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Injectable Polyurethane Composite Scaffolds Delay Wound Contraction and Support Cellular Infiltration and Remodeling in Rat Excisional Wounds

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Abstract

Injectable scaffolds present compelling opportunities for wound repair and regeneration due to their ability to fill irregularly shaped defects and deliver biologics such as growth factors. In this study, we investigated the properties of injectable polyurethane biocomposite scaffolds and their application in cutaneous wound repair using a rat excisional model. The scaffolds have a minimal reaction exotherm and clinically relevant working and setting times. Moreover, the biocomposites have mechanical and thermal properties consistent with rubbery elastomers. In the rat excisional wound model, injection of settable biocomposite scaffolds stented the wounds at early time points, resulting in a regenerative rather than a scarring phenotype at later time points. Measurements of wound width and thickness revealed that the treated wounds were less contracted at day 7 compared to blank wounds. Analysis of cell proliferation and apoptosis showed that the scaffolds were biocompatible and supported tissue ingrowth. Myofibroblast formation and collagen fiber organization provided evidence that the scaffolds have a positive effect on extracellular matrix remodeling by disrupting the formation of an aligned matrix under elevated tension. In summary, we have developed an injectable biodegradable polyurethane biocomposite scaffold that enhances cutaneous wound healing in a rat model.

Keywords

polyurethane; injectable scaffolds; wound healing; regeneration; tissue engineering

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Introduction

The increases in immobile aging, diabetic amputee, and paralyzed patients afflicted with large, chronic wounds and fistulas as well as trauma victims with large cutaneous defects create a need for development of injectable biomaterials to promote restoration of tissue integrity. Such scaffolds could offer new options for both cutaneous and fascial indications while adding options for site-specific customization.¹ Furthermore, a biomaterial that is applied as a liquid and cures *in situ* can flow to fill the contours of irregularly shaped defects that may not conform to a preformed implant. Maximizing the contact surface area between the material and surrounding tissue should enhance cellular infiltration and integration of the scaffold.

Several requirements are critical to the success of injectable biomaterials, such as flowability for a sufficient time (the working time) to enable injection and curing within minutes of injection (the setting time) thus avoiding long surgical procedures. The injected material should not have adverse effects on surrounding host tissue due to the reactivity of specific components or to the release of heat through a reaction exotherm.² The viscosity of the injected material should be high enough to be retained at the injection site and to minimize extravasation into surrounding tissues where it may have an adverse effect.³ The reproducibility of properties such as porosity, degradation, and setting time in the clinical environment is also a significant challenge. Injectable porous biomaterials must have a suitable pore structure for cell migration, nutrient exchange, and tissue ingrowth.⁴

Injectable hydrogels, such as poly(ethylene glycol) (PEG), collagen, fibrin, chitosan, alginate, and hyaluronan, have been shown to support bone ingrowth *in vivo*, particularly when combined with angiogenic or osteogenic growth factors.⁵⁻¹¹ However, hydrogels lack the tough, elastomeric properties of thermoplastic polymers that are appropriate for cutaneous applications. Furthermore, the microstructure of synthetic hydrogels is typically smaller than the average size of cellular populations (5-15 μm)¹², thus requiring resorption or displacement of the matrix by cells that results in slow infiltration of the scaffold. We have recently developed injectable, allograft bone/polyurethane (PUR) composite scaffolds for bone regeneration with tunable working and setting times of 5 and 15 minutes.¹³ Materials were prepared by mixing allograft bone particles, a flowable lysine triisocyanate (LTI)-PEG prepolymer, a flowable polyester triol, and triethylene diamine catalyst. An LTI-PEG prepolymer was used in the scaffold synthesis rather than monomeric LTI because it has been shown previously that injection of LTI *in vivo* could be toxic.¹³ The porosity of the cured scaffolds varied from 30 – 70%, and the pore size ranged from 177 – 700 μm . When injected into 3-mm femoral condylar plug defects in rats, the composites exhibited cellular infiltration and new bone formation at 3 weeks. While these composites are not suitable for cutaneous wound healing due to the allograft bone component, our previous studies have shown that pre-formed PUR scaffolds implanted in both subcutaneous¹⁴ and excisional¹⁵ wounds in Sprague-Dawley rats supported cellular infiltration and ingrowth of new tissue.

In the present study, we have synthesized injectable PUR composite scaffolds incorporating polysaccharide particles and evaluated their performance *in vitro* and *in vivo*. We hypothesized that the degradable scaffolds would function as an initial temporary matrix that

both provides a surface for attachment and proliferation of cells and also stents the wound to minimize the undesirable outcomes of contraction and scarring. Either hyaluronic acid (HA), a 1,500 – 2,200-kDa glycosaminoglycan found in the extracellular matrix, or carboxymethylcellulose (CMC), a plant-derived 90-kDa polysaccharide, was added to the reactive PUR to control the foaming reaction through absorption of excess moisture from the wound bed. Rheological and physical properties of the scaffolds were measured *in vitro*, and their potential to support cellular infiltration and new tissue ingrowth were evaluated in excisional wounds in Sprague-Dawley rats.

Materials and Methods

Materials

Glycolide and D,L-lactide were purchased from Polysciences (Warrington, PA). TEGOAMIN33, a tertiary amine catalyst composed of 33 wt% triethylene diamine (TEDA) in dipropylene glycol, was obtained from Goldschmidt (Hopewell, VA). Polyethylene glycol (PEG, 200 Da) was supplied by Alfa Aesar (Ward Hill, MA). Glycerol and the sodium salts of carboxymethyl cellulose (CMC; 90-kDa) and hyaluronic acid (HA; 1,500 – 2,200-kDa) were purchased from Acros Organics (Morris Plains, NJ). Lysine triisocyanate (LTI) was obtained from Kyowa Hakko USA (New York), and stannous octoate catalyst was obtained from Nusil technology (Overland Park, KS). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO). Prior to use, glycerol and PEG were dried at 10 mm Hg for 3 h at 80°C, and ϵ -caprolactone was dried over anhydrous magnesium sulfate. All other materials were used as received.

