Mitigation of hypertrophic scar contraction via an elastomeric biodegradable scaffold

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Abstract

Hypertrophic scar (HSc) occurs in 40–70% of patients treated for third degree burn injuries. Current burn therapies rely upon the use of bioengineered skin equivalents (BSEs), which assist in wound healing but do not prevent HSc contraction. HSc contraction leads to formation of a fixed, inelastic skin deformity. We propose that BSEs should maintain their architecture in the wound bed throughout the remodeling phase of repair to prevent HSc contraction. In this work we study a degradable, elastomeric, randomly oriented, electrospun micro-fibrous scaffold fabricated from the copolymer poly(L-lactide-co-ε-caprolactone) (PLCL). PLCL scaffolds displayed appropriate elastomeric and tensile characteristics for implantation beneath a human skin graft. In vitro analysis using human dermal fibroblasts demonstrated that PLCL scaffolds decreased myofibroblast formation as compared to an in vitro HSc contraction model. Using a validated immune-competent murine HSc contraction model, we found that HSc contraction was significantly greater in animals treated with standard of care, Integra, as compared to those treated with collagen coated-PLCL (ccPLCL) scaffolds. Finally, wounds treated with ccPLCL were significantly less stiff than control wounds at d30 in vivo. Together, these data suggest that scaffolds which persist throughout the remodeling phase of repair may represent a clinically translatable method to prevent HSc contraction.

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1. Introduction

Dermal scarring affects more than 80 million people worldwide annually [1]. For example, over 4.4 million people are injured in motor vehicle accidents, thousands of our nation’s warriors are wounded in military excursions, and over 2.4 million patients are burned [2,3]. The World Health Organization states that “there is no doubt that the social and medical costs of burns are significant. Economic impact of burns includes lost wages, and the costs related to deformities from burns, in terms of emotional trauma and lost skill.” In severe burns (~28,000 patients/year in the United States), the incidence of hypertrophic scar (HSc) is 40–70% [14]. HSc are firm, raised, red, itchy scars that are disfiguring and can have a severe impact on quality of life. 70% of HSc occur across joints or other areas of high tension in the body, resulting in a scar contractures that restrict range-of-motion [5]. Current preventative therapies for scar contracture are ineffective, and patients requiring intervention undergo at least four corrective surgeries on average [15].

HSc develops during the first 4–8 weeks following injury and continues to mature and contract throughout the remodeling phase of repair for as long as six months post trauma; however, commercially available bioengineered skin equivalents (BSE) degrade and are remodeled in the wound bed within 1–4 weeks [6]. There are currently three BSEs on the market which are approved for...
third degree burn treatment by the Food and Drug Agency as well as the Center for Medicare and Medicaid Services: Epicel, TransCyte, and Integra. Of these, Integra is the most widely used for treatment due to its cost and ease of use. Integra Dermal Regeneration Template (Integra LifeSciences, Plainsboro, NJ) is a bovine collagen-based lyophilized matrix covered by a silicone dressing. The biologic layer remains in the wound bed, while the silicone layer is removed prior to application of skin graft. Previous work studying collagen-based scaffolds for dermal regeneration has shown that prolonging the half-life of the material in the wound bed has a profound impact on minimizing wound contraction; however, the longest scaffold half-life tested in these studies was 2–4 weeks [7]. We hypothesize that to improve mitigation of HSc, the scaffold should be present in the wound bed throughout the remodeling phase of repair when HSc occurs.

In comparison to biologic biomaterials, synthetic biomaterials have advantages in that they possess tunable mechanical properties and biodegradation rates. In searching for a suitable synthetic elastomer for this application, we looked for favorable characteristics of mechanical strength, elasticity, and biodegradability. We selected the copolymer poly(l-lactide-co-ε-caprolactone) (PLCL), synthesized from a 50:50 ratio of poly(lactic acid) (PLA) and poly(ε-caprolactone) (PCL) [8–12]. The PLCL has been extensively studied for tissue engineering applications [13]. Although neither PLA nor PCL is elastomeric, PLCL displays elastic characteristics due to the phase separation of the crystalline PLA and the amorphous PCL segments, creating hard and soft domains somewhat akin to that observed in elastomeric polyurethanes [8–10]. Synthetic materials have additional advantages over biologics with respect to ease of handling, long shelf-life, low cost, and well-defined physicochemical properties.

Application of mechanical load has been shown to initiate HSc formation in mice [14]. Thus, it has been suggested that stress-shielding cells from transmission of mechanical load could assist in mitigating HSc [15]. Mechanical tension and inflammatory cytokines (primarily transforming growth factor-beta (TGFβ)) secreted following dermal injury cause resident fibroblasts to differentiate into myofibroblasts [16]. Myofibroblasts are distinguished from fibroblasts by (1) the presence of a contractile apparatus, similar to that of smooth muscle cells, and (2) the neo-expression of an actin isoform found in vascular smooth muscle cells, α-smooth muscle actin (αSMA) [17]. When de novo αSMA is incorporated into stress fibers, myofibroblasts produce strong contractile force and physically contract the wound or scar bed. To achieve tissue level contraction, single myofibroblasts join stress fibers at sites of adherens junctions, resulting in cytoskeletal alignment and the formation of a coordinated cellular syncytium [18]. This conglomeration allows myofibroblasts to multiply their contractile forces along the axis of cell alignment, and coincides with the alignment of the extracellular matrix (ECM). This is observed in scar tissue as linear arrays of ECM, as compared to randomly oriented ECM in uninjured skin.

