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Anterior cruciate ligament regeneration using braided biodegradable scaffolds: in vitro optimization studies

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Abstract

The anterior cruciate ligament (ACL) is the most commonly injured intra-articular ligament of the knee, and limitations in existing reconstruction grafts have prompted an interest in tissue engineered solutions. Previously, we reported on a tissue-engineered ACL scaffold fabricated using a novel, three-dimensional braiding technology. A critical factor in determining cellular response to such a graft is material selection. The objective of this in vitro study was to optimize the braided scaffold, focusing on material composition and the identification of an appropriate polymer. The selection criteria are based on cellular response, construct degradation, and the associated mechanical properties. Three compositions of poly-α-hydroxyester fibers, namely polyglycolic acid (PGA), poly-L-lactic acid (PLLA), and polylactic-*co*-glycolic acid 82:18 (PLAGA) were examined. The effects of polymer composition on scaffold mechanical properties and degradation were evaluated in physiologically relevant solutions. Prior to culturing with primary rabbit ACL cells, scaffolds were pre-coated with fibronectin (Fn, PGA-Fn, PLAGA-Fn, PLLA-Fn), an important protein which is upregulated during ligament healing. Cell attachment and growth were examined as a function of time and polymer composition. While PGA scaffolds measured the highest tensile strength followed by PLLA and PLAGA, its rapid degradation in vitro resulted in matrix disruption and cell death over time. PLLA-based scaffolds maintained their structural integrity and exhibited superior mechanical properties over time. The response of ACL cells was found to be dependent on polymer composition, with the highest cell number measured on PLLA-Fn scaffolds. Surface modification of polymer scaffolds with Fn improved cell attachment efficiency and effected the long-term matrix production by ACL cells on PLLA and PLAGA scaffolds. Therefore based on the overall cellular response and its temporal mechanical and degradation properties in vitro, the PLLA braided scaffold pre-coated with Fn was found to be the most suitable substrate for ACL tissue engineering.

Keywords: Polymer composition; Anterior cruciate ligament (ACL); Poly-α-hydroxyester; Degradation; Fibronectin; Ligament tissue engineering

1. Introduction

The anterior cruciate ligament (ACL) is the most commonly injured ligament of the knee. Due to its intrinsically poor healing potential and limited vascularization, injuries to the ACL do not heal and surgical intervention is usually required. In the United States

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alone, over 100,000 ACL reconstruction surgeries are performed annually [1]. Current treatment options in ACL reconstruction include autografts, allografts, and synthetic grafts. The wide utilization of allografts is limited by the potential for infectious disease transfer and immunogenic response [2]. The clinical realization of synthetic alternatives such as the Gore Tex prosthesis, the Stryker–Dacron ligament and the Kennedy ligament augmentation device (LAD) [1,3–6] has been hindered by mismatch in mechanical properties which often result in fatigue, creep, and unwanted wear [6–8]. The long-term clinical outcome of these grafts was suboptimal due to poor abrasion resistance, high incidence of fatigue failures, and limited integration between the graft and host tissue [1,3–6]. While autologous grafts based on either the patellar tendon or hamstring tendon remain the clinical gold standard, they are limited by the scarcity of tissue, donor site pain and morbidity, osteolysis, and possible inadequate fixation between the graft and bone.

The aforementioned disadvantages associated with both biological and synthetic grafts have prompted a growing interest in tissue-engineered solutions for ACL reconstruction [1,7,9–12]. The ideal ACL replacement scaffold should be biodegradable, porous, biocompatible, exhibit sufficient mechanical strength, and promote the formation of ligamentous tissue [9]. Our approach is to utilize biodegradable polymers to develop a three-dimensional (3-D) ACL construct using novel braiding techniques that will permit the controlled fabrication of constructs with a wide range of geometries and mechanical properties [9]. Previously, we reported on a tissue-engineered ACL scaffold fabricated using a 3-D braiding technology [9]. This braided scaffold was comprised of three regions: femoral tunnel attachment site, ligament region, and tibial tunnel attachment site. The attachment sites had high angle fiber orientation at the bony attachment ends and lower angle fiber orientation in the intra-articular zone. The scaffold was pre-designed with optimal pore diameter and porosity for soft tissue growth, and was composed of poly-α-hydroxyester fibers with an average diameter similar to that of type I collagen fiber.