Synthesis of PUR scaffolds and reactive intermediates

PEG (200 Da) was reacted with an excess of LTI (NCO:OH equivalent ratio = 3:1) to form an LTI-PEG prepolymer (21,000 cP) in which the PEG molecules were end-capped with LTI.¹³ PEG with molecular weight > 200 Da did not yield a flowable prepolymer, and thus could not be injected. PEG was added dropwise to LTI in a 100 mL reaction flask with stirring under argon for 24 h at 45°C. The prepolymer was then dried under vacuum at 80°C for 14 h. A polyester triol (900 Da) with a backbone comprising 60% caprolactone, 30% glycolide, and 10% lactide was synthesized by reacting the monomers (ϵ -caprolactone, glycolide, and D,L-lactide) with a glycerol starter in the presence of stannous octoate catalyst.¹⁶ This polyester triol composition and molecular weight were chosen to maintain both good flowability of the reactive mixture as well as a favorable degradation rate of the cured PUR scaffold *in vivo*.¹⁷ The reaction was carried out under dry argon at 140°C for 48 h, and the resulting polyester triol was dried under vacuum at 80°C for 24 h.

PUR scaffolds were synthesized by reactive liquid molding of the LTI-PEG prepolymer with a hardener component^{13,14} and a polysaccharide filler (carboxymethyl cellulose [CMC] or hyaluronic acid [HA]). The hardener comprised 100 parts polyester triol (polyol), 1.5 parts per hundred parts polyol (pphp) water, 0.625 pphp TEGOAMIN33 catalyst, 0.375 pphp 30% bis(2-dimethylaminoethyl)ether (DMAEE) blowing catalyst in poly(propylene glycol), and 4.0 pphp calcium stearate pore opener. The polysaccharide was combined with the hardener and mixed by hand for 30 s. The prepolymer was added to the hardener and

polysaccharide and mixed by hand for 1 min. The resulting mixture then rose freely for 10 – 20 min and cured. The targeted index (the ratio of NCO to OH equivalents times 100) was 115.

Scaffold characterization

Core densities and porosities were determined from mass and volume measurements of triplicate cylindrical foam cores.^{14,18} The scaffold pore size distribution was assessed by scanning electron microscopy (Hitachi S-4200 SEM, Finchampstead, UK) after gold sputter coating with a Cressington Sputter Coater. Temperature profiles of the reactive mixtures during foaming were measured using a digital thermocouple at the centers of the rising foams. Scaffold degradation was evaluated by incubating triplicate 20 mg samples in 1 ml phosphate buffered saline (PBS) (pH 7.4) at 37°C for up to 24 weeks. At various time points, the samples were rinsed in deionized water, dried under vacuum for 48 h at room temperature, and weighed.

Reactivity of scaffold components

The reactivities of the LTI-PEG prepolymer with the polyester triol, HA, and CMC were measured using attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR; Bruker Tensor 27 FTIR, Billerica, MA). Prepolymer; TEGOAMIN33 and DMAEE catalysts; and either polyol, HA, or CMC were mixed together for 1 min and then placed in contact with the ATR crystal. The area of the isocyanate peak (wavelength 2150 – 2350 cm) was monitored as a function of time.

Mechanical properties

Mechanical properties of wet scaffolds were measured using a TA Instruments Q800 Dynamic Mechanical Analyzer (DMA) in compression mode (New Castle, DE). Samples were tested either shortly after fabrication or after 7 days of incubation in PBS prior to mechanical testing. Stress-strain curves were generated by compressing wet cylindrical 7 × 6 mm samples at 37°C at a rate of 0.1 N/min until they reached 50% strain. The Young's modulus was determined from the slope of the initial linear region of each stress-strain curve. The scaffolds could not be compressed to failure due to their elasticity, so the compressive stress was measured one minute after the application of 50% strain.¹⁴

Rheological properties during cure

The cure profiles of the HA and CMC scaffolds were measured using a TA Instruments parallel plate AR 2000ex rheometer operating in dynamic mode with 25 mm disposable aluminum plates (New Castle, DE). LTI-PEG prepolymer was added to a mixture of hardener and polysaccharide (0, 15, or 30 wt%) and mixed by hand using a spatula for 1 min. The sample was then loaded onto the bottom plate of the rheometer. An oscillation time sweep was run with a controlled strain of 1% and a frequency of 6.28 rad/s in order to obtain the cure profile of each PUR scaffold. The storage modulus (G') and loss modulus (G'') were determined as a function of time. The working time was determined to be the G' -crossover point. To measure the setting time, the surface of the foam was contacted with a

spatula at regular intervals of 30 sec. The tack-free time, which approximates the setting time, was determined to be the time at which the foam did not stick to the spatula.