To encourage random cell alignment, and hence disordered ECM deposition, we electrospun PLCL into a randomly oriented microfibrous scaffold [19–21]. We first studied the behavior of human dermal fibroblasts on this scaffold using the fibroblast populated collagen lattice (FPCL) assay (22), which has been used to model HSc contraction [16,22,23]. This was followed by in vivo evaluation using a murine HSc contraction model recently established in our laboratory; our model is the only immune-competent murine model that mirrors the human condition in terms of causality [24]. Together our in vitro and in vivo data suggest that slowly degrading electrospun scaffolds prevent myofibroblast activation associated with HSc, and provide the necessary longevity to prevent HSc contraction in vivo.

2. Materials and methods

2.1. Synthesis of PLCL

PLCL (50% LA, 50% CL) was synthesized as described elsewhere [8]. Briefly, L-lactide (100 mmol; Purac; Lincolnshire, IL, USA) and ε-caprolactone (100 mmol; Sigma; St. Louis, MO, USA) were polymerized at 150 °C for 24 h in the presence of stannous octoate (1 mmol, Sigma) as a catalyst. After being dissolved in chloroform, the polymer was precipitated in methanol, then dried under a vacuum for 72 h and stored in vacuum pack at −20 °C.

2.2. Fabrication and analysis of electrospun PLCL

PLCL scaffolds were fabricated using continuous single fiber electrospinning to deposit a 3D matrix of fibers on a rotating grounded mandrel using a custom spinning apparatus. PLCL was dissolved 14% (w/w) overnight in dichloromethane. Random fibers were spun at a flow rate of 3 mL/h with a voltage of 8 kV at a distance of 13 cm from the mandrel, which was rotating at ~70 revolutions per minute. Ambient temperature was 22 °C with 43% humidity. Following spinning, fibers were removed from the mandrel and residual solvent was removed by air drying for 72 h. Fiber characteristics and scaffold thickness were analyzed using scanning electron microscopy (FEI XL30 SEM-HEI, Hillsboro, OR, USA).

2.3. Oxygen plasma treatment & collagen coating methods

Samples were placed inside of a plasma asher (Emitech K-1050X, Montigny-le-Bretonneux, France) and treated with reactive oxygen plasma for 45 s at 100 W to impart hydrophilicity prior to cell culture and prepare for covalent collagen coating. Following treatment, samples were immediately immersed in sterile water and subsequently sterilized in 70% ethanol for 20 min. Samples were rinsed thoroughly with water following sterilization. Covalent collagen coating was performed by EDC/NHS chemistry as previously described [25]. This well-characterized method is biocompatible, non-cytotoxic, and does not include a linker-arm. Carbodiimide is not incorporated into the covalent-linkage, allowing the collagen to directly coat scaffold. This method does not modify scaffold morphology and generates a uniform collagen coating covering fibers throughout the depth of the scaffold. Scaffolds in collagen coated PLCL (ccPLCL) groups were covalently coated with bovine type-1 collagen (Nuragen, Advanced Biomatrix, San Diego, CA, USA) prior to in vivo implantation. Contact angle analysis before oxygen plasma treatment, after oxygen plasma treatment, and after collagen coating treatment was carried out on PLCL films using a goniometer as previously described [26].

2.4. Permeability measurement

In order to examine the impact of collagen coating on scaffold permeability, effective hydraulic permeability of PLCL and ccPLCL scaffolds was measured according to ASTM protocol F2952 using a custom-built flowmeter as previously described [27]. In brief, a “scaffold sandwich” was constructed between two silicone gaskets using a 100 μm scaffold and a fine stainless steel mesh. The “sandwich” was placed between mount pieces and a watertight seal was formed by applying light pressure using a threaded screw housing unit. A 50 mL pipette was suspended over a 5 mm gap between clamps. Samples were analyzed on a fluid pressure using a threaded screw housing unit. A 50 mL pipette was suspended over a 5 mm gap between clamps. Samples were analyzed on a fluid pressure meter as previously described [28].

2.5. Static tensile testing of electrospun scaffolds as compared to skin and scar tissues

Static tensile testing was carried out as described in ASTM D3822-07. Human and murine skin and scar tissues were tested as previously described [24]. In brief, scar and uninjured skin samples were gathered from human and murine donors. Uninjured murine tissue samples were collected from the dorsum of 10–12 week-old C57BL/6 mice. Murine scar tissue was taken from contracted d30 skin grafted mice. Tissue from five murine donors was used, with three biological replicates per donor. Human skin samples were donated from discarded human tissue from Duke Hospital operating rooms under exemption by Duke institutional review board. Uninjured human skin was gathered from breast resection, while scar tissue was taken from excised keloid, radiated forearm, and rejected skin graft. Tissue from the human donors was used with 3–5 biological replicates per donor. All human and murine tissues were kept moist on damp gauze between collection and mechanical testing, and analyzed within 1–5 h of collection. Prior to testing, underlying tissue was removed and samples were cut into uniform strips. PLCL, ccPLCL, Integra, and tissue samples were cut to 5 cm × 5 mm strips using a scalpel and surgical scissors and loaded with a 5 mm gap between clamps. Samples were analyzed on a microstrain analyzer (MSA) (RSA II, TA Instruments, New Castle, DE, USA) at a rate of 0.1 mm/s at room temperature (23 °C) until failure. The initial elastic modulus (within the first 0–200% strain) was analyzed for each sample. The lower elastic modulus was selected for analysis because this strain range best mimics strains that...
are experienced in the body. Ultimate tensile strength and elongation at break were taken from the data set of each sample.