Material selection in tissue-engineering scaffolds is one of the primary factors dictating the overall cellular response and resultant matrix formation on the scaffolds. The FDA has approved the use of poly-α-hydroxyesters such as polyglycolic acid (PGA), poly-L-lactic acid (PLLA), and polylactic-*co*-glycolic co-polymer (PLAGA) for a variety of clinical applications. This family of degradable polymers has been researched for use in tissue engineering [9,13–15], as they do not elicit a permanent foreign body reaction and are gradually reabsorbed and replaced by natural tissue. A significant challenge in the utilization and optimization of degradable material resides in achieving a balance between scaffold degradability, structural integrity needed for cell culture, overall scaffold mechanical properties, and the rate of cell matrix production.

Protein modification of biomaterials has been reported to improve cell adhesion [16–20] and control the subsequent cellular response to material surfaces [21,22]. Due to their varied degrees of surface hydrophilicity, poly-α-hydroxyesters in general support relatively lower levels of cell adhesion [17,18,23,24]. Current strategies in improving cell attachment and augmenting subsequent cellular response include pre-coating these surfaces with molecules such as laminin, fibronectin (Fn) or grafting the Fn-related arginine–glycine–aspartic acid (RGD) tripeptide on biomaterials [19,25–27]. Ko et al. pre-coated the surface of PLA film with an engineered protein which contains the RGD sequence, and found that the resultant surface increased the attachment of porcine endothelial cells [27]. Eid et al. pre-coated porous PGA disks with RGD peptides and implanted the construct in vivo using a 5 mm rat cortical bone model [25], and found a peptide dose-dependent increase in bone healing. These studies demonstrate that modifying the biomaterial surface with cell adhesion molecules such as Fn has a beneficial effect on cellular response and tissue repair. In addition to being one of the most abundant extracellular glycoproteins found in the body, Fn is reported to be up-regulated during ligament formation and wound healing [28–32]. The utilization of Fn in ACL tissue-engineering scaffolds has not been explored.

The aim of this study was to optimize in vitro the 3-D circular braided scaffold designed for ligament tissue engineering, focusing on material selection and the identification of an appropriate polymer composition based on cellular response, construct degradation, and the associated mechanical properties. Specifically, this study evaluated the effects of polymer composition, polymer surface modification via Fn adsorption on scaffold material properties and cell proliferation. Three compositions of poly-α-hydroxyester fibers with varying rates of degradation were considered, namely PGA, PLAGA, and PLLA. The attachment and growth of ACL cells on these three types of polymers were examined. It was hypothesized that cellular response will be dependent on polymer composition, and the Fn-coated polymeric surfaces would promote cell adhesion in this tissue-engineered system.

2. Materials and methods

2.1. Scaffold fabrication

Fibers of polyglycolic acid (PGA, 45–50% crystalline, 60 denier), polylactic-*co*-glycolic acid 82:18 (PLAGA, $M_w \approx 160,000$, 70 denier), and poly-L-lactic acid (PLLA, $M_w \approx 250,000$, 70 denier) were purchased from

Albany International Research Corporation (Mansfield, MA). Three-dimensional (3-D) fibrous scaffolds were fabricated using a 3-D braiding machine [9]. In this braiding method, multi-filament yarns (30 filaments per yarn) of PGA, PLAGA, or PLLA were plied to produce yarn bundles with a density of 10 multi-filament yarns per yarn bundle. Yarn bundles were then placed in a custom built circular braiding loom with a 3×8 carrier arrangement. Sequential motion of the carriers (alternating tracks) resulted in the formation of 3-D circular braids with 24-yarn bundles. The final dimensions of the 3-D circular braids were approximately $4 \times 16 \times 1\,mm^3$ (Fig. 1A).

