Biostable electrospun microfibrous scaffolds mitigate hypertrophic scar contraction in an immune-competent murine model

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Abstract

Burn injuries in the United States account for over one million hospital admissions per year, with treatment estimated at four billion dollars. Of severe burn patients, 30–90% will develop hypertrophic scars (HSc). In this study, we evaluate the impact of an elastomeric, randomly-oriented biostable polyurethane (PU) scaffold on HSc-related outcomes. In vitro, fibroblast-seeded PU scaffolds contracted significantly less and demonstrated fewer SMA+ myofibroblasts compared to fibroblast-seeded collagen lattices. In a murine HSc model, collagen coated PU (ccPU) scaffolds significantly reduced HSc contraction as compared to untreated control wounds and wounds treated with the clinical standard of care. Our data suggest that electrospun ccPU scaffolds meet the requirements to reduce HSc contraction including reduction of in vitro HSc related outcomes, diminished scar stiffness, and reduced scar contraction. While clinical dogma suggests treating severe burn patients with rapidly biodegrading skin equivalents, our data suggest that a more long-term scaffold may possess merit in reducing HSc.

Statement of Significance

In severe burns treated with skin grafting, between 30% and 90% of patients develop hypertrophic scars (HSc). There are no therapies to prevent HSc, and treatments are marginally effective. This work is the first example we are aware of which studies the impact of a permanent electrospun elastomer on HSc contraction in a murine model that mimics the human condition. Collagen coated polyurethane scaffolds decrease SMA+ myofibroblast formation in vitro, prevent stiffening of scar tissue, and mitigate HSc contraction. Unlike current standards of care, electrospun, polyurethane scaffolds do not lose architecture over time. We propose that the future bioengineering strategy of mitigating HSc contraction should consider a long-term elastomeric matrix which persists within the wound bed throughout the remodeling phase of repair.

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1. Introduction

Burn injuries in the US bring more than one million patients to the hospital each year; treatments for these injuries cost over four billion dollars [1]. Previous studies have reported diverging instances of hypertrophic scar (HSc) formation following burns ranging from 30% to 90% depending on the history of patient, likelihood of infection, and location of the burn [2]. When HSc arises, approximately 70% occur across joints or other areas of tension in the body [3]. HSc are firm, raised, red scars that progressively contract over six months to two years to form fixed scar contractions [4]. Contractures are painful, disfiguring, and debilitating; when contractures occur across a joint, they result in loss of range-of-motion [3,5]. Preventative treatments such as splinting and pressure garments remain marginally effective [6].
Current standard-of-care for deep burn wounds includes burn excision followed by placement of a skin graft over the wound site to enable re-epithelialization [7]. In extensive burn wounds, skin grafts may be placed atop artificial skin substitutes known as bioengineered skin equivalents (BSE). The most commonly used commercially available BSE, Integra, has proven invaluable in assisting wound healing, but does not prevent HSc contraction. Integra consists of a lyophilized matrix of bovine collagen and shark cartilage glycosaminoglycans [8]. While these natural polymers give Integra remarkably high biocompatibility, they also impart poorly-controllable mechanical properties and rapid degradation within the wound bed [9–11]. This is problematic in HSc as contraction can continue for months to years [4].

HSc contraction occurs secondary to the persistence of highly contractile cells in the wound bed known as myofibroblasts [12]. Myofibroblasts derive from fibroblasts through a process that is thought to be mediated by soluble factors, most importantly TGF-β1, and increased mechanical tension in the wound bed [4,12]. Myofibroblasts differ from residual fibroblasts in their expression of several contractile proteins, most predominantly α-smooth muscle actin (αSMA) [13]. αSMA is similarly found in smooth muscle cells and interacts with non-muscle myosin II to promote matrix contraction [12]. Matrix contraction occurs as randomly oriented ECM collagen is aligned by myofibroblasts [14,15]. The linear ECM then uniformly transmits forces to cells in a feed-forward mechanism, which promotes further differentiation of fibroblasts into myofibroblasts [12]. Conversely, randomly oriented collagen fibers native to the uninjured dermis encourage random cell alignment through contact guidance and do not transmit as much force as aligned collagen fibers [16].

Randomly-arranged nano- and micro-scale fibrous scaffolds can be fabricated by electrospinning [17]. Electrospun scaffolds are ideal candidates for tissue engineering because fiber diameter can be precisely tuned to alter topographical cues [17,18]. Topography close to biological scale provides a passive approach without bioactive agents to modulate cell behavior [19]. While topography in the nano-range has been heralded for impact on cellular processes, the pore size of nano-scale electrospun scaffolds limits cellular infiltration and nutrient transport in vivo [20]. Generally, a pore size on the order of several μm to several hundred μm is thought to be necessary for cellular penetration [21].

Previous work in our laboratory studying a microfibrous biodegradable scaffold suggested that loss of scaffold architecture prior to the completion of the remodeling phase of repair may be a major cause for HSc contraction [22]. In order to further investigate this hypothesis, we chose to study the impact of the same design using a non-degradable material. In searching for the appropriate material for this application, we looked for a medical-grade, resistance to stress cracking at 500% strain, and optimal mechanical properties and rapid degradation within the wound bed [4,12]. Myofibroblasts differ from residual fibroblasts in their expression of several contractile proteins, most predominantly α-smooth muscle actin (αSMA) [13]. αSMA is similarly found in smooth muscle cells and interacts with non-muscle myosin II to promote matrix contraction [12]. Matrix contraction occurs as randomly oriented ECM collagen is aligned by myofibroblasts [14,15]. The linear ECM then uniformly transmits forces to cells in a feed-forward mechanism, which promotes further differentiation of fibroblasts into myofibroblasts [12]. Conversely, randomly oriented collagen fibers native to the uninjured dermis encourage random cell alignment through contact guidance and do not transmit as much force as aligned collagen fibers [16].

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Our scaffold design is based upon the hypothesis that a randomly-oriented elastomeric scaffold persisting throughout the remodeling phase of repair will provide structural support to the healing tissue and prevent HSc contraction. This design employs three key characteristics: (1) random orientation of fibers to encourage random cell alignment; (2) viscoelastic mechanical properties appropriate for placement beneath skin graft; and (3) persistence within the wound bed for the duration of the remodeling phase of repair. The objective of this work was to push the boundaries of scaffold longevity and explore whether a permanent material would improve our ability to mitigate HSc contraction.

2. Materials and methods

2.1. Scaffold fabrication

Scaffolds were electrospun as previously described [26]. Briefly, Chronoflex AL80A polyurethane (Advansource Biomaterials) was dissolved in a 3:1 mixture of chloroform:ethanol at 11.25% w/v and dispensed at 6 mL/h through a 24 ga needle with a voltage of 8 kV. Random fibers were collected 13 cm away on a rotating (~70 RPM) cylindrical mandrel. Pore sizes were measured along the longest axis of the pore.

2.2. Scaffold surface modification

Samples were cut to their final size prior to treatment with reactive oxygen plasma (Emitech K-1050X) at 100 W for 45 s and subsequent ethanol sterilization for 20 min in 70% ethanol, followed by 3 × 5 min washes in sterile water. Sterile scaffolds were collagen coated using EDC/NHS as previously described (full coating protocol available in Appendix A) [27]. Bovine type-1 collagen (Advanced Biomatrix) was covalently coated to PU scaffolds (ccPU) a minimum of 24 h prior to implantation and/or analysis. Presence of collagen coating was confirmed via confocal microscopy following immunnochemical staining using anti-collagen type1 primary antibody (1:200, Abcam) for 2 h at room temperature followed by Alexa Fluor 488 secondary antibody (1:200, LifeTechnologies) for 1 h at room temperature. Contact angle analysis of PU oxygen plasma treated PU (OP-PU), and ccPU films was conducted using a goniometer as previously described [22].

2.3. Permeability measurement

Scaffold hydraulic permeability was conducted according to ASTM F2952 using a flowmeter as previously described [28]. Modifications included a 50 mL horizontal pipette, a height of 32 cm, and an inner diameter of 12.7 mm. Circular scaffolds (1.9 cm diameter, 110 μm thick) were inserted into the flowmeter. Following 15 mL of equilibration with PBS, time was measured at 5 mL intervals for 15 mL total. The Darcy coefficient (k), average pore diameter, and average fiber diameter were calculated as previously described [28].

2.4. In vitro culture and contraction analysis

Normal human dermal fibroblasts (NHDF, Lonza) were cultured in high glucose DMEM supplemented with 10% Premium Select fetal bovine serum, 25 μg/mL gentamicin, and 1 × GlutaMAX, non-essential amino acids, sodium pyruvate, and β-mercaptoethanol (LifeTechnologies). NHDF (passage six or less) were seeded on sterile OP-PU or ccPU scaffolds (60 μm thick, 8 mm diameter) in a 48-well suspension plate at a density of 200,000 cells in 500 μL culture media with 5 ng/mL TGFβ and incubated (37 °C, 5% CO₂) for 1 h in order to allow attachment to the scaffolds. An additional 500 μL media was then added to each well. The media was changed on d 2.5, and the trial was concluded on d 5. The same process was used without TGFβ to drop seed PU scaffolds for ECM deposition analysis, with analyses on d 0, 4, 7, 10, and 14. Seeded scaffolds were fixed in 4% paraformaldehyde for 15 min at 23 °C and washed in increasing concentrations of
ethanol. Scaffolds were then air dried, sputter coated (Denton Vacuum), and viewed under SEM (FEI).