2.6. Fatigue testing of PLCL and ccPLCL scaffolds

Fatigue properties of scaffolds were measured to determine whether the samples would be able to withstand continuous cyclic loading, such as that encountered across joints in the body. PLCL and ccPLCL samples were loaded on the MSA and immersed in 37 °C PBS using a heated sample cup. Samples were allowed to equilibrate for 1 h, then extended to 10% strain, and oscillated over 10% strain at 1 Hz for 24 h according to ASTM protocol D3479/D3479M-12.

2.7. Cell culture

Adult normal human dermal fibroblasts (NHDFs) (Lonza, Basel, Switzerland) were cultured in high glucose Dulbecco’s Modified Eagle’s Medium (GIBCO 11960-044) (Invitrogen, Grand Island, NY, USA) supplemented with 10% Premium Select fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA, USA), 25 μg/mL gentamicin (Invitrogen), and 1× GlutaMAX, non-essential amino acids, sodium pyruvate, and L-mercaptoethanol (Invitrogen), at 37 °C and 5% CO2. NHDFs were passaged a maximum of six times prior to experimentation.

2.8. Contraction studies

Media was prepared fresh on d1 (10% FBS + 5 mg/mL TGFβ), fresh media was added at d2.5, and the trial was stopped at d5 for analysis. Following sterilization, PLCL and ccPLCL scaffolds (8 mm round, 60 μm thick) were washed with PBS and left in 10% PBS media for 24 h prior to cell culture studies. Prior to seeding, scaffolds were rinsed thoroughly with PBS, placed in a 48 well suspension plate, and seeded with 200,000 cells in 500 μL media. Cells were allowed to attach for 1 h before addition of 500 μL media.

FPCLs were constructed as previously described [28,29]. In brief, FPCLs were prepared in triplicate by combining the following materials in the corresponding order: 50 μL of 5xPBS (pH 8.5, 23 °C) was combined with 200 μL bovine type I collagen (6 mg/mL, pH 5, 23 °C) (Nutralgen, Advanced Biomatix, San Diego, CA, USA), and 750 μL cell suspension (590,000 cells/mL). FPCLs were cast in small, pre-warmed, triplicate 35 mm × 10 mm tissue culture polystyrene dishes (250μL/dish). Dishes were placed in a 37 °C cell culture incubator for 1 h to allow FPCLs to solidify before slowly adding 2.5 mL media. FPCLs were allowed to remain attached to the cell culture dish throughout the study [30].

2.9. Viability analysis

Cellular viability analysis was performed using LifeTechnologies Live/Dead Viability/Cytotoxicity Kit for Mammalian Cells (Invitrogen). Live/dead staining on d5 NHDF-seeded PLCL and ccPLCL scaffolds was conducted according to manufacturer instructions. d5 scaffolds were mounted and imaged on an inverted fluorescence microscope (Eclipse TE2000-U, Nikon, Tokyo, Japan) within 3 h. Images were analyzed by counting labeled cells using Image J software (NIH).

2.10. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, permeabilized, and stained in a blocking solution containing the antibodies, 0.03 g/mL bovine serum albumin (BSA, Sigma), 10% goat serum (Sigma), and 0.3% Triton X-100 (Sigma) in PBS. Samples were blocked for 3 h prior to staining. Primary stain for sSMA (1:100, Abcam, Cambridge, MA, USA) was conducted for 18 h at 4 °C, the samples were then washed before incubation with Alexa Fluor 594 anti-mouse secondary antibody (1:200, Invitrogen) for 4 h at room temperature. Cell nuclei were stained with 4,6-Diamidino-2-phenylindole (DAPI) (1:5000, Invitrogen) and the actin cytoskeleton was stained with phalloidin 488 (1:200, Invitrogen). The samples were then mounted in Fluoro-Gel (Electron Microscopy Sciences, Hatfield, PA, USA) for fluorescent imaging.

Images for analysis of sSMA presence in immunocytochemistry were acquired using an inverted confocal microscope (Zeiss LSM 510, Oberkochen, Germany) with 10× magnification. Three images from each of three replicates were collected for each condition, resulting in a minimum of 2000 cells for each condition for analysis using Image J software. Nuclei quantification and area of sSMA positive stress fibers were analyzed using a size exclusion to disregard non-cell debris and sSMA localized to the cytosol or focal adhesions. Quantification of sSMA positive stress fibers was achieved by dividing the area of sSMA by the number of nuclei present in the image and normalizing to staining in FPCLs as 100.