In vivo cutaneous repair in rats

All surgical procedures were reviewed and approved by the local Institutional Animal Care and Use Committee. NIH guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) have been observed. The capacity of the scaffolds to facilitate dermal wound healing was evaluated in an excisional wound model (6.25cm² square wounds) in adult male Sprague-Dawley rats. All materials were sterilized by gamma irradiation at 5 kGy prior to surgery. The treatment groups investigated were untreated wounds (negative control) and PUR scaffolds with 15 wt% HA or CMC polysaccharide. We have previously observed that PUR scaffolds without polysaccharide fillers over-expanded and formed large voids *in vivo*, so this treatment group was not investigated in the rat study. Preliminary experiments showed that 15% polysaccharide was sufficient to control foaming, and no additional benefits were observed at 30%. To investigate the effects of the bioactivity of the polysaccharide on healing, both HA (which was anticipated to possibly have biological effects and enhance healing) and CMC (which was considered a relatively inert material) were tested. For the HA and CMC treatment groups, the materials were applied as a reactive liquid immediately after mixing the LTI-PEG prepolymer with the hardener and polysaccharide. The polyurethane expanded by gas foaming to fill the defects and cured *in situ*. When the scaffolds expanded beyond the wound dimensions, they were trimmed to be flush with the skin surface. Each wound and scaffold was covered with nonadherent, absorbent, Release gauze (Johnson & Johnson) and covered with a Tegaderm outer dressing (3M, St. Paul, MN). Wounds were harvested at days 7, 17, 26, and 35 after surgery. Four replicates of each treatment group were harvested at each time point. The wounds were fixed in neutral buffered formalin for 24 h, transferred into 70% ethanol for 48 h, embedded in paraffin, and sectioned at 5µm. Hematoxylin & eosin (H&E), Gomori's trichrome, picrosirius red, TUNEL, myeloperoxidase, Ki67, α-SMA, and procollagen I immunostaining were performed on the tissue sections.

Statistical analysis

Single factor analysis of variance (ANOVA) was used to evaluate the statistical significance of results. P values less than 0.05 were considered statistically significant.

Results

Setting properties of PUR foams

The specific reaction rates for the second order reactions between LTI-PEG prepolymer and water, polyol, HA, and CMC were determined using ATR-FTIR (Figure 1B). Water was the most reactive, with a rate constant of 600 g mol⁻¹ min⁻¹ (data not shown). The rate constant measured for the polyester triol (9.14 g mol⁻¹ min⁻¹) was 21 times larger than that measured for CMC (0.438 g mol⁻¹ min⁻¹) and 7 times larger than that measured for HA (1.29 g mol⁻¹ min⁻¹). These data show that the water and polyester triol components are the most reactive in the system and considerably more reactive than the polysaccharides. The higher reactivity of HA compared to CMC is consistent with the structures shown in Figure 1A. Each repeat

unit of HA has one primary OH group, while CMC has only carboxylic acids and secondary OH groups, which are at least 2 – 4 times less reactive than primary OH groups.¹⁹

The rheological properties of PUR, PUR + CMC, and PUR + HA scaffolds are shown in Figure 2A-C. The G-crossover point was considered to be the gel point and thus the working time of the foam. The working time was 5.8 ± 0.7 min for the PUR foam, 6.2 ± 0.5 min for the PUR + CMC foam, and 5.5 ± 0.6 min for the PUR + HA foam. Although the working time can be adjusted by altering the concentrations of the catalysts, it was maintained constant in the present study. The tack-free time was 16 ± 3 min for the PUR foam, 19 ± 3 min for the PUR + CMC foam, and 15 ± 4 min for the PUR + HA foam. The addition of the polysaccharide had no significant effect on either the working or tack-free time.

The temperature profiles of PUR, PUR + CMC, and PUR + HA foams are shown in Figure 2D. The temperature within the reactive mixture was recorded with a digital thermocouple at the center of the rising foams, which were insulated to minimize the effects of heat loss from the exterior surface. Starting at room temperature, the maximum increase in temperature was 7.3 ± 1.7 °C for the PUR foam, 7.1 ± 1.4 °C for the PUR + CMC foam, and 6.7 ± 1.1 °C for the PUR + HA foam. The addition of the polysaccharide did not significantly affect the temperature profile.

Physical properties of PUR scaffolds

Physical properties of the PUR scaffolds before and after incubating in an aqueous environment for 7 days are shown in Table 1. On day 0, the properties of PUR + HA and PUR + CMC scaffolds were not significantly different from each other, but both had significantly higher densities (45%), lower porosities (4%), and smaller pore sizes (13%) than the blank PUR scaffolds. However, by day 7 there were no significant differences in porosity, pore size, or density between the three groups, presumably due to dissolution of the polysaccharides.

Degradation of PUR scaffolds

A representative SEM image of a PUR + HA scaffold is shown in Figure 3A.1. The interconnected pores of the scaffolds permit cellular infiltration.¹⁴ Panels A.2 and A.3 show images of 100 – 200 μm particles embedded in a PUR + HA scaffold at low and high magnification, respectively. As shown in Panel A.4, the particles were almost completely dissolved after 24 h *in vitro* incubation time in buffer, resulting in the formation of additional pores. Alcian blue staining was used to confirm the presence of HA particles embedded in the scaffolds. PUR (negative control) and PUR + HA scaffolds were stained with Alcian blue at pH 2.5 and pH 1.0. At pH 1.0, Alcian blue only stains highly sulfated glycosaminoglycans, while at pH 2.5 the dye stains HA blue. PUR scaffolds did not stain at either pH and PUR + HA scaffolds did not stain at pH 1.0, but PUR + HA scaffolds stained blue at pH 2.5, thereby confirming the presence of HA in the scaffolds. Taken together, these data suggest that dissolution of HA (or CMC) embedded in the PUR scaffolds can create additional pores *in vivo*. To investigate the effects of polysaccharide loading on scaffold degradation, the degradation rates of the PUR and PUR + CMC (15 and 30%) scaffolds in PBS at 37°C were recorded for up to 24 weeks (Figure 3B). Under *in vitro*

conditions, the primary mechanism of degradation was hydrolysis of the ester bonds within the polyester soft segment.¹⁷ The polysaccharides caused a high initial mass loss within the first few days, which is consistent with the SEM data in Figure 3.A.4. After this time period, the rates of polymer degradation were similar.