Fibroblast populated collagen lattices (FPCL) were created as previously described [29,30]. Briefly, bulk FPCLs were prepared in triplicate by combining 50 μL of 5× PBS (pH 8.5, 23 °C) with 200 μL bovine type I collagen (6 mg/mL, pH 5, 23 °C) and a 750 μL suspension of NHDFs in culture media with 5 ng/mL TGFB (590,000 cells/mL). FPCLs were cast (250 μL/dish) in triplicate in pre-warmed TCP dishes (35 × 10 mm). TCPs dishes were incubated at 37 °C for 1 h to allow for FPCL solidification, followed by the slow addition of 2.5 mL of PBS. For floating FPCL studies, FPCLs were detached from the dishes and imaged using a laser scanner at d 0, 1, 3, and 7 following detachment. Scaffold and FPCL diameter were quantified with ImageJ (National Institute of Health) as compared to a known distance. For fixed FPCL studies, FPCLs were allowed to remain attached to the dish throughout the study.

2.5. Staining, imaging, and quantification of in vitro HSc-related outcomes

On d 5 after seeding, cells seeded in floating FPCLs and scaffolds were fixed, permeabilized, and stained in a blocking solution containing 0.03 g/mL BSA, 10% goat serum, and 0.3% Triton X-100 in PBS. Samples were blocked for 3 h prior to staining. Primary stain for αSMA (1:100, Abcam) was conducted for 18 h at 4 °C, followed by incubation with Alexa Fluor 594 anti-mouse secondary antibody (1:200, LifeTechnologies) for 4 h at 23 °C. Cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, 1:5000, LifeTechnologies) and the actin cytoskeleton was stained with phalloidin 488 (1:200, LifeTechnologies). Live/dead assay was conducted with LifeTechnologies Live/dead viability/cytotoxicity kit for mammalian cells according to manufacturer instructions.

Images for quantification of αSMA presence were acquired under confocal microscopy (Zeiss LSM 510, Zeiss Microscopy) at 10× and 40× magnification. Three 10× images from each of three replicates were collected for each condition, resulting in a minimum of 2000 cells for each condition for analysis using ImageJ.

2.6. In vivo HSc contraction studies

Murine housing and experimental protocols were approved by, and conducted in accordance with, guidelines from the Institutional Animal Care and Use Committee of Duke University. All murine surgeries were performed as previously described by trained surgeons at Duke University. Each animal received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (National Institute of Health Publication 80-23, revised 1996). All surgery was performed under iso-flurane anesthesia. After sacrifice, tissues were excised and fixed in 10% formalin. Tissues were paraffin embedded and cut into 4μm sections.

2.7. Mechanical testing

Discarded human scar and unwounded skin samples were obtained under Duke Institutional Review Board guidelines (IRB awarded 02/01/2010). Human skin and scar tissues were tested as previously described [31]. Integra was gently scraped from its outer silicone layer using a razor blade and forceps. Samples were cut into 1 cm × 5 mm rectangles for mechanical analysis. Tissue/scaffold constructs were explanted on post-operative d 30 from mice treated with skin graft alone and ccPU scaffolds. Collected explants were cut into 3 pieces (Supplementary Fig. S4). Static tensile testing was conducted at 23 °C according to ASTM D3822-07 using a microstrain analyzer (MSA) (TA Instruments RSA II). Sandpaper was attached to the metal grips of the MSA using double sided, water-proof carbon tape to prevent sample slippage. Samples were tested until failure at a rate of 0.1 mm/s. Ultimate tensile strength (UTS) and elongation at break (EAB) were obtained directly from stress-strain analysis for each sample. Fatigue testing was conducted according to ASTM D3479/D3479M-12. PU and ccPU samples were loaded onto the MSA in PBS and heated to 37 °C in a sample cup. A temperature-controlled oven surrounding the sample cup was used to maintain constant temperature during fatigue experiments. An initial 10% strain was applied and samples were allowed to equilibrate for 1 h. Samples were then subjected to a cyclical 10% strain at 1 Hz over 24 h (15,000 cycles). Storage modulus, loss modulus, and tanδ were obtained.

2.8. Histological staining, imaging, and analysis of in vivo specimens

In vivo tissue specimens were preserved in 10% formalin and subsequently embedded in paraffin wax for histological analysis. Sections were stained with hematoxylin–eosin. Slides were visualized using a Nikon eclipse E600 microscope. Sections for immunohistochemical analysis were treated stained as previously described anti-F4/80 antibody (1:1500 dilution, eBioscience) and anti-CD31 antibody (1:50, Abcam) [31]. Quantification of CD31 images was performed using five high powered field (HPF) images per section at 40× magnification across a minimum of five mice per treatment group. Only positively stained vessels inside the perimeter of the scaffolds were quantified.