3. Surgical methods

Female C57BL/6 mice, 10-12 weeks-old, weighing 18–23 g (Jackson Laboratories, Bar Harbor, ME), were used as wild type mice throughout the study. Mice were housed individually for the first three weeks, and in groups of four for the final week of study. Female mice were selected to reduce the risk of aggression-related injury when transferred to group housing; only one sex was used in order to reduce secondary variables related to hormonal differences between male and female mice. Mice were housed under protocols approved by the Institutional Animal Care and Use Committee of Duke University. Surgical methods were performed as described in our previous work; in all instances, skin graft refers to the use of a split thickness skin graft fashioned from the skin tissue of two donor mouse ears [24]. In brief, a third degree burn was created on the dorsum of the donor mouse, the burn was left for 3d, and a 14 mm diameter circle over the burn site was excised for recipient skin grafting. Treatment groups included: (1) skin graft without placement of scaffold material, (2) skin graft over Integra, (3) skin graft over PLCL, (4) skin graft over ccPLCL. Scaffolds 110 μm in thickness ± collagen coating were cut into circles using a 1.2 cm diameter arch stainless steel punch (VWR International, Radnor, PA) prior to sterilization. Integra was cut using a 1.2 cm punch immediately before surgery and removed from the silicone backing. Integra, sterile PLCL, or sterile ccPLCL scaffolds were laid into the wound bed and sutured just beneath the skin edges with four mattress stitches of 6-0 silk suture. Donor ear skins were laid over the excised burn wound after the implantation of the relevant scaffold to fashion a skin graft. The murine skin grafts were then secured with a padded bolster. The bolster was removed on post-operative d3.

3.1. Analysis of electrospun scaffold mechanical properties and degradation on d30 in vivo

The mice were euthanized and tissue was collected on post-operative d30 in all mice. The collected tissues from d30 mice treated with ccPLCL, Integra, or skin graft alone (scar tissue) were cut into three pieces (Fig. 5A). The two peripheral tissue specimens were preserved in 10% formalin and embedded in paraffin wax for histological analyses. The center piece was immediately taken to MSA for static tensile testing. Tissue explants were kept moist between collection and mechanical testing, and analyzed within 2 h of collection.

PLCL was extracted from d30 excised tissue samples by incubation with chloroform overnight and tested using nuclear magnetic resonance (NMR) and gas permeation chromatography as previously described [8]. The component composition of PLCL was analyzed using a 400 MHz 1H NMR (Varian, USA). Spectra were obtained using 1% (w/v) solutions in CDC13 and the compositions calculated from these relative intensities. Molecular weights were determined using a gel permeation chromatography (GPC, Viscotek TDAmax, Malvern Instruments Ltd, UK). Chloroform was used as the mobile phase at a flow rate of 1 mL/min. Calibration was performed using polystyrene standards to determine number-average and weight-average molecular weights (Mn and Mw).

3.2. Immunohistochemistry

Routine staining was carried out as previously described [24]. Primary antibodies used included: F4/80 (1:1500, ebioscience, San Diego, CA, USA) and anti-CD31 (1:50, Abcam). Secondary antibodies included: biotinylated rabbit anti-rat (1:200, Vector Laboratories, Burlingame, CA, USA), biotinylated goat anti-rabbit (1:50, Vector Laboratories), avidin-biotin complex reaction (Vector Laboratories), DAB substrate solution (Biocare Medical, Concord, CA, USA). Stained slides were visualized by use of a Nikon eclipse E600 microscope and images were captured with a Nikon DXM 1200 digital camera under the same settings. Quantification of CD31 images was performed using five HPF images per section across a minimum of five mice. Only positively stained vessels inside the perimeter of the scaffolds were quantified. Fiber diameter of ccPLCL scaffolds at d30
in vivo was calculated using H&E images, one HPF per mouse and a minimum of ten measurements were used.

### 4. Statistical analysis

Unless otherwise stated, the following applies to all experimental results. Gaussian data have been presented as mean ± standard error of the mean. Two-way ANOVA followed by students t-test was carried out to discern differences between groups, with significance at p < 0.05. In the case of non-Gaussian results, data were presented in box and whisker format. Significance was analyzed via two-way ANOVA followed by Mann–Whitney test. Mechanical and permeability analysis experiments were carried out with n ≥ 5, in vitro experiments were carried out in triplicate at least three separate times, and in vivo experiments were conducted with n ≥ 5 per treatment group.

### 5. Results

#### 5.1. Scaffold characteristics

Scaffolds were spun to thickness of 60 ± 10 μm for in vitro studies and 100 ± 11 μm for in vivo studies; fiber diameter was 5.6 ± 0.70 μm for all samples (Fig. 1AB). We chose to use a fiber diameter in this range because it has been associated with anti-fibrotic events in coronary tissue [31]. Covalent collagen coating uniformly covered fibers throughout the depth of the scaffolds (Fig. 1C) and did not significantly modify fiber diameter (6.5 ± 0.86 μm) or scaffold morphology (Fig. 1D). Contact angle analysis before oxygen plasma treatment, after oxygen plasma treatment, and after collagen coating treatment on PLCL films showed progressive hydrophilicity of 72 ± 6°, 60 ± 1°, and 44 ± 8°, respectively (Fig. 1E).