Mechanical properties of PUR scaffolds

The compressive Young's modulus and compressive stress of the scaffolds under physiological conditions (wet at 37°C) before and after incubation for 7 days are summarized in Table 2. When compressed for extended periods of time, the scaffolds exhibited less than 5% permanent deformation, which is consistent with the properties of rubbery elastomers. Furthermore, the materials did not fail under compression, so compressive stress-strain tests were carried out to 50% strain, where the compressive stress was measured as reported previously.²⁰ The initial modulus and strength of scaffolds containing filler were higher (but not significantly) than those of blank scaffolds. After incubating in PBS for 7 days, the modulus and strength of all three scaffolds decreased, but only the changes in the modulus of the polysaccharide-filled scaffolds were significant ($p < 0.005$ for PUR + HA and $p < 0.02$ for PUR + CMC).

Measurements of excisional wounds

Injectable PUR scaffolds with 15% CMC or HA were tested for their effects upon dermal wound healing in a rat excisional wound model. No frank necrosis of the surrounding tissue was seen at the early time points, suggesting that the mild exotherm resulting from the PUR reaction did not adversely affect the host tissue. In addition, the level of apoptosis in the scaffold-treated groups was not greater than in the blank wounds at any of the time points (Figure 4B). The average length of the wound gap, granulation tissue thickness, and percent re-epithelialization of the wounds in the three treatment groups at each time point are summarized in Figure 4. At days 7 and 17, the thickness of the wounds in the HA and CMC treatment groups was less than the thickness of the blank wounds; however, only the thickness of the wounds in the HA group at day 17 was significantly less than the blank ($p < 0.015$). At day 7, the length of the blank wounds was significantly less than those of the HA and CMC groups ($p < 0.045$, $p < 0.015$, respectively), providing evidence that the PUR scaffolds stented the wound. Blank (contracted) wounds were fully epithelialized by day 26, while HA and CMC treatment groups (stented) were not fully epithelialized by day 35.

Analysis of proliferating and apoptotic cells in an excisional wound model

Ki67 staining was performed to assess the level of cell proliferation within the wound bed (Figure 5A). After 7 days, we found no difference in the number of Ki67⁺ cells in the blank wounds compared to the scaffold treatment groups. From day 7 to day 17, the number of proliferating cells remained constant in the CMC and HA treatment groups but decreased by 67% in the blank treatment group. Thus at day 17, the number of Ki67⁺ cells was significantly higher in the scaffold treatment groups than in the blanks. The number of Ki67⁺ cells decreased slightly from day 17 to day 26, but the level of proliferation in the scaffold treatment groups remained significantly higher than in the blank wounds. From day 26 to day 35, the number of Ki67⁺ cells decreased by 40% in the scaffold treatment groups

and remained constant in the blank treatment group. At day 35, the number of Ki67⁺ cells in the scaffold treatment groups was comparable to that observed for the blank wounds.

TUNEL staining was used to measure cell apoptosis in the wound site (Figure 5B). At day 7, the number of cells stained with TUNEL was higher in the blank wounds than in the wounds with scaffolds, but the difference was not statistically significant. From day 7 to day 17, the number of cells stained with TUNEL decreased by 40% in the blank wounds and remained relatively constant in the scaffold treatment groups. The level of apoptosis did not change in any of the treatment groups from day 17 to day 26. There were no significant differences in the number of cells stained with TUNEL among the three treatment groups at any of the time points.

Analysis of contraction in an excisional wound model

Staining for α -smooth muscle actin (α -SMA) was performed in order to examine the formation of myofibroblasts in the wound site. Representative images of sections stained for α -SMA are displayed in Figure 6. In the blank wounds, the number of myofibroblasts was greatest at days 17 and 26 and decreased almost completely by day 35. In contrast, fewer myofibroblasts were present at days 17 and 26 in the HA and CMC treatment groups. Myofibroblast formation in these groups was delayed and remained higher at the day 35 interval than in the blank group. Myofibroblasts were oriented parallel to the epidermis in the blank wounds, forming lines of tension in the skin as is characteristic of wounds undergoing scarring and contraction. In contrast, myofibroblasts were randomly oriented around pieces of PUR in the HA and CMC groups. These results show that myofibroblast formation was delayed in the HA and CMC groups and that fragments of PUR scaffolds disrupted the linear alignment of myofibroblasts.

During the nascent phases of cutaneous wound repair, the provisional loose connective tissue matrix develops a very robust capillary network, which causes the healing wound to appear red due to the fragile capillaries that bleed easily. If healing progresses through its expected phases, the number of new capillaries peaks and subsequently begins to decline. By days 26 and 35 in the life of the wound, the capillary density is regressing, which is consistent with the histological sections in Figure 6. The remodeling phase is underway and is converting the newly formed tissue within the wound bed into a dense irregular connective tissue that is characterized by a higher density of matrix proteins (predominantly collagens) and a lower number of capillaries. Taken together, the histological sections in Figure 6 are consistent with a maturing wound that is progressing past the granulation tissue stage that is typical of chronically impaired wound healing.

Analysis of collagen production in the excisional wound model

Picrosirius red staining and procollagen I immunostaining were performed in order to analyze the temporal and spatial production, accumulation, and organization of collagen in the rat excisional wounds. Representative images of sections stained with picrosirius red are shown in Figure 7. Picrosirius red staining supports the observation that collagen fiber formation in the HA and CMC treatment groups was more randomly oriented than in the blank wounds. At days 17, 26, and 35, collagen fibers in blank wounds were organized and

aligned parallel to the epidermis. In contrast, collagen fibers surrounding polymer remnants in the HA and CMC scaffolds were randomly oriented. Representative images of sections stained for procollagen I are displayed in Figure 8A, and the number of procollagen I-producing cells is quantified in Figure 8B. At day 17, there were significantly more procollagen I-producing cells in the HA group than in the blanks ($p < 0.02$). At day 26, there were significantly fewer procollagen I-producing cells in the HA group than in the blanks ($p < 0.02$). At day 35, there were significantly fewer procollagen I-producing cells in the CMC group than in the blanks ($p < 0.045$).