3. Results

3.1. Scaffold characteristics

Scaffolds were spun to thickness of 60 ± 10 μm for the in vitro studies and 110 ± 10 μm for the in vivo studies. Scaffolds demonstrated uniform structure and random morphology (Fig. 1a). Collagen coating uniformity was confirmed via immunohistochemical staining (Fig. 1b). Contact angle analysis was used to analyze changes in surface hydrophilicity. PU films demonstrated significantly reduced contact angle from 92 ± 2.8° to 48 ± 2.2° following oxygen plasma treatment, and to 33 ± 2.4° with subsequent collagen coating, indicating increases in sample hydrophilicity following surface modification (Fig. 1c). Fiber diameter of PU scaffolds (5.2 ± 0.1 μm, 4.9 ± 1.4 μm) was not significantly different from collagen coated PU (ccPU) scaffolds (5.2 ± 0.1 μm, 5.2 ± 1.5 μm) as measured by scanning electron microscope (SEM) and flowmeter, respectively (p = 0.79, p = 0.52). We chose to electrospin scaffolds with 5 μm diameter fibers to encourage cellular attachment and orchestrate pores large enough for infiltration in vivo [32]. Pore size is a critical variable in the design of electrospun scaffolds: pores which are too small inhibit cell and nutrient penetration, whereas pores which are too large do not allow for cell attachment [33]. However, the accurate measurement of pore diameters in electrospun scaffolds is a documented issue [20,28]. The flowmeter is thought to obtain measurements an order of magnitude smaller than mercury porosimetry and SEM because it is considered an “effective measure” of pore size, capturing the presence of “faux pores” and blind pouches within the scaffolds [26]. Pore size obtained by flowmeter (3.7 ± 1.0 μm PU, 3.7 ± 2.0 μm ccPU) was
significantly smaller than that obtained by SEM measurement (34 ± 3.6 μm PU, 33 ± 3.5 μm ccPU) (p = 0.002). Pores within this size range are comparable to sizes described in the literature using mercury porosimetry to study a 5 μm diameter scaffold composed of polycaprolactone (PCL) [20,28]. The flowmeter also demonstrated that effective permeability (k) of PU (k = 13 ± 0.7 μm²) and ccPU scaffolds (k = 14 ± 0.8 μm²) was not significantly different (p = 0.22). Taken together these data demonstrate that covalent collagen coating increased scaffold hydrophilicity, but did not significantly modify fiber diameter, pore size, or permeability of electrospun scaffolds.

3.2. Scaffold and tissue mechanical properties

In choosing PU to fabricate HSc contraction-inhibiting scaffolds, we considered the mechanical properties of human skin and scar tissue as design parameters: while uninjured skin is viscoelastic in nature, scar tissue is stiff and inelastic [34]. The mechanical properties of PU and ccPU scaffolds were analyzed using MSA and compared to those of human skin, human scar, and Integra (Fig. 2a, detailed statistical analysis can be found in Supplementary Table 1). The elastic modulus of PU scaffolds (2.4 ± 0.13 kPa), Integra (6.5 ± 0.91 kPa), and ccPU scaffolds (8.3 ± 0.67 kPa) were significantly lower than human skin (17 ± 1.6 kPa), and human scar (63 ± 14 kPa) (Fig. 2b). Elastic moduli in this range suggest that these materials will not inhibit patient motion if placed across a joint. Unlike Integra (0.26 ± 0.023 MPa), the ultimate tensile strengths of PU (2.2 ± 0.11 MPa) and ccPU (4.8 ± 1.9 MPa) were greater than or equal to that of human skin (2.6 ± 0.40 MPa) and scar tissue (2.7 ± 0.50 MPa), suggesting that these materials will not fail due to tensile stress placed upon the skin (Fig 2c). During normal joint motion, skin may be strained up to 100% [35].
The elongation at break of Integra (75 ± 4.4%) was significantly lower than human skin (200 ± 15.6%), and human scar (144 ± 16.2%). Conversely, PU and ccPU scaffolds displayed elongation at break values of 660 ± 92% and 1300 ± 130%, respectively (Fig 2d). While strains of this magnitude are not likely to be experienced in the body, it is critical that implants designed for implantation beneath skin graft are capable of withstanding physiologically relevant strains. Scaffold elasticity is similarly important in designing implants for placement across joints, thus tensile fatigue testing was carried out on PU and ccPU scaffolds. Both PU and ccPU demonstrated negligible changes in storage modulus, loss modulus, and tan$\Delta$ values over 15,000 cycles at 1 Hz, suggesting that these materials can withstand the repetitive extension and relaxation of joint movement (Fig. 2e and f). Taken together, these data suggest that PU and ccPU scaffolds are mechanically more appropriate than Integra to withstand the physiological strains placed on skin grafts.