2.2. Scaffold characterization

The as-made scaffolds were characterized in terms of architecture (pore diameter, porosity, surface area) and mechanical properties (tensile modulus, maximum tensile load). Mercury porosimetry (Micromeritics Autopore III, Micromeritics, Norcross, GA) was used to measure the porosity, average pore diameter and total surface area of the braided polymeric scaffolds ($n = 3$). In this method, porosity was determined by measuring the volume of mercury infused into the structure during analysis, and pore diameter is correlated to the applied pressure. Pore diameter, distribution, and geometry were also confirmed via scanning electron microscopy (SEM, AMRAY3000, MA) performed at 20 keV.

In addition, the braided polymer scaffolds ($n = 6$) were tested under tension using an Instron testing system (Model 4442, Instron Inc., MA) with a maximum load of 500 N. The testing was performed with a gauge length of 10 mm at a strain rate of 2%/s. The tensile modulus, ultimate tensile strength, and maximum tensile load were determined as a function of polymer composition.

2.3. Cells and cell culture

Anterior cruciate ligament (ACL) tissue samples were isolated from New Zealand white rabbits (1 kg), and primary ACL fibroblasts were then obtained by enzymatic digestion of the tissue following the methods of Nagineni et al. [33]. Briefly, under aseptic conditions, a straight midline longitudinal incision extending from the distal femur to the tibia was made in the rabbit knee. After retraction of skin and subcutaneous fascia, the patella tendon was removed, and a deeper incision was made into the joint capsule in order to expose the femoral condyle and the tibial plateau. The ACL was identified and excised from the joint, and the tissue was cut into smaller pieces and serial digested in a 0.1% collagenase solution (Sigma, St. Louis, MO). The cells were maintained in minimum essential media (α-MEM) supplemented with 10% fetal bovine serum (Mediatech, Herndon, VA), 1% L-glutamine and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). The cultures were grown to confluence at 37 °C and 5% CO_2.

2.4. Scaffold surface modification and cell seeding

Prior to in vitro culture, the braided PGA, PLAGA and PLLA scaffolds were sterilized by first soaking the scaffolds in 70% alcohol for 5 min. After drying, the samples were UV irradiated for 15 min on each side. To improve cell attachment, the scaffold surface was modified by coating the sample with human recombinant fibronectin (Fn, Sigma, St. Louis, MO). In this process, the polymeric scaffolds were pre-soaked in a reconstituted Fn solution for 30 min. The Fn solution concentration used for this study was 10 μg/ml, and the amount of Fn absorbed on the scaffold surface was determined by the Coomassie Brilliant Blue assay (Bio-Rad Laboratories, CA). The final Fn density on all three types of scaffolds was measured to be 8 μg per scaffold. After surface modification, the Fn-adsorbed scaffolds (PGA-Fn, PLAGA-Fn, PLLA-Fn) were then seeded with ACL cells at a density of 80,000 cells/scaffold. ACL cells seeded on uncoated scaffolds (PGA, PLAGA, PLLA) and tissue culture polystyrene (TCPS) served as control groups for this study. The seeded scaffolds were cultured in supplemented α-MEM for 1, 3, 7, and 14 days at 37 °C and 5% CO_2. Media was exchanged every 2 days and for each time point, solution pH was also measured.

2.5. Cell attachment and growth

The attachment and growth of ACL cells on the polymeric scaffolds were examined as a function of culturing time, surface modification, and polymer composition. Cell attachment morphology and growth on the scaffolds were monitored using electron microscopy ($n = 2$). At the end of each time point, the sample was harvested and fixed overnight in Karnovsky's fixative and subsequently dehydrated through a series of ethanol dilutions [34]. To minimize surface charging effects, the samples were sputter-coated with gold (Denton Desk-1 Sputter Coater, NJ). Cell growth and morphology were examined by SEM (20 keV, Amray 3000, MA).

Cell proliferation on the scaffolds ($n = 3$) was quantified using the Promega CellTiter 96® Assay (Promega, Madison, WI). This colorