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HB-EGF embedded in PGA/PLLA scaffolds via subcritical CO_2 augments the production of tissue engineered intestine

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ABSTRACT

The ability to deliver sustained-release, biologically active growth factors through custom designed tissue engineering scaffolds at sites of tissue regeneration offers great therapeutic opportunity. Due to the short *in vivo* half-lives of most growth factors, it is challenging to deliver these proteins to sites of interest where they may be used before being degraded. The application of subcritical CO_2 uses gas-phase CO_2 at subcritical pressures ranging from 41 to 62 bar (595–913 PSI) which avoids foaming by reducing the amount of CO_2 dissolved in the polymer and maintains completely reversible plasticization. In the current study, heparin-binding EGF-like growth factor (HB-EGF) was embedded into polyglycolic acid (PGA)/Poly-L-latic acid (PLLA) scaffolds via subcritical CO_2 exposure for the production of tissue engineered intestine (TEI). PGA fiber morphology after subcritical CO_2 exposure was examined by scanning electron microscopy (SEM) and the distribution of HB-EGF embedded in the scaffold fibers was detected by HB-EGF immunofluorescent staining. *In vivo* implantation of HB-EGF-embedded scaffolds confirmed significantly improved TEI structure as a result of local delivery of the trophic growth factor. These findings may be critical for the production of TEI in the treatment of patients with short bowel syndrome in the future.

1. Introduction

Patients with short bowel syndrome (SBS) lack the adequate intestinal function needed to sustain their nutritional needs. The production of tissue-engineered intestine (TEI) using a patient's own intestinal cells may restore functional intestinal absorptive area while avoiding the complications of current therapeutic options such as small bowel transplantation. The smallest transplantable mucosal units are intestinal "organoids" which are clusters of 20–40 cells isolated from intestinal mucosa. These organoids contain the putative intestinal stem cells (ISCs) and surrounding cells that comprise the stem cell niche. Transplantation of these intestinal organoids has been shown to generate TEI that resembles native intestine in both structure and function with variable results [1–5].

In the 1980s, Thompson et al. first described the augmentation of neomucosal growth after exposure to luminal factors or to systemically administered urogastrone, a peptide that shares an intestinal receptor with epidermal growth factor (EGF) [6,7]. Based on the concept that intestinal growth is regulated by nutrients and enteric secretions, as well as local and systemic growth factors, subsequent studies compared luminal versus systemic administration of trophic peptides. The route of growth factor delivery has been shown to impact mucosal growth, demonstrating that local delivery is optimal [8–12]. This work led to studies of the administration of growth factors to augment the production of TEI [13–16]. Very few studies have examined the effects of locally delivered growth factors on tissue-engineered neomucosa. The application of three-dimensional, porous polymer constructs for tissue engineering applications has received considerable attention as a method of aiding and defining new tissue growth [17,18]. Due to the short *in vivo* half-lives of most growth factors, it is desirable to deliver the protein to the site of interest where it may be used before it is degraded [19]. For example, one successful approach to

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deliver VEGF within a polymer implant was to produce a porous PLGA matrix with dispersed alginate microspheres [20]. The growth factor was encapsulated within the alginate spheres and was released in an active state upon exposure of the construct to an aqueous buffer. Another mechanism used to release growth factors at the desired site was to encapsulate the protein within the PLGA matrix itself [20].

Supercritical CO_2 fluid mixing technology is performed at or below physiological temperatures without any harsh solvents, allowing bioactive factors to be encapsulated and retained in biodegradable polymer constructs without compromising their biological activity [21]. The use of CO_2 avoids concerns surrounding halogenated solvents that are often necessary to achieve mixtures of resorbable polymers with other compounds [22–24]. However, the use of supercritical CO_2 with bulk polymeric implants carries a substantial penalty: foaming, or the loss of the original implant form [25]. Therefore, an alternative treatment that preserves form is based on the use of gas-phase CO_2 at subcritical pressures ranging from 41 to 62 bar (595–913 pounds per square inch; PSI). This avoids foaming by reducing the amount of CO_2 dissolved in the polymer and maintains conditions that allow plasticization to be completely reversible. Subcritical CO_2 has been used successfully to embed the anti-cancer drug paclitaxel into polylactic acid with proven sustained chemotherapeutic activity [26]. Dormer et al. reported the use of subcritical CO_2 with sinter microspheres for the delivery of transforming growth factor (TGF)-beta3 and bone morphogenetic protein (BMP)-2 to human bone marrow stromal cells *in vitro*, and found that subcritical CO_2 had only slight adverse effects, as well as some desirable effects, on protein availability and bioactivity [27].

In the current study, heparin-binding EGF-like growth factor (HB-EGF) was embedded into polyglycolic acid (PGA)/Poly-L-latic acid (PLLA) scaffolds via subcritical CO_2 exposure in an effort to augment the growth of TEI. We have previously shown that HB-EGF promotes intestinal epithelial cell (IEC) proliferation and migration [28–30], decreases IEC apoptosis [31,32], and protects ISC from injury [33], supporting the use of HB-EFG in the current studies. The release kinetics and bioactivity of HB-EGF released from PGA/PLLA scaffolds were characterized, and the effects of embedding scaffolds with HB-EGF were tested in *in vitro* cell cultures and *in vivo* scaffold implantation.