#### 5.2. Permeability analysis

PLCL scaffolds exhibited a mean Darcy permeability constant, \( \tau \), of 20.0 ± 1.2 μm²/s, compared to 19.7 ± 1.6 μm²/s for ccPLCL scaffolds with no statistically significant difference (Fig. 1F). The corresponding average pore sizes were 4.54 ± 0.13 μm and 4.50 ± 0.18 μm, respectively. Similarly, measured pore size using SEM images found an insignificant difference in average pore size of PLCL scaffolds (40.8 ± 2.8 μm) and ccPLCL scaffolds (36.3 ± 2.3 μm), further confirming that the collagen coating did not affect scaffold pore size. The discrepancy between derived average pore size and SEM measured pore size has been previously described when calculating pore size of electrospun scaffolds using this technique [27]. Sell et al. postulated that these difference may stem from the presence of “faux pores” and blind pouches within the scaffolds which are not visible via SEM measurement but affect the effective pore size gathered from flowmeter measurement.

#### 5.3. Mechanical properties

The mechanical properties of PLCL and ccPLCL scaffolds were analyzed using MSA and compared to those of human skin, human scar, and Integra (Fig. 2A). The elastic modulus of uncoated PLCL (3.6 ± 0.22 kPa) was significantly lower than that of Integra (6.5 ± 0.91 kPa), human skin (17 ± 1.6 kPa), and scar (55 ± 14 kPa). Elastic modulus of ccPLCL (8.3 ± 0.67 kPa) was not significantly different from that of Integra, suggesting that the combined process of oxygen plasma treatment and collagen coating increased the PLCL scaffold stiffness (Fig. 2B). Oxygen plasma treatment is known to generate a higher crosslinking density within the first few thousand angstroms of polymer surface, which results in a local increase in hardness and likely attributes to the modest increase in elastic modulus of ccPLCL [32].

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Fig. 1. Characterization of PLCL Scaffolds. Scaffolds were imaged using SEM; fiber diameter values were measured from top-view images (A) while scaffold overall thickness was measured via cross-sectional imaging (B). Scaffolds were covalently coated with Bovine type 1 collagen, collagen was stained using AlexaFluor488 and imaged via confocal microscopy (C) to analyze coating efficiency. Collagen coating did not fill pores or alter scaffold topography when imaged on confocal (C) or SEM (D). Contact angle was analyzed on PLCL films, following collagen coating films were significantly more hydrophilic than untreated PLCL films. Permeability analysis was carried out on PLCL scaffolds 110 μm thick, the Darcy coefficient of permeability was not significantly different between untreated PLCL and ccPLCL. Scale bar is set to 100 μm for all images.
The ultimate tensile stress (UTS) for both PLCL (0.97 ± 0.10 GPa) and ccPLCL scaffolds (1.3 ± 0.17 GPa) was significantly greater than that of Integra (0.26 ± 0.020 GPa). While PLCL showed lower UTS than that of human skin (2.6 ± 0.040 GPa), or human scar (2.7 ± 0.50 GPa), ccPLCL did not (Fig. 2C). In contrast, the elongation at break (EB) for PLCL (1100 ± 93 kPa) and ccPLCL (1300 ± 170 kPa) scaffolds was significantly higher than the values obtained for Integra (75 ± 4.4 kPa), human skin (200 ± 16 kPa), and human scar (140 ± 16 kPa) (Fig. 2D). The storage modulus, loss modulus, and tan δ of PLCL and ccPLCL scaffold subjected 10% strain at 1 Hz showed negligible deterioration over 24 h, or 15,000 cycles (Fig. 2E). This fatigue testing condition was chosen to emulate joint range of motion at the pace of walking [33].

Taken together, the mechanical properties of PLCL and ccPLCL scaffolds relative to human skin and scar — lower modulus, comparable UTS, and higher elongation at break — suggest that they would be suitable materials for placement beneath a skin graft.

5.5. In vivo application of PLCL & ccPLCL scaffolds

All data presented below are described in terms of percentage of original wound size, where 100% is the wound size at d3 (after removal of the post-operative bolster), and a fully contracted wound would be described as 0% of its original size. All samples were applied beneath the skin graft. Murine wounds treated with skin grafts alone contracted to 47 ± 2.0% at d30 (Fig. 4A), while wounds treated with Integra contracted to 28 ± 1.8% (Fig. 4B). Wounds treated with uncoated PLCL scaffolds remained at 68 ± 11% at day 21. However, partial scaffold extrusion and skin graft death began at d21 and continued until the end of the study, resulting in wound contraction down to 95 ± 5.8% at d30 (Fig. 4C). In contrast, wounds treated with ccPLCL scaffolds showed significantly decreased contraction, down to 95 ± 5.8% at d30 (Fig. 4D). Fig. 4E shows the rate of wound contraction over the test period. Upon extraction of the tissue from the mice on d30, ccPLCL scaffolds were observed to integrate with host tissue beneath the skin grafts, indicating that it out-performed all other groups in delaying HSc contraction (Fig. 4F).