3.3. In vitro NHDF response to scaffolds

In vitro HSc-related outcomes were compared between PU scaffolds, ccPU scaffolds, and FPCLs. The FPCL is the gold-standard assay for studying how myofibroblast contractility affects wound and scar contraction. Myofibroblast behavior observed in FPCLs closely mimics in vivo observations in healing HSc including myofibroblast formation and collagen production [36–38]. FPCLs can be used to model initial wound bed contraction (floating matrices) or HSc contraction (fixed matrices) [39]. In this work, fibroblasts were seeded in FPCLs as in vitro models of fibroblast-driven matrix contraction and myofibroblast formation in un-treated wounds. Normal human dermal fibroblasts (NHDF) were seeded in FPCLs, PU, or ccPU scaffolds and assayed for HSc related outcomes. NHDF remained viable in PU (94 ± 3.7%) and ccPU scaffolds (94 ± 2.2%) at d 5 in vitro (Fig 3a). NHDF seeded in floating PU scaffolds contracted their matrix significantly less over seven days (96 ± 1.2% total area) than cells in floating FPCLs (22 ± 5.2% total area) (Fig 3b). Immunocytochemistry demonstrated that significantly less $\alpha$SMA was present in stress fibers of cells seeded in PU scaffolds (26 ± 10%) and ccPU scaffolds (20 ± 6.1%) as compared to those seeded in FPCLs (normalized to 100%), suggesting reduced myofibroblast activity or conversion (Fig 3c). A decrease in $\alpha$SMA expression does not necessarily relate to a decrease in myofibroblast number, but could also represent the use of an alternative contractile apparatus, or decreased activity within the myofibroblast

![Fig. 3. In vitro analysis of scaffold impact on HSc-related outcomes. Live/dead analysis of cells in PU and ccPU scaffolds indicated high cell viability (a). NHDF-seeded floating FPCLs rapidly contracted whereas PU scaffolds maintained their original size over 7 d ($p = 0.0001$ days 1, 3, 7) (b). Fixed FPCLs demonstrated significantly greater $\alpha$SMA incorporation into stress fibers when compared with PU and ccPU scaffolds ($p = 0.018$, $p = 0.005$) (c). Confocal microscopy was used to image FPCL (d), PU scaffolds (e), and ccPU scaffolds (f) on d 5 after seeding. Red stained stress fibers represent $\alpha$SMA, green staining represents actin filaments, and blue staining indicates cell nuclei. SEM photomicrographs demonstrate ECM production by NHDFs seeded PU scaffolds at d 7 (a), d 10 (b), and d 14 (c). Cell culture experiments were carried out in triplicate with a minimum of three distinct experiments. Data presented as mean ± SEM, significance determined via ANOVA followed by unpaired t-test with Welch’s correction; $p \leq 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
population [13]. Confocal imaging revealed that NHDF seeded in FPCLs (Fig. 3d) displayed larger area and more distinct actin stress fibers as compared to NHDF seeded in PU (Fig. 3e) and ccPU scaffolds (Fig. 3f). NHDFs attached to PU scaffolds and deposited ECM over the course of 14 days in vitro (Fig. 3g–i). In future studies it would be interesting to study whether the structure and production of ECM by cells cultured in PU and ccPU scaffolds differed from that produced in FPCLs. Confocal z-stack imaging demonstrated that NHDF infiltrated through the depth of electrospun scaffolds in vitro (Supplementary Fig. S1). Taken together, these data suggest that PU scaffolds mitigate the αSMA+ myofibroblast conversion and scaffold contraction associated with HSc as compared to an untreated wound model.

3.4. In vivo response and mechanical properties

HSc contraction in wounds treated with skin graft alone, Integra, PU scaffold, or ccPU scaffold was studied over 30 days in vivo (Fig. 4a). The temporal change in wound size was quantified, where 100% is the initial size of the wound and 0% represents a fully contracted wound (Fig. 4b, detailed statistical analysis can be found in Supplementary Table 1). Mice treated with skin graft alone demonstrated wound contraction of 45 ± 2.0% while Integra treated wounds contracted to 28 ± 1.5% at d 30. At d 21, PU scaffold-treated skin grafts retained 75 ± 3.8% of their original size, but partial scaffold extrusion and skin graft death were noted. At d 30, the PU treated wounds had contracted to 53 ± 8.0% of original size, large areas of scaffold had extruded, and partial-full skin graft necrosis was noted. Additional studies were carried out on PU with excision at d 7 and d 14 to explore the mechanism behind skin graft necrosis and scaffold extrusion at the later time points. ccPU scaffolds demonstrated improved tissue interaction. While several ccPU treated mice demonstrated small areas of scaffold extrusion at wound edges (Supplementary Fig. S2a), the majority of mice healed with healthy skin grafts. ccPU treated mice displayed significantly less contraction (70 ± 4.4%) than all other treatment groups at d 30.