2. Materials and methods

2.1. Materials for scaffold preparation

PGA Biofelt (2 mm thickness and 60 mg/cm^3 density) was purchased from Biomedical Structures (Warwick, RI). Poly-L-latic acid (PLLA) and fluorescein isothiocyanate (FITC) conjugated bovine serum albumin (BSA) were from Sigma-Aldrich (St. Louis, MO). Chloroform and collagen type I were from Fisher Scientific (Pittsburgh, PA). HB-EGF was from R&D Systems (Minneapolis, MN).

2.2. Preparation of scaffolds

Scaffold preparation is depicted in Fig. 1. Flat scaffolds (0.8 × 1.6 cm) were cut directly from PGA Biofelt and used for scanning electron microscopy (SEM), *in vitro* cell culture, and measurement of surface area as depicted by FITC-BSA distribution. Tubular scaffolds (0.5 × 3.0 cm) were prepared by wrapping PGA flat sheets around stainless steel mandrels for suture retention strength (SRS) tests. Similarly, tubular scaffolds (0.5 × 1.0 cm) were prepared for *in vitro* release kinetics, bioactivity, compression tests, and *in vivo* implantation. All flat and tubular scaffolds were coated with 5% PLLA in chloroform. Once the solvent was completely

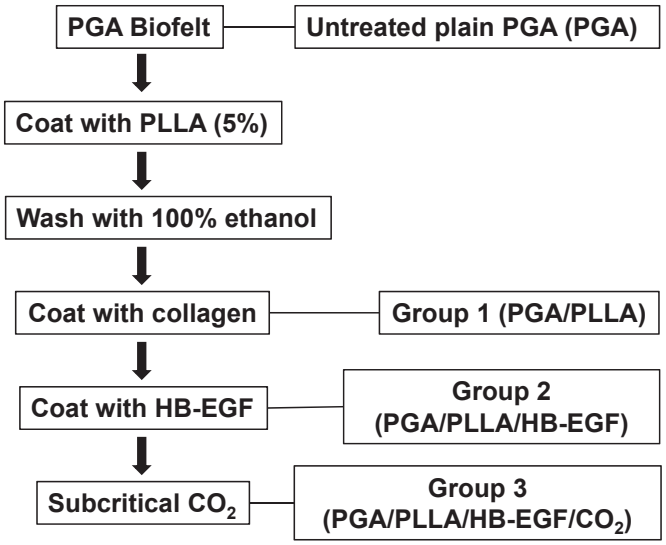

Fig. 1. Scaffold preparation for *in vitro* and *in vivo* studies. Three groups of scaffolds were fabricated: PGA coated with PLLA (PGA/PLLA), PGA coated with PLLA and embedded with HB-EGF (PGA/PLLA/HB-EGF), and PGA coated with PLLA and embedded with HB-EGF followed by subcritical CO_2 exposure (PGA/PLLA/HB-EGF/CO_2). In some *in vitro* studies, untreated plain PGA scaffolds were used as a control.

evaporated, scaffolds were soaked in 100% ethanol for 30 min in order to improve the hydrophilicity of PGA/PLLA scaffolds (Video clip 1 is available online), followed by 3 washes with PBS. Scaffolds were then soaked in 0.4 mg/ml collagen type I for 30 min followed by 3 washes with PBS, lyophilized, and sterilized with ethylene oxide. All flat and tubular scaffolds were divided into three groups based on the following treatments:

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2016.06.039.

1) PGA coated with PLLA (PGA/PLLA), 2) PGA coated with PLLA and embedded with HB-EGF (PGA/PLLA/HB-EGF), and 3) PGA coated with PLLA and embedded with HB-EGF followed by subcritical CO_2 exposure (PGA/PLLA/HB-EGF/CO_2). Scaffolds from the second and third groups were hydrated with 10 μg per scaffold of HB-EGF in 100 μl of PBS for *in vitro* studies and 2 μg per scaffold of HB-EGF in 100 μl of PBS for *in vivo* studies. Additionally, scaffolds from the third group were placed in sterile 50 ml conical bottom tubes with porous caps and exposed to subcritical CO_2 in a stainless steel vessel (Parr Instruments Co., Moline, IL) at 900 PSI for 1 h followed by a slow release of CO_2 at 15 PSI per minute for 60 min. All prepared scaffolds were stored at −30 °C and brought to RT 10–20 min prior to use.

2.3. Scanning electron microscopy (SEM) and confocal microscopy

Flat scaffolds from the three groups, in addition to plain PGA scaffolds, were cut to 2 × 5 mm strips and 4 strips from each group were affixed to a stub and sputter-coated with gold (Emitech K550X, Quorum Technologies Ltd, Ashford, Kent, England). Samples were examined using a scanning electron microscope (Hitachi S-4800, Hitachi High Technologies America, Inc., Dallas, TX) at a voltage of 10–20 kV. To visualize the distribution of HB-EGF in the scaffolds, HB-EGF-embedded scaffolds were incubated in 10 μg/ml of goat derived anti-HB-EGF primary antibody (R&D Systems) for 1 h followed by 3 washes with PBS and incubation with donkey anti-goat IgG secondary antibody for 1 h (Invitrogen, Grand Island, NY). After 3 washes with PBS, the scaffolds were examined with a confocal microscope (Zeiss LSM 710, Thornwood, New York).