5.6. Physicochemical properties of scaffolds at d30 after implantation

Tensile testing of explanted ccPLCL from d30 mouse studies displayed a similar elastic modulus to scaffolds prior to implantation (Fig. 5B). d30 ccPLCL explants exhibited an elastic modulus (5.7 ± 2.2 kPa) significantly lower than tissue alone in both mouse skin (36 ± 3.3 kPa) and scar (80 ± 17 kPa) samples, although not significantly different from that prior to implantation (9.7 ± 0.20 kPa).

The molecular weight of the scaffolds decreased by 49% from Mₙ = 151 kDa to Mₙ = 73.2 ± 6.5 kDa over the implanted period of
Fig. 3. Cellular interactions with scaffolds. (A–F) Overlay of DAPI (blue, left), F-actin (green, center), and αSMA (red, right) in (A–C) FPCLs and (D–F) PLCL scaffolds. (G) Significantly less αSMA was present in immunostaining in PLCL scaffolds than in FPCLs. (H) Cells remained viable in PLCL scaffolds. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. Wound contraction and scaffold incorporation at day 30. (A–D) Gross appearance of wounds treated with (A) skin graft alone, (B) Integra beneath skin graft, (C) PLCL scaffold, and (D) ccPLCL scaffold, at days 7, 9, 14, 21, and 30 following surgery. (E) Wound contraction curves derived from measurements of wounds shown in A–D. (F) ccPLCL scaffolds beneath skin graft immediately following excision from wound bed on d30.
30 days. NMR analysis shows that the lactide (LA) moiety was noticeably more rapidly decreased than the caprolactone (CL) units. The molar ratio was as expected since PLA is known to degrade more rapidly than PCL. The mole fraction of LA decreased from 50% to 44% in 30 days, while that of CL increased from 50% to 56%. Table 1 displays results of NMR and GPC analysis.

5.7. ccPLCL scaffolds incorporate into host tissue and maintain architecture in vivo

Fig. 6A-D shows representative images of histological staining in Integra and ccPLCL scaffold treated wounds on d30. Fiber diameter of ccPLCL scaffolds at d30 in vivo (5.5 ± 0.33 μm) was not significantly different from initial fiber diameter prior to implantation. Throughout all samples, H&E stained Integra and ccPLCL scaffolds on d30 exhibited acute inflammation (Fig. 6A,B). ECM alignment appeared more prevalent in Integra treated samples than in ccPLCL treated samples (Fig. 6A,B, data not quantified). Negligible fibrous capsule formation was present in ccPLCL treated mice; no fibrous capsule was visible in Integra treated mice as the implant was no longer discernable within the wound bed. CD31 staining demonstrated vessel ingrowth into ccPLCL scaffolds and Integra. Multinuclear giant cells and neutrophils were visible at the boundary of the ccPLCL scaffolds with the tissue (Fig. 6B). F4/80 staining confirmed the presence of macrophage within the giant cells in wounds treated with Integra and ccPLCL (Fig. 6C). Cells penetrating into the ccPLCL scaffold and Integra granulation tissue included histocytes and foreign body giant cells. Quantification of CD31 outlined vessels showed that angiogenesis into ccPLCL (6.1 ± 0.54 vessels/HPF) and Integra (6.5 ± 0.90 vessels/HPF) was not significantly different; vessels were observed spanning both scaffold materials as well as passing through the material (Fig. 6D).

<table>
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<th>Analysis of explanted scaffold material</th>
<th>Mn (kDa)</th>
<th>Mn (%)</th>
<th>Mw (kDa)</th>
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6. Discussion

This study highlights the role of scaffold longevity in the list of relevant design parameters for BSEs aimed at mitigating HSc contraction. In this work, we employed a synthetic, biodegradable, elastomeric, electrospun scaffold to study the effect of scaffold longevity on the development of HSc contraction in vivo. In developing a scaffold for use in HSc prevention, we focused on the properties of healthy skin as design parameters. First, we considered the random nature of the ECM which inspired us to choose electrospinning as our fabrication method. Next, we considered the mechanical properties of human skin, including its tensile and viscoelastic characteristics, guiding us to select a viscoelastic synthetic polymer. Finally, we hypothesized that the optimal scaffold should last through the remodeling phase of repair when HSc occurs, which prompted us to study a slow-degrading PLA-PCL blend. To improve cell–scaffold interactions, we covalently attached bovine collagen to the scaffold prior to in vivo implantation. To ensure adequate time for HSc stabilization in skin-grafted murine wounds, we followed all treatment groups out for 30 days following implantation [24]. While wound healing studies are often conducted over 14 days, this extended timeline allowed us to gain an early determination of the effects of scaffold degradation on scar contraction. In comparison to previous work using biodegradable polymers for BSE fabrication, this biodegradable elastomer demonstrates appropriate mechanical properties for implantation beneath skin grafts and is capable of repetitively undergoing physiologically relevant strain and relaxation without entering plastic deformation. Most importantly, this synthetic elastomer possesses a degradation rate on the scale of six months in vivo, allowing it to maintain its architecture throughout the remodeling phase of repair [6,34].