The enhanced biocompatibility of ccPU scaffolds is likely a result of improved scaffold interactions with resident cell types [40]. Following PU scaffold extrusion, rapid wound contraction
occurred suggesting that PU scaffolds could have been acting as an internal splint [41]. If this is indeed the case, Integra could be providing a similar splinting effect to the wound prior to its degradation [42].

While uninjured skin is viscoelastic in nature, scar tissue is stiff and inelastic. There are five classical classifications of HSc in which scars are distinguished based on color, elevation, and stiffness. A reduction in HSc phenotype is accompanied by reduced scar stiffness: a grade 5 HSc has been shown to be up to seven times stiffer than normal skin tissue, while a grade 1 HSc is not significantly stiffer than normal skin [43]. Scaffolds/tissue explants were harvested on d 30 and subjected to static tensile testing until failure. d 30 ccPU explants possessed an elastic modulus (2.9 ± 0.80 kPa) significantly lower than ccPU scaffolds prior to implantation (8.3 ± 0.67 kPa), uninjured murine skin (36 ± 3.3 kPa), and d30 contracted murine scar explants (80 ± 17 kPa) (Fig. 4c, detailed statistical analysis can be found in Supplementary Table 1). Taken together, these data suggest the presence of a reduction in scar stiffening and improved healing phenotype in ccPU treated mice.

3.5. Histological analysis

In order to investigate the cause of extrusion and skin graft death in PU scaffold-treated mice, histology was conducted before (d 7, d 14) and after (d 30) skin graft death. Hematoxylin and eosin (H&E) staining (Supplementary Fig. S3, Fig. 5a and b) demonstrated that cellular infiltration into PU scaffolds increased between d 7, 14, and 30 in mice with necrosing grafts (qualitative observation). Multinucleated giant cells were present in PU scaffolds at both time points; presence of macrophage was confirmed via the pan macrophage stain, F4/80 (Fig. 5a, b and Supplementary Fig. S3). F4/80 quantification at d 14 and d 30 demonstrated a negligible difference in inflammatory reaction to PU scaffolds (25 ± 3.5/HPF at d 14, 5.7 ± 1.2/HPF at d 30) as compared to Integra (22 ± 5.4/HPF at d 14, 5.4 ± 0.20/HPF at d 30), and an overall significant drop in macrophage presence at d 30 as compared to d 14 (Fig. 5a–d). A similar trend was present upon quantification of CD31+ vessels (Fig. 5a–d): significantly more CD31+ vessels were present in PU (6.4 ± 0.54/HPF) and Integra treated wounds (12 ± 0.87/HPF) at d 14 than were found at d 30 in wounds treated with PU scaffolds (3.2 ± 0.20/HPF) and Integra (6.5 ± 0.90/HPF).

Gross observation of ccPU scaffold-treated wounds demonstrated improved bio-incorporation as compared to PU scaffolds (Fig 4a). Histological analysis confirmed that cellular infiltration was qualitatively higher in ccPU scaffolds than in uncoated PU scaffolds at d30 (Fig. 5b and e). All ccPU scaffolds demonstrated some degree of buckling beneath skin grafts (Supplementary Fig. S2b), in these areas an aggravated acute inflammatory response could be observed (Supplementary Fig. S2c). In the case of small areas of scaffold extrusion, clusters of αSMA positive cells could be discerned outside the scaffold perimeter directly surrounding extrusion points (Supplementary Fig. S2c). Within the scaffold and in areas free of extrusion, αSMA positive cells were not present (data not shown). In areas free of bunching or extrusion, the acute inflammatory response was decreased and marked by the presence of multinucleated giant cells and neutrophils present along the periphery of the scaffold (Fig. 5e), confirmed by F4/80 and H&E staining. The number of CD31+ vessels per HPF in ccPU scaffolds at d 30 (4.7 ± 0.20) was significantly higher than d 30 PU scaffolds (3.2 ± 0.20/HPF) and Integra (6.5 ± 0.90/HPF) (Fig. 5b, d and e).

4. Statistical analysis

Unless noted otherwise in figure captions, the following is true of all data analyzed. Data were tested for normal distribution using the Shapiro–Wilk normality test. In the case of normal distribution, data are presented as mean ± standard error of the mean. Two-way
ANOVA followed by unpaired t-test was carried out to detect statistical significance between groups, with significance considered as \( p < 0.05 \). Results without normal distribution (either due to sample number or result variability) are presented in box-and-whisker format where each data point represents the average of three technical replicates. Statistical significance was determined via Mann–Whitney test with significance considered as \( p < 0.05 \). Mechanical analysis was conducted on \( n \geq 5 \) samples; \( \text{in vitro} \) samples were analyzed in technical triplicates within at least 3 biological replicates; \( \text{in vivo} \) samples were analyzed with \( n \geq 5 \) mice per treatment group.

