10% insulin microsphere (arrow indicates insulin crystals).

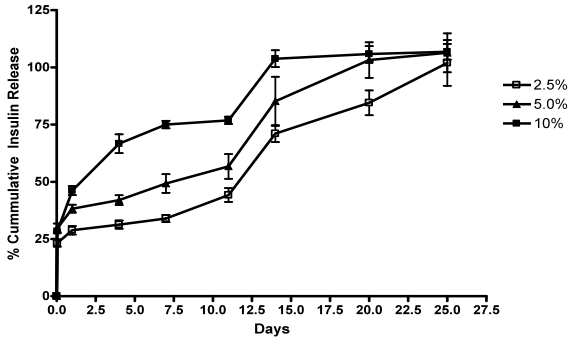

Figure 2. Cummulative insulin release profiles for 2.5, 5, and 10% insulin loaded PLGA microspheres.

Biphasic release kinetics (Fig. 2) are attributed to the bulk erosion process of PLGA matrices.

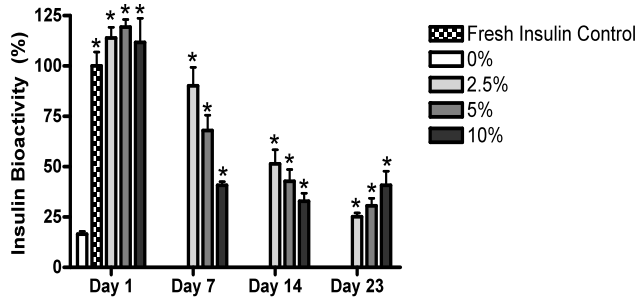

Figure 3. Insulin bioactivity measurements for PLGA microsphere formulations with 0-10% w/w crystalline insulin.

Insulin bioactivity was assessed by measuring the levels of phosphorylated AKT in rat myoblasts, after stimulation with insulin or placebo. There was only a minor and gradual decrease in AKT phosphorylation over time (Fig. 3). HaCaT cell growth velocity was greatest with insulin stimulated cells versus insulin free conditions (Fig 4).

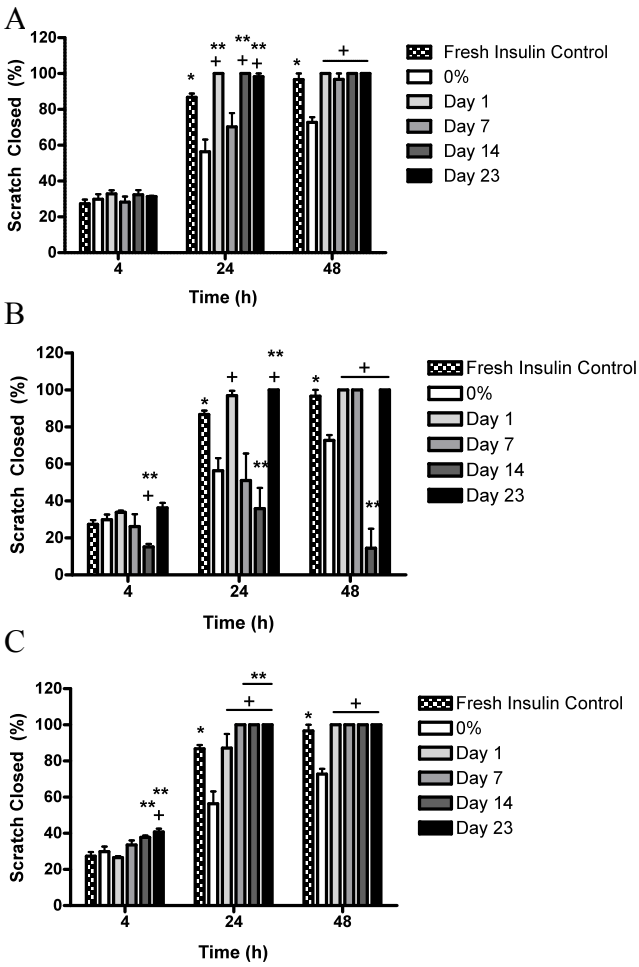

Figure 4. HaCaT cell scratch repair assays evaluating of insulin-protein bioactivity released from 2.5 (A), 5 (B), and 10% (C) insulin loaded PLGA microspheres into supernatants sampled on days 1, 7, 14 and 23 days of sustained release.

CONCLUSIONS

Extended and sustained topical delivery of active insulin from a stable protein crystal-based reservoir shows promise in promoting tissue healing.

REEFERENCES

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