The presence of the scaffold had a modifying impact on collagen I production and deposition. Blank wounds developed a linear pattern of contraction and scarring and were highly cellular. By comparison, scaffold-treated wounds at day 35 revealed reduced cellularity and fewer collagen I secreting cells. Furthermore, the orientation of the cells and collagen fibers was more random in the presence of scaffolds. These data support the hypothesis that the scaffold hinders or alters the expected scarring and contraction pattern observed in blank wounds.

Discussion

In this study, we have shown that the physical, mechanical, and rheological properties of polyurethane composites render them suitable for use as injectable scaffolds in the setting of cutaneous wound repair. The materials exhibited working and cure times of 5 – 7 and 15 – 19 min, respectively, which are compatible with the temporal practicalities imposed by the clinical setting. Due to their compressive properties, which approach those of intact skin, the scaffolds stented the wounds at early time points and promoted granulation tissue formation while preventing wound contraction. As a result of wound contraction, unstented wounds resurfaced more rapidly. Collagen synthesis and organization, as well as myofibroblast formation, were altered by the presence of the scaffolds with a net positive impact.

Injectable scaffolds are advantageous because they can fill large, irregularly shaped wounds and cavities. Moreover, scaffolds have the added potential to serve as delivery vehicles for additives such as antibacterial and growth factors. Nevertheless, several challenges associated with injectable biomaterials have been described in recent reviews.^{21,22} The reactants and catalyst must not be cytotoxic, and the reaction exotherm must be minimal to avoid necrosis of surrounding tissues. Achieving interconnected pores while retaining robust mechanical properties presents an additional challenge. In a recent study, injectable poly(propylene fumarate) (PPF) scaffolds with pores ranging from 50 - 500 μm , <61% porosity, and elastic modulus of 20 – 40 MPa were fabricated via a gas-foaming process using an NVP crosslinker and benzoyl peroxide initiator.²³ However, the effects of the solvents and initiator on *in vivo* biocompatibility remain untested to date.

Natural and synthetic polymers including collagen, chitosan, fibrin, and poly(lactic-co-glycolic acid) are currently used in cutaneous wound healing in the form of hydrogels, sheets, sponges, and electrospun scaffolds.²⁴ These polymers are advantageous due to their biocompatibility and biodegradability, but they present potential drawbacks such as low modulus and strength, small pore size, and low porosity.¹² Low mechanical properties result

in undesirable outcomes such as contraction and scarring, and small pores and low porosity lead to slow infiltration and delayed vascularization.²⁴ Scaffolds with >90% porosity are desirable because they can easily support infiltration of new tissue and transport of nutrients and waste.²⁵ A previous study has reported optimal pore sizes for fibroblast infiltration and new tissue ingrowth ranging from 90 – 360 μm ²⁶, while another study has shown that the viability of seeded fibroblasts was highest for pore size <160 μm .^{27,28} Another study resulted in low viability of fibroblasts in scaffolds with pores ranging from 50 – 80 μm compared to scaffolds with larger pores.²⁵

Nanofibrous scaffolds have potential for use in cutaneous wound healing because they mimic the structure and function of natural ECM.²⁴ Despite their small pores, their high surface area to volume ratio results in excellent permeability for oxygen and nutrients.²⁴ Delivery of recombinant human platelet-derived growth factor (rhPDGF) from nanofibrous PLGA scaffolds has been reported to enhance wound healing in rats²⁹, and another study has examined the use of bioactive poly-N-acetyl-glucosamine nanofibrous membranes in cutaneous wounds in diabetic mice.³⁰ The nanofibers enhanced keratinocyte migration, cell proliferation, and angiogenesis compared to a cellulose control.³⁰ However, pre-formed implants such as nanofibrous scaffolds cannot be injected, and thus cannot fill and conform to deep tissue defects.

We have previously reported an injectable polyurethane scaffold for use in bone tissue engineering applications, wherein a matrix comprising allograft bone particles and a two-component reactive polyurethane supported cellular infiltration, extracellular matrix synthesis, and new bone formation in femoral plug defects in rats.¹³ In this study, we have applied the findings from our previous work on injectable allograft bone biocomposites to create injectable scaffolds for use in cutaneous wound repair and regeneration. With a working time of <7 min and a setting time of <19 min, the scaffolds can be mixed and injected in a clinically relevant period of time. The pore size ranged from 320 – 370 μm , which is consistent with values reported previously for polyurethane scaffolds^{13,14,31}, and are comparable to those known to facilitate infiltration of cells such as fibroblasts (90 – 360 μm ²⁶) and osteoblasts.³² While blank, HA-, and CMC-filled scaffolds showed no significant differences in pore size, the polysaccharide-filled scaffolds exhibited higher density and modulus and lower porosity than the blank scaffolds. However, by day 7 the density, modulus, and porosity of the polysaccharide-filled scaffolds were not significantly different than those properties measured for the blank scaffolds. These data are consistent with the SEM images in Figure 3A and the degradation data in Figure 3B suggesting that the polysaccharide had leached from the scaffolds by day 7, resulting in lower modulus and density. They are also in agreement with a previous study showing a reduction in the modulus of PUR scaffolds incorporating 7 – 8% tobramycin after 7 days due to leaching of the tobramycin from the scaffolds.³³