5. Discussion

In this study we demonstrate that randomly electrospun, elastomeric PU and ccPU scaffolds possess appropriate mechanical properties for placement beneath skin graft and decrease HSc-related outcomes \( \text{in vitro} \). Further, we show for the first time that a biostable, elastomeric electrospun microfibrillar scaffold decreases HSc contraction and scar stiffening in a clinically relevant murine model.

The majority of commercial BSEs developed for wound healing focus on promoting healing and preventing chronic wound states; however, in severely burned patients treated with skin grafts, the concern for HSc contraction is paramount. HSc contraction is an incremental process that gradually occurs over 6–18 months and leads to stiff, shrunken scars. Due to the extended timeline associated with development of HSc contraction, we hypothesized that a permanent BSE capable of persisting throughout the remodeling phase of repair would be best suited to combat disease progression. While traditionally employed natural polymers, such as collagen, are attractive for use as BSEs due to their inherent biocompatibility, their degradation rates are difficult to optimize in practice \[44\]. Synthetic polymers offer well-defined degradation rates and mechanical properties. The biostable PU used in this work possesses excellent resistance to degradation \( \text{in vivo} \), even when subjected to 500% strain \[25\]. These properties allow it to persist throughout the remodeling phase of repair while maintaining the elasticity necessary for placement across a patient’s joint \[24,25\].

Mechanical load can be transmitted to wounds on the macroscale via joint motion, or on the cellular level via the ECM. Application of mechanical load on both of these levels has been shown to instigate formation of an HSc-like phenotype \( \text{in vitro} \) and \( \text{in vivo} \) \[4,38\]. An intricate force transmission relationship, termed mechanotransduction, exists between the ECM, the cytoskeleton, and the cell nucleus \[45\]. Mechanical forces and topographical cues from the ECM are thus passed to cells and dictate their behavior \[46\]. In the ECM of unwounded skin, collagen fibers are arranged in a random “basketweave” orientation; however, in contracted scar tissue, collagen fibers align linearly along lines of tension \[14,15\]. Coinciding with the alignment of the ECM, myofibroblasts align, join stress fibers via adherens junctions, and multiply their contractile forces along the axis of cell alignment \[47\]. Myofibroblast alignment results in a feed-forward loop that promotes fibroblast-to-myofibroblast transition and scar contraction \[47\].

When the structure of the ECM is lost due to injury, fibroblasts experience a dramatically different mechanical environment, resulting in increased ECM synthesis and remodeling \[38,48,49\]. Beyond passively encouraging random cell alignment, we hypothesized that randomly-oriented electrospun fibers may provide “stress shielding” to resident fibroblasts similar to the protective environment provided by uninjured ECM. If randomly oriented PU scaffolds indeed provide stress shielding, it could lead to a decrease in the transmission of mechanical cues between cells that encourage myofibroblast differentiation \[38\]. Further studies are warranted to explore the mechanism behind how these scaffolds are impacting cellular responses \( \text{in vitro} \) and \( \text{in vivo} \).

To study the \( \text{in vivo} \) applicability of our scaffolds in mitigating HSc contraction, we utilized a murine model of HSc contraction which was recently developed in our laboratory \[31\]. While swine arguably represent the best model for human wound healing, with 78% agreement between human and porcine wound healing, swine are expensive to purchase and maintain \[50\]. However, our immune-competent murine model allows for introductory studies of HSc contraction in a system which replicates conditions and methods used for burn treatment in humans. This model provides the benefit of an expedited form of scar contraction, occurring over two to three weeks in the rodents before stabilizing, as compared to six months to two years in humans. By \( d \geq 30 \) in this murine model, skin grafts have stabilized and the primary contraction of the graft has completed, corresponding in the overall absence of \( \alpha \)-SMA positive myofibroblasts from the wound area (unpublished data). However, the absence of myofibroblasts does not dictate the cessation of HSc or graft contraction \[51\]. Recent studies have shown that \( \alpha \)-SMA-deficient myofibroblasts are capable of producing contractile forces through other contractile proteins, and that \( \alpha \)-SMA is not required for myofibroblast formation and function, or wound closure \( \text{in vivo} \) \[13,51\].

HSc is a complex disease where the proliferative and contractile components of HSc may be observed independent of one another or together in the same scar, and it should be noted that our animal model focuses on the contractile component of the disease. Observations from human HSc specimens suggest that a robust inflammatory response may underlie the excessive fibrosis characteristic of proliferative HSc \[52,53\]. Therefore, it is possible that the chronic inflammation typically associated with biostable implants could result in an increase in the proliferative component of HSc. In this case, pharmacological modulation inflammatory cytokines could be explored to improve wound healing and scar quality \[54\]. Electrospun PU scaffolds have been used as a platform for drug delivery in our laboratory the past, and delivery of anti-inflammatory compounds via ccPU scaffolds may be a future route for mitigation of both the contractile and proliferative components of HSc \[55,56\].

