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Tailoring the porosity and pore size of electrospun synthetic human elastin scaffolds for dermal tissue engineering

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ABSTRACT

We obtained low and high porosity synthetic human elastin scaffolds by adapting low (1 mL/h) and high (3 mL/h) flow rates respectively during electrospinning. Physical, mechanical and biological properties of these scaffolds were screened to identify the best candidates for the bioengineering of dermal tissue. SHE scaffolds that were electrospun at the higher flow rate presented increased fiber diameter and greater average pore size and over doubling of overall scaffold porosity. Both types of scaffold displayed Young's moduli comparable to that of native elastin, but the high porosity scaffolds possessed higher tensile strength. Low and high porosity scaffolds supported early attachment, spreading and proliferation of primary dermal fibroblasts, but only high porosity scaffolds supported active cell migration and infiltration into the scaffold. High porosity SHE scaffolds promoted cell persistence and scaffold remodeling *in vitro* with only moderate scaffold contraction. The scaffolds persisted for at least 6 weeks in a mouse subcutaneous implantation study with fibroblasts on the exterior and infiltrating, evidence of scaffold remodeling including de novo collagen synthesis and early stage angiogenesis.

1. Introduction

Efficacious dermal substitutes assist in the successful treatment of large and deep burns [1], as they effectively close the wound and help to guide cells during granulation tissue formation, fibroblast-driven remodeling, angiogenesis and re-epithelization [2]. Collagen-based scaffolds currently dominate the dermal substitute field but are limited by lack of elasticity, substantial scaffold contraction and scar formation during the repair process [3,4]. Elastin-containing dermal substitutes have the potential to improve the elasticity and functionality of severe scars by replacing the missing elastic network or by signaling the up-regulation of elastic tissue biosynthesis during the wound healing process [5].

Electrospinning is a valuable tool in dermal substitute engineering as it generates thin, continuous polymer fibers that are morphologically similar to native ECM fibers, and promote cellular interactions leading to new tissue formation [6–10]. Electrospinning employs a strong electric field to process a polymer solution into a fibrous construct with highly interconnected pores [6]. However, the resulting scaffolds have inherently small pore sizes that do not allow for cellular infiltration and ingrowth [11–16] that would be essential for the replacement and repair of many tissues, including the dermis [17–24]. Fibroblasts are the principal cellular component of the skin responsible for dermal homeostasis and repair. Dermal substitute scaffolds promote fibroblast adhesion, growth and infiltration, which accelerates and enhances dermal and epidermal regeneration [17–24].

We have previously demonstrated that recombinant human tropoelastin [25], the soluble precursor of elastin, can be electrospun into three-dimensional, fibrous and highly elastic scaffolds [26–28] but a lack of cell infiltration was the major factor limiting the potential of these scaffolds as dermal substitutes. Our initial studies showed that an increase in the electrospinning flow rate results in the creation of open-weave scaffolds with large pore sizes that allow for fibroblast infiltration into the scaffold. Here we present an assessment of the physical, mechanical and biological properties of these highly porous, open-weave elastin-based scaffolds, with emphasis on long-term cell growth and scaffold re-modeling *in vitro* and scaffold tolerance and evidence of persistence *in vivo*.

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2. Materials & methods

2.1. Materials

Recombinant human tropoelastin isoform SHELΔ26A (Synthetic Human Elastin without domain 26A) corresponding to amino acid residues 27–724 of GenBank entry AAC98394 (gi 182020) was purified from bacteria on a multi-gram scale as previously described [29,30]. GMP clinical grade tropoelastin with the same sequence was sourced from Elastagen Pty Ltd for *in vivo* work. Primary human dermal fibroblasts were obtained from biopsies of healthy patients undergoing breast or abdomen reduction surgery at the Concord Repatriation General Hospital (Concord, NSW), in accordance with the approval of the hospital research and ethics committee. All reagents were purchased from Sigma–Aldrich unless specified otherwise.