An abundance of fibroblasts was observed in our histological sections. Proliferative assays were performed to demonstrate that the scaffolds supported cellular attachment and proliferation, which provides compelling evidence that the scaffold was non-toxic and biocompatible as it degraded and was replaced by new matrix. In the *in vivo* experiments, there were no significant differences in the level of apoptosis among the three treatment

groups, suggesting that the polyurethane scaffolds and their degradation products are noncytotoxic and do not harm the surrounding tissue, consistent with previous studies.^{14,34-36} In addition to its role as an initial temporary matrix that provides a surface for cell attachment, the scaffolds also stented the wounds by providing resistance to the contractile forces exerted by the cells within and surrounding the implant. The Young's modulus of the scaffolds measured under compressive deformation approaches that of human skin, which has been reported as 35 kPa for the dermis³⁷, and rat skin, which we measured to be 400 ± 150 kPa. The injectable polyurethane networks are rubbery elastomers at physiological temperatures with glass transition temperatures (T_g) less than 10°C, and they sustain compressive strains exceeding 50% without mechanical failure.¹⁴ The wound healing (Figure 4), cell proliferation (Figure 5), and matrix deposition (Figure 7) data are consistent with the notion that the scaffold delays contraction and scarring due to the fact that its initial mechanical properties are approaching those of native skin. Cutaneous wound repair goes through predictable stages, characterized by an initial acute inflammatory phase that leads to ingrowth of granulation tissue followed by a progressive transition to sustained matrix production and remodeling. Rapid wound closure often leads to excessive matrix production and the very undesirable outcomes of scarring and wound contraction, which were not observed with these scaffolds. Matrix production was visibly dampened and the alignment of collagen fibers was more random compared to control wounds. Thus, by resisting the contractile forces that are generated in the host tissue, the scaffolds promote cellular infiltration and remodeling rather than excessive matrix deposition and scarring.

Excisional wounds treated with PUR scaffolds were significantly less contracted than blank wounds at day 7 as demonstrated by measures of wound gap (Figure 4C). While the cross-sectional area of granulation tissue was similar among all groups, unstented wounds tended to form a thickened eschar, although the differences were not significant (Figure 4B). These indicators suggest that the scaffolds stented the wounds at early time points, thus leading to a restorative rather than a scarring/contracting phenotype at later time points. Although wounds treated with injectable scaffolds showed prolongation of the proliferative phase, the effects on myofibroblast accumulation and orientation are potentially advantageous features. Myofibroblasts normally generate unwanted contractile forces that promote wound contraction and fibrosis. The architectural disruption of myofibroblast alignment led to a more reticular arrangement of collagen fibers. Although the upper surface of the scaffolds was approximately flush with the surface of the skin, epidermal resurfacing of the wounds was delayed. While this response was likely due in part to the greater length of the stented wounds, either the wound covering or the properties of the upper surface of the scaffold may benefit from further modification.

At later time points in the study, the marked difference in the alignment of collagen fibers and cells within the composite scaffolds suggests that the transient presence of the scaffold disrupted the formation of a uniformly aligned extracellular matrix under elevated tension. Ideally the scaffold should degrade at a rate comparable to that of new tissue ingrowth. While Figure 3 shows ~20% mass loss of the polyurethane after 24 weeks *in vitro*, we have recently shown that lysine-derived polyurethane scaffolds undergo oxidative degradation to soluble break-down products mediated by macrophages *in vivo*.¹⁷ As a result, the scaffold was almost completely resorbed after 4 weeks post-implantation in rat excisional wounds.¹⁷

Biostable polyurethane foams have been developed as coverings to minimize fibrous encapsulation of breast implants.^{38,39} However, the polyurethane foams slowly degraded *in vivo* into small pieces after periods longer than 18 months post-implantation, thereby inducing fibrous encapsulation of the implant and an intense foreign-body response to the foam fragments. The delayed appearance of myofibroblasts in the injectable scaffolds was also consistent with an altered mechanical environment, particularly in light of the evidence that cell-generated tension in the context of relatively stiff extracellular matrix can lead to the activation of latent TGF- β , which promotes matrix accumulation and differentiation of the myofibroblast phenotype.⁴⁰

Although not tested in the present investigation, the injectable polyurethane scaffolds also have the potential to accelerate wound healing through the local delivery of biologics such as recombinant human platelet-derived growth factor (rhPDGF)¹⁵ or antibiotics.^{33,41} Delivery of rhPDGF-BB from polyurethane scaffolds implanted in excisional wounds in rats accelerated both ingrowth of new tissue as well as degradation of the scaffolds.¹⁵ In another study, delivery of vancomycin from polyurethane scaffolds implanted in a contaminated femoral segmental defect in rats decreased bacterial counts in both bone and soft tissue.⁴¹ Biologics can be added to the polyester triol component prior to mixing with the prepolymer, thereby facilitating clinical ease of use and customization at the point of care.

Conclusions

In this study, an injectable, biodegradable polyurethane scaffold supported cellular infiltration and ingrowth of new tissue in a rat excisional wound model. The two-component polyurethanes exhibited working times and setting times ranging from 5 – 7 and 15 – 19 minutes, respectively. A micron-sized polysaccharide powder, either hyaluronan or carboxymethylcellulose, added to the liquid polyurethane controlled excessive expansion after injection. The cured scaffolds delayed wound contraction at early time points, with the favorable outcomes of enhanced cellular proliferation and reduced alignment of scar collagen. The biocompatibility, ease of use, clinically relevant working and setting times, support of cellular infiltration, positive impact on matrix remodeling, and potential to deliver biologics at the point of care may present compelling opportunities for injectable polyurethanes as void fillers for healing of cutaneous tissue defects.