While traditional BSEs are attractive, their degradation rates and mechanical properties are difficult to design in practice. We postulate that the mechanical properties of human skin are critical to consider during the design of a scaffold for HSc prevention. Scaffolds with tensile elastic moduli greater than human skin may inhibit joint motion, similar to how stiffened scar inhibits motion [24,35]. Therefore, BSEs should possess an elastic modulus less than or equal to that of human skin. Further, possessing elongation at
Integra-treated wounds at d30 in vivo. (For interpretation of the references to color in (HPF). There is no signi
cant difference in vascularity in ccPLCL Scaffold compared to the in vitro wound contraction model, the FPCL. Once seeded in the FPCL, fibroblasts are able to contract the collagen fibrils in the gel similarly to how they would contract scar granulation tissue. The fraction of cells which converted into myofibroblasts in this model was analyzed by immunocytochemical staining for αSMA, which is a marker for myofibroblast formation. Using this quantitative method, we found significantly more αSMA in the FPCL than was present in PLCL scaffolds. These data suggest that electrospun PLCL scaffolds mitigate cellular processes associated with HSc.

Fig. 6. Histological analysis of implanted materials at d30. (A, left) Low power view of Integra treated wounds shows the presence of skin grafts (epidermis, e) and underlying dermis (d) and dermal scar (s); no evidence of Integra is identified. (A, right) ccPLCL treated wounds with overlying skin graft (e), dermis (d) and ccPLCL scaffold (p, outlined); architecture of PLCL scaffold remains intact. (B, left) High power view of epidermis and underlying scar in Integra treated wound no evidence of Integra architecture. (B, right) Cellular infiltration into PLCL scaffold at d30, ccPLCL (p) architecture can be noted by the existence of white space where the scaffold is present. (C) Presence of foreign body giant cells in both Integra treated wound (left) and ccPLCL treated wounds (right) is confirmed by F4/80 pan-macrophage membrane stain (brown). Foreign body giant cells are noted by the presence of black arrows. (D) Angiogenesis into wounds is analyzed by staining the endothelial lining of neo-vessels in the wound bed using CD31 stain (brown). Black arrows indicate examples of representative CD31+ vessels. (E) Quantitative analysis of CD31 stained sections is graphically represented. Number of CD-31 positive vessels were counted in five 40x high power fields (HPF). There is no significant difference in vascularity in ccPLCL Scaffold compared to Integra-treated wounds at d30 in vivo. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

HSc has typically been studied in immune-compromised mice, in which multiple models have been developed; however, due to the importance of the immune system in wound healing, we have chosen not to use these models [37-42]. Two porcine models are available to study HSc: the female red Duroc pig, and the Yorkshire pig [43]. Swine arguably represent the best model for human wound healing, with 78% agreement between human and porcine wound healing, however pre-clinical studies in swine are costly [44]. We chose to test PLCL and ccPLCL scaffolds in our recently developed murine model of HSc contraction [24]. While the murine model employed in this work directly follows the human condition in terms of treatment and contracting scar, it should be noted that the model does not reflect the proliferative component or timeline of human HSc. The term HSc, as it applies to humans, describes a constellation of scar symptoms (red, raised, itchy, contracted scars), whereas the murine model only exhibits the contraction phenotype. Over time the murine skin grafts become flat and pale, as is observed in some but not all human HSc [45,46]. The difference between these two scarring states may be due to a diminished level of mechanical tension; human skin exists in a resting state of tension, unlike murine skin, which is loose. Further, the model employed in these studies is an expedited HSc contraction model where contraction ensures for 14d and then stabilizes, whereas in humans contraction can last for 6–12 months. The PLCL copolymer used in these studies has been shown to persist in canine patients for up to 12 months, suggesting that it will last through the contraction phase of scarring if translated to be used in humans [34].

While uncoated PLCL scaffolds were rejected between d21–d28, ccPLCL scaffolds integrated into the wound tissue and prevented HSc contraction. This is likely because synthetic polymer implants do not offer integrin binding sites and often require surface treatment prior to implantation. PLCL scaffolds are hydrophobic in nature, encouraging the likelihood for random protein adsorption and eventual extrusion. The extrusion process is caused by spontaneous and uncontrollable adsorption of proteins from the blood, lymph, and wound exudate to hydrophobic implant surfaces [47]. Implantation of synthetic polymer structures into open wounds...
without integrin binding sites can lead to foreign body encapsulation and extrusion. To decrease the likelihood of random protein adsorption and encourage cell—scaffold interactions through integrin binding sites, collagen was covalently attached to the surface of PLCL scaffolds. There is concern that protein coating of electrospun polymers may restrict effective aqueous fluid flow, thus disrupting transport of fluids and nutrients through scaffolds in vivo [48]. However, despite the presence of a protein coating, a negligible change in effective permeability was found between ccPLCL and PLCL scaffolds. Following in vivo implantation, ccPLCL scaffolds integrated into the tissue beneath the skin graft. The beneficial impact of the collagen coating could be explained by favorable changes in scaffold hydrophilicity or the introduction of cell-binding motifs. These data are in agreement with the literature on the importance of introducing integrin binding sites to the surfaces of intra-dermal, hydrophobic, synthetic polymer implants.