2.2. Electrospinning and cross-linking tropoelastin

A 20% (w/v) solution of tropoelastin was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) and loaded into a syringe equipped with a blunt 18 gauge needle. Flow rates of 1 mL/h and 3 mL/h were modulated using a syringe pump. The needle was connected to a 20 kV positive power supply (Gamma High Voltage Research, Inc.) and directed at a grounded, 30 mm diameter circular, brass collector at a collector distance of 20 cm. Electrospun tropoelastin scaffolds were chemically cross-linked to stabilize their structures in aqueous environments. Scaffolds were placed in an open stage desiccator and cross-linked by vapor from a separate 25% (v/v) aqueous glutaraldehyde (GA) solution. Unreacted GA in the scaffolds was quenched by immersion into 0.2 M glycine solution overnight. Scaffolds were then washed repeatedly in PBS. Cross-linked tropoelastin is referred to as synthetic human elastin (SHE) scaffolds or fibers.

2.3. Fiber width quantification

Cross-linked electrospun SHE scaffolds were fixed with 2.5% (w/v) glutaraldehyde in 0.1 M phosphate buffer (PB) for 1 h at room temperature. Samples were washed 3X with PB and post-fixed with 1% (v/v) osmium tetroxide in PB. The scaffolds were dehydrated in increasing concentrations of ethanol and dried overnight in hexamethyldisilazane. Uncross-linked and cross-linked samples were sputter-coated with 20 nm gold and imaged using a Philips XL-30 Scanning Electron Microscope (SEM). The widths of 10 fibers within an image for a total of 10 images per sample ($n = 3$ per treatment) were quantified using ImageJ (National Institutes of Health) [31].

2.4. Pore diameter and porosity quantification

Pore diameters were quantified from SEM images of electrospun SHE scaffolds. The diameters of the longest axes in each of 5 pores within an image for a total of 5 images per sample ($n = 3$ per treatment) were quantified using ImageJ.

Scaffold porosity was quantified from hematoxylin and eosin (H&E)-stained scaffold cross-sections [32] with modifications. SHE scaffolds were fixed in 10% (w/v) buffered formalin solution and embedded in paraffin. 5 μm sections were deparaffinized in xylene and stained with H&E. Section images were converted to binary format using ImageJ, where the fibers appeared black and pores white. The percentage of the white area relative to the total scaffold section surface area in each image was defined as the percent porosity.

2.5. Mechanical properties of SHE scaffolds

Tensile mechanical properties of SHE scaffolds (dimensions 3 × 1 cm) were tested using an Instron 5543 with a 50 N load cell at a constant strain rate of 3 mm/min until failure ($n = 2–3$). Samples were immersed in PBS at 37 °C during testing. The Young's modulus was obtained from the slope of the stress-strain curve generated over the linear portion of the strain range after the initial toe region. Tensile strength was defined as the ultimate stress at break. The calculation of strain was based on the cross-sectional area of each of the samples.

2.6. Fibroblast isolation and cell culture

Human skin dermis was separated from the epidermis by incubation in dispase, diced into small sections and incubated in 1 mg/mL type I collagenase solution at 37 °C. The digested dermal mixture was filtered through a 100 μm cell strainer to harvest cells. Dermal fibroblasts up to passage 5 were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 1% (v/v) L-glutamine and 50 μmol/mL gentamicin.

Fibroblasts (5×10^4 /cm^2) were seeded onto three-dimensional (3D) SHE scaffolds or a thin layer of electrospun SHE fibers on glass coverslips in a static cell culture system. 3D scaffolds were kept retained in cell culture wells using hollow well inserts for 3 h post-seeding. Scaffolds were then transferred to larger wells with no rings and cell culture media was changed every 2 days. Loosely adherent or unbound cells were removed by washing twice with PBS prior to further analysis. Cells were analyzed at 30 min and days 1, 5, 8, 14, 21, 28 and 35 post-seeding.

2.7. Cell visualization and quantification

Samples for histological analysis were fixed in 4% (w/v) paraformaldehyde overnight at 4 °C and embedded in paraffin. 5 μm sections were deparaffinized in xylene and mounted with SlowFade Gold antifade reagent containing DAPI.