In this work, we designed a biostable ccPU scaffold to study the effect of random microfibrillar architecture on HSc contraction without introducing extraneous variables associated with scaffold degradation. Our data demonstrate a significant decrease in murine HSc contraction and stiffening following treatment with our permeant ccPU scaffold; however, biointegration with the ccPU material is sub-optimal. Histological analysis of infrequent areas of ccPU scaffold bunching or extrusion beneath skin grafts demonstrated punctate areas of inflammation and myofibroblast activation surrounding the scaffold area. These areas were not present in the biodegradable scaffold from our previous work; rather, there were areas where the scaffold had been dismantled to allow cellular remodeling of the tissue \[22\]. The data from this study aligns with those of previous investigators supporting the existence of an intricate balance between scaffold longevity and wound remodeling, where maintenance of scaffold architecture may be critical to prevent HSc related outcomes in vitro as well as \( \text{in vivo} \) \[22,57–59\].

A comparative study of random synthetic microfibrillar scaffolds with distinct degradation rates may be able to further explore this delicate balance and the mechanism by which scaffolds with microfibrillar architecture mitigate HSc contraction. As we continue to learn about the progression of HSc and work to develop treatments to mitigate its occurrence, it is wise to consider similar clinical indications from which we can draw upon retrospective studies of patient outcomes to aid in our design. Clinically, burn wounds experience a high degree of infection and bacterial burden \[60\]. Similar to patients experiencing bowel per-
forations along with hernia placement, these infections result in the creation of a “contaminated field” prior to scaffold or mesh implantation. Therefore, application of a permanent hernia mesh to a contaminated field can be considered as similar to the application of a permanent scaffold to an infected burn wound. Hernia wounds treated with slowly degrading meshes have shown significantly decreased rates of recurrence, pain, and infection as compared to those treated with permanent meshes [61]. Based on the similarity of the bio-burden present in contaminated hernia mesh placement and large area burns, clinical experience suggests a similar relationship may develop in the use of permanent biomaterials for treatment of burn wounds as is present in the treatment of hernias. Combining clinical experiences in hernia mesh use with the data we have gathered studying degradable and non-degradable scaffolds for treatment of murine HSc contraction, we advocate for the further development of slowly degrading scaffolds for prevention of HSc [22].

6. Conclusions

Electrospun scaffolds have previously been studied to combat scarring and HSc; however, to our knowledge none of the materials used have been biostable, synthetic elastomers [22,55,62,63]. A biostable scaffold, such as PU, provides the opportunity to examine cell-scaffold interactions without introduction of acidic degradation products, loss of mechanical properties, or loss of scaffold architecture over time. However, permanent dermal implants often encounter issues with infection or extrusion; therefore, the permanent nature of this material restricts its clinical translation. Considering this biostable PU as a model system, our results suggest the need to reach a balance between scaffold degradation and tissue regeneration when designing long-term implants as targeted BSEs for the prevention of HSc contraction.

Disclosures

The authors declare no competing financial interests.

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Appendix A. Collagen coating protocol


1. Wash the prepared surface with coupling buffer 1.
2. Defrost vials of EDC, NHS, and ethanolamine immediately before use. The fresh solutions can only be used for one activation round.
3. Immediately activate the sample surface with the EDC & NHS for 10 min. (Note: activation time can be used to regulate the amount of immobilized ligand.)
4. Wash quickly but thoroughly with coupling buffer 1.
5. Introduce collagen to the sample surface for 15 min in a concentration of 200 μg/mL in coupling buffer 1.
7. The remaining NHS–ester groups are blocked with a 1 M ethanolamine at slightly alkaline pH for 7 min.
8. Immediately after coupling, wash with a mild acidic regeneration fluid such as 50–100 mM HCl.
9. Wash 3 times in PBS.
10. Store in cell culture media in tissue culture incubator overnight prior to cell seeding.

Appendix B. Materials

Coupling buffer 1: maleic acid (Sigma) (pH 5.5) diluted in water at 10 mM, adjusted to pH 5.5, and passed through a 0.45 μm filter. Stored at 4 °C.

EDC (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride; Sigma) rapidly weighed under gas, dissolved in water at 0.4 M, and passed through 0.45 μm filter. Divide stock into small aliquots and store at −20 °C. Solid EDC is hygroscopic and labile and must be kept under dry gas. Thaw immediately before using.

NHS (N-hydroxysuccinimide, Sigma) is dissolved in water at 0.1 M, passed through 0.45 μm filter, and divided into small aliquots, and stored at −20 °C.

Ethanolamine hydrochloric acid (Sigma: E6133) is dissolved in water at 1 M and pH adjusted to 8.5 with HCl, passed through 0.45 μm filter, and subsequently divided into small aliquots and stored at −20 °C.

Appendix C. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2015.12.025.

References