Upon removal of wound tissue from the mice, ccPLCL scaffolds could be discerned beneath skin grafts. Tensile testing of explanted ccPLCL from d30 mouse studies displayed a similar elastic modulus to scaffolds prior to implantation (Fig. 5B). d30 ccPLCL explants also exhibited an elastic modulus significantly lower than tissue alone in both mouse skin and scar samples. These data suggest that the scaffold is preventing stiffening associated with HSc scar formation. Scar formation is also associated with alignment of the ECM, as is seen in d30 Integra treated samples via H&E staining. Conversely, cells in ccPLCL scaffolds display random nuclear orientation and ECM alignment at d30 via H&E staining (Fig. 6B). The presence of a foreign body reaction, characterized by neutrophil and macrophage infiltration, can be seen in both treatment groups. The acute inflammation present in these sections is confirmed by F4/80 staining and is commonly observed following intra-dermal implantation of a foreign body. Vessel infiltration is not significantly different between Integra and ccPLCL scaffolds, suggesting that both materials are integrating well with the host tissue and allowing lymphogenesis and/or angiogenesis to occur. Overall, the histological data suggest the presence of an acute inflammatory reaction in ccPLCL treated mice along with vessel infiltration into the scaffold and maintenance of randomly oriented ECM. These outcomes are all necessary to maintain skin graft health and prevent the formation and subsequent contraction of HSc.

ccPLCL scaffolds significantly prevented HSc contraction in vivo as compared to Integra or placement of a skin graft alone. Integra treated mice maintained 75% of the original wound area until day eight, after which scars rapidly contracted (Fig. 4E). The contraction seen after day eight could be associated with partial necrosis of skin grafts on Integra-treated wounds due to the immediate placement of skin grafts over Integra [49]. While collagen-based scaffolds can be successful following immediate skin graft placement, Integra requires a two-step implantation procedure [50]. During clinical use, Integra is placed and allowed to integrate into the wound bed for approximately two weeks prior to placement of the skin graft. This delay allows a period for vascular infiltration of the Integra matrix; however, this method increases risk of infection and requires a second operation [51]. In general, collagen scaffolds, such as Integra, are associated with wound contraction and scarring [52]. Collagen is degraded and remodeled by collagenases and other proteases within the wound bed with a half-life on the order of days to weeks depending on the crosslinking method. It is conceivable that after day eight of implantation, Integra begins to lose its architecture and mechanical properties, allowing rapid scar contraction [53]. A similar rapid contraction after day eight has been shown in the literature following treatment of full thickness dermal wounds with electrop spun collagen scaffolds in a guinea pig model [54]. Together, these data suggest that rapid scar contraction after day eight is related to the longevity of collagen scaffolds in the wound bed, rather than the fabrication technique. Wounds also rapidly contracted following extrusion of uncoated PLCL scaffolds, which began after d21 in vivo (Fig. 4E). Rapid wound contraction is commonly seen following removal of splinting materials from murine wounds, suggesting that Integra and non-incorporated scaffolds may act as temporary splinting materials to keep the wound open until they either extrude or lose their architecture via degradation [55].

HSc contraction develops progressively over the course of several weeks in mice and multiple months in humans; therefore, it is feasible that a loss of scaffold architecture prior to the completion of the remodeling phase of repair could allow for delayed cellular alignment and the formation of a mechanically-coordinated cellular syncytium, thus increasing the likelihood of HSc contraction. The structural support provided by collagen-based scaffolds, such as Integra, has been suggested to rely upon the pore architecture of the scaffold, rather than the elastic modulus of the material [53]. Our data support the hypothesis that elastic modulus of the implant does not dictate its ability to resist wound contraction; the elastic modulus of ccPLCL electrospun scaffolds is within the same order of magnitude as Integra when tested in the early strain region. However, the effects on wound contraction between Integra and ccPLCL scaffolds are drastically different at d30 in vivo. At d30 following implantation, the architecture of ccPLCL scaffolds remains clearly defined in the wound bed histology, whereas the d30 the architecture of Integra is not visible via histological staining. Lyophilized collagen-GAG based scaffolds, similar to Integra, degrade over the order of several days to several weeks [56]. This short degradation time can be exacerbated by patient-to-patient variability in the wound healing process, leading to the possibility of premature degradation prior to proper healing. Indeed, rapid degradation and resorption of a BSE prior to the completion of the remodeling phase of repair leads to increased likelihood of scar formation [57]. We hypothesize that the critical difference leading to the success of ccPLCL scaffolds in preventing HSc contraction is the residence time of the scaffold architecture in the wound bed. The PLCL material employed in this study will retain its structure in the wound bed throughout the remodeling phase of repair, having only lost 51% of its number-average molecular weight at 30 days in vivo. Future studies are required to confirm this hypothesis and will explore longer time points to evaluate the response of the tissue to degradation of the polymer.

7. Conclusions

Loss of scaffold architecture prior to the completion of the remodeling phase of repair is likely a major cause for HSc contraction. This study suggests that the relative maintenance of integrity, rather than the absolute mechanical properties of the ccPLCL scaffold, is responsible for improved mitigation of HSc contraction.

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