Cell proliferation over 8 days was quantified by examining DAPI-stained nuclei in 20 SHE scaffold cross-sections (each at least 50 μm apart) for each time point ($n = 2–3$ per treatment/timepoint).

2.8. Detection of ECM proteins secreted by cells

Electrospun SHE scaffolds ($n = 3$) were fixed in 4% (w/v) paraformaldehyde and embedded in paraffin. 5 μm sections were deparaffinized in xylene and immunoprobed for the expression of collagen I and fibronectin. Immunohistochemistry studies were controlled by comparison with samples that were devoid of cells and no primary antibody and isotype controls.

Collagen I target retrieval was achieved by incubation with 100 mM glycine HCl and 50 mM ammonium chloride in 0.1 M Tris Buffer Saline (TBS) containing 0.3 M NaCl and 0.05% (v/v) Tween-20 for 30 min at room temperature, followed by incubation in 0.2% (w/v) hyaluronidase in TBS (without Tween-20) at 37 °C for 15 min. Endogenous peroxidase activity was blocked by incubation with 1% (v/v) hydrogen peroxide in methanol for 20 min at room temperature. Non-specific binding was blocked with 10% (v/v) goat serum in TBS for 20 min at room temperature. Samples were incubated with polyclonal anti-collagen I antibody (AbCam) for 1 h at room temperature, followed by Dako EnVision HRP labeled polymer (anti-rabbit) for 30 min at room temperature. Color was developed with Dako Liquid DAB + Substrate chromagen system for 5 min at room temperature followed by multiple water washes. Samples were counterstained with hematoxylin and embedded in DPX mountant for Microscopy-10.

Fibronectin target retrieval was achieved by incubation in Dako target retrieval solution (pH 9) for 12 min at 95 °C. Endogenous peroxidase activity was blocked by incubation with 1% (v/v) hydrogen peroxide in methanol for 20 min at room temperature. Non-specific binding was blocked with 10% (v/v) goat serum in TBS for 20 min at room temperature. Samples were incubated with monoclonal anti-fibronectin antibody (Thermo Scientific) for 1 h at room temperature, followed by Dako EnVision HRP labeled polymer (anti-mouse) for 30 min at room temperature.

2.9. Quantification of scaffold contraction

High porosity SHE scaffold contraction was quantified by measuring the diameter of circular scaffolds seeded with fibroblasts up to 35 days, expressed as a percentage of the original scaffold diameter prior to cell seeding (% original scaffold diameter) ($n = 3$ per time point).

2.10. Subcutaneous implantation of SHE scaffolds in mice

A mouse model of subcutaneous implantation was used to evaluate the tolerance, persistence and cellularization of SHE scaffolds. Pathogen-free, female and male BALB/c mice, aged 8 weeks and weighing 21 ± 1.9 g (female)/25 ± 2.7 g (male) were purchased from the Australian Animal Resources Center. All animals were acquired, housed and studied under a protocol approved by SSWAHS Animal Welfare Committee in Sydney, Australia. Each mouse ($n = 7$) was anesthetized individually by intra-peritoneal injection of a mixture of ketamine (75 mg/ml) and xylazine (10 mg/ml) at 0.01 ml/g of body weight. The dorsal hair was shaved and skin was cleaned with betadine solution and washed with sterile saline. An incision of about 1 cm in length was made and dissected to create a subcutaneous pouch into which the SHE scaffold was inserted. The wounds were then closed with 6-0 silk sutures and covered using IV3000 wound dressings (Smith & Nephew) for 5 days. Carprofen (5 mg/kg) was given at the time of anesthesia and then on the following day, post surgery for analgesia. After surgery, each mouse was caged individually for the first two days and then two mice per cage thereafter with free access to water and food. Skin biopsies were collected for histological analysis at 6 weeks post-implantation.

2.11. Statistical analysis

Data are expressed as mean ± standard deviation. Statistically significant differences were determined by one- or two-way analysis of variance (ANOVA) and Bonferroni post-test. Statistical significance was accepted at $p < 0.05$ and indicated in the figures as *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$).