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References

1. Praemer, A.; Furner, S.; Rice, D. Musculoskeletal conditions in the United States. American Academy of Orthopaedic Surgeons; Park Ridge, IL: 1992. p. 85-124.
2. Deramond H, Wright NT, Belkoff SM. Temperature elevation caused by bone cement polymerization during vertebroplasty. Bone. 1999; 25(2 Suppl):17S–21S. [PubMed: 10458268]

3. Padovani B, Kasriel O, Brunner P, Peretti-Viton P. Pulmonary embolism caused by acrylic cement: a rare complication of percutaneous vertebroplasty. *AJNR Am J Neuroradiol*. 1999; 20(3):375–7. [PubMed: 10219399]
4. Khan Y, Yaszemski MJ, Mikos AG, Laurencin CT. Tissue engineering of bone: Material and matrix considerations. *Journal of Bone & Joint Surgery: American Edition*. 2008; 90(Supplement 1):36–42.
5. Augst AD, Kong H-J, Mooney DJ. Alginate Hydrogels as Biomaterials. *Macromolecular Bioscience*. 2006; 6(8):623–633. [PubMed: 16881042]
6. Chen RR, Mooney DJ. Polymeric growth factor delivery strategies for tissue engineering. *Pharmaceutical Research*. 2003; 20(8):1103–1112. [PubMed: 12948005]
7. Kipshidze N, Chawla P, Keelan MH. Fibrin Meshwork as a Carrier for Delivery of Angiogenic Growth Factors in Patients With Ischemic Limb. *Mayo Clinic Proceedings*. 1999; 74(8):847–848. [PubMed: 10473371]
8. Labhasetwar V, Bonadio J, Goldstein S, Chen W, Levy RJ. A DNA controlled-release coating for gene transfer: Transfection in skeletal and cardiac muscle. *Journal of Pharmaceutical Sciences*. 1998; 87(11):1347–1350. [PubMed: 9811488]
9. Mizuno K, Yamamura K, Yano K, Osada T, Saeki S, Takimoto N, Sakurai T, Nimura Y. Effect of chitosan film containing basic fibroblast growth factor on wound healing in genetically diabetic mice. *Journal of Biomedical Materials Research Part A*. 2003; 64A(1):177–181. [PubMed: 12483711]
10. Simmons CA, Alsberg E, Hsiong S, Kim WJ, Mooney DJ. Dual growth factor delivery and controlled scaffold degradation enhance in vivo bone formation by transplanted bone marrow stromal cells. *Bone*. 2004; 35(2):562–569. [PubMed: 15268909]
11. Zawko SA, Truong Q, Schmidt CE. Drug-binding hydrogels of hyaluronic acid functionalized with β -cyclodextrin. *Journal of Biomedical Materials Research Part A*. 2008; 87(4):1044–1052. [PubMed: 18257063]
12. Raeber GP, Lutolf MP, Hubbell JA. Molecularly engineered PEG hydrogels: a novel model system for proteolytically mediated cell migration. *Biophys J*. 2005; 89(2):1374–88. [PubMed: 15923238]
13. Dumas JE, Zienkiewicz K, Tanner SA, Prieto EM, Bhattacharyya S, Guelcher S. Synthesis and Characterization of an Injectable Allograft Bone/polymer Composite Bone Void Filler with Tunable Mechanical Properties. *Tissue Eng Part A*. 2010; 16(8):2505–18. [PubMed: 20218874]
14. Hafeman A, Li B, Yoshii T, Zienkiewicz K, Davidson J, Guelcher S. Injectable biodegradable polyurethane scaffolds with release of platelet-derived growth factor for tissue repair and regeneration. *Pharm Res*. 2008; 25(10):2387–99. [PubMed: 18516665]
15. Li B, Davidson JM, Guelcher SA. The effect of the local delivery of platelet-derived growth factor from reactive two-component polyurethane scaffolds on the healing in rat skin excisional wounds. *Biomaterials*. 2009; 30(20):3486–94. [PubMed: 19328544]
16. Guelcher SA, Patel V, Gallagher KM, Connolly S, Didier JE, Doctor JS, Hollinger JO. Synthesis and in vitro biocompatibility of injectable polyurethane foam scaffolds. *Tissue Engineering*. 2006; 12(5):1247–1259. [PubMed: 16771638]
17. Hafeman AE, Zienkiewicz KJ, Zachman AL, Sung HJ, Nanney LB, Davidson JM, Guelcher SA. Characterization of the degradation mechanisms of lysine-derived aliphatic poly(ester urethane) scaffolds. *Biomaterials*. 2011; 32(2):419–29. [PubMed: 20864156]
18. Hafeman AELB, Yoshii T, Zienkiewicz K, Davidson JM, Guelcher SA. Injectable biodegradable polyurethane scaffolds with release of platelet-derived growth factor for tissue repair and regeneration. *Pharmaceutical Research*. 2008; 25(10):2387–2399. [PubMed: 18516665]
19. Szycher, M. *Szycher's Handbook of Polyurethanes*. CRC Press; Boca Raton: 1999.
20. Guelcher S, Srinivasan A, Hafeman A, Gallagher K, Doctor J, Khetan S, McBride S, Hollinger J. Synthesis, In vitro degradation, and mechanical properties of two-component poly(ester urethane)urea scaffolds: Effects of water and polyol composition. *Tissue Engineering*. 2007; 13(9):2321–2333. [PubMed: 17658992]
21. Kretlow JD, Klouda L, Mikos AG. Injectable matrices and scaffolds for drug delivery in tissue engineering. *Advanced Drug Delivery Reviews*. 2007; 59:263–273. [PubMed: 17507111]
22. Khan Y, Yaszemski MJ, Mikos AG, Laurencin CT. Tissue engineering of bone: material and matrix considerations. *J Bone Joint Surg Am*. 2008; 90(Suppl 1):36–42. [PubMed: 18292355]

23. Kim CW, Talac R, Lu L, Moore MJ, Currier BL, Yaszemski MJ. Characterization of porous injectable poly-(propylene fumarate)-based bone graft substitute. *J Biomed Mater Res A*. 2008; 85(4):1114–9. [PubMed: 17941027]
24. Zhong SP, Zhang YZ, Lim CT. Tissue scaffolds for skin wound healing and dermal reconstruction. *Wiley Interdiscip Rev Nanomed Nanobiotechnol*. 2010; 2(5):510–25. [PubMed: 20607703]
25. Lawrence BJ, Madhally SV. Cell colonization in degradable 3D porous matrices. *Cell Adh Migr*. 2008; 2(1):9–16. [PubMed: 19262124]
26. Wang H, Pieper J, Peters F, van Blitterswijk CA, Lamme EN. Synthetic scaffold morphology controls human dermal connective tissue formation. *J Biomed Mater Res A*. 2005; 74(4):523–32. [PubMed: 16028236]
27. Adekogbe I, Ghanem A. Fabrication and characterization of DTBP-crosslinked chitosan scaffolds for skin tissue engineering. *Biomaterials*. 2005; 26(35):7241–50. [PubMed: 16011846]
28. Yang J, Shi G, Bei J, Wang S, Cao Y, Shang Q, Yang G, Wang W. Fabrication and surface modification of macroporous poly(L-lactic acid) and poly(L-lactic-co-glycolic acid) (70/30) cell scaffolds for human skin fibroblast cell culture. *J Biomed Mater Res*. 2002; 62(3):438–46. [PubMed: 12209930]
29. Jin Q, Wei G, Lin Z, Sugai JV, Lynch SE, Ma PX, Giannobile WV. Nanofibrous Scaffolds Incorporating PDGF-BB Microspheres Induce Chemokine Expression and Tissue Neogenesis In Vivo. *PLoS ONE*. 2008; 3(3):e1729. [PubMed: 18320048]
30. Scherer SS, Pietramaggiore G, Matthews J, Perry S, Assmann A, Carothers A, Demcheva M, Muise-Helmericks RC, Seth A, Vournakis JN. Poly-N-acetyl glucosamine nanofibers: a new bioactive material to enhance diabetic wound healing by cell migration and angiogenesis. *Ann Surg*. 2009; 250(2):322–30. others. [PubMed: 19638916]
31. Kavlock KD, Pechar TW, Hollinger JO, Guelcher SA, Goldstein AS. Synthesis and characterization of segmented poly(esterurethane urea) (PEUUR) elastomers for bone tissue engineering. *Acta Biomater*. 2007; 3:475–484. [PubMed: 17418651]
32. Murphy CM, Haugh MG, O'Brien FJ. The effect of mean pore size on cell attachment, proliferation and migration in collagen-glycosaminoglycan scaffolds for bone tissue engineering. *Biomaterials*. 2009; 31(3):461–6. [PubMed: 19819008]
33. Hafeman AE, Zienkiewicz KJ, Carney E, Litzner B, Stratton C, Wenke JC, Guelcher SA. Local delivery of tobramycin from injectable biodegradable polyurethane scaffolds. *J Biomater Sci Polym Ed*. 2010; 21(1):95–112. [PubMed: 20040156]
34. Guelcher SA, Srinivasan A, Dumas JE, et al. Synthesis, mechanical properties, biocompatibility, and biodegradation of polyurethane networks from lysine polyisocyanates. *Biomaterials*. 2008; 29:1762–75. [PubMed: 18255140]
35. Zhang J-Y, Beckman EJ, Hu J, Yuang G-G, Agarwal S, Hollinger JO. Synthesis, biodegradability, and biocompatibility of lysine diisocyanate-glucose polymers. *Tissue Eng*. 2002; 8(5):771–785. [PubMed: 12459056]
36. Adhikari R, Gunatillake PA, Griffiths I, Tatai L, Wickramaratna M, Houshyar S, Moore T, Mayadunne RT, Field J, McGee M. Biodegradable injectable polyurethanes: synthesis and evaluation for orthopaedic applications. *Biomaterials*. 2008; 29(28):3762–70. others. [PubMed: 18632149]
37. Paillet-Matteia C, Beca S, Zahouani Z. In vivo measurements of the elastic mechanical properties of human skin by indentation tests. *Medical Eng and Physics*. 2007 In Press.
38. Seare WJ Jr. Alloplasts and biointegration. *J Endourol*. 2000; 14(1):9–17. [PubMed: 10735567]
39. Abramo AC, De Oliveira VR, Ledo-Silva MC, De Oliveira EL. How texture-inducing contraction vectors affect the fibrous capsule shrinkage around breasts implants? *Aesthetic Plast Surg*. 2010; 34(5):555–60. [PubMed: 20354696]
40. Hinz B. The myofibroblast: paradigm for a mechanically active cell. *J Biomech*. 2010; 43(1):146–55. [PubMed: 19800625]
41. Li B, Brown KV, Wenke JC, Guelcher SA. Sustained release of vancomycin from polyurethane scaffolds inhibits infection of bone wounds in a rat femoral segmental defect model. *J Control Release*. 2010; 145:221–230. [PubMed: 20382191]

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Figure 8.

Table 1

Physical properties of PUR scaffolds

PUR Sample	Day 0			Day 7		
	Density (kg/m ³)	Porosity (vol %)	Pore Size (μm)	Density (kg/m ³)	Porosity (vol %)	Pore Size (μm)
Blank	110 ± 2	90.9 ± 0.1	370 ± 90	105 ± 2	91.4 ± 0.1	320 ± 70
PUR+HA	158 ± 9	87.0 ± 0.7	330 ± 70	100 ± 7	91.8 ± 0.6	330 ± 80
PUR+CMC	161 ± 8	86.7 ± 0.6	320 ± 80	116 ± 18	90.4 ± 1.5	340 ± 90

Table 2

Mechanical properties of PUR scaffolds

PUR Sample	Day 0		Day 7	
	Young's Modulus (kPa)	Compressive Stress (kPa)	Young's Modulus (kPa)	Compressive Stress (kPa)
Blank	30 ± 4	7.7 ± 1.0	19 ± 8	6.8 ± 0.6
PUR+HA	50 ± 20	10 ± 2	11 ± 4	8 ± 3
PUR+CMC	60 ± 30	10 ± 7	14 ± 4	9 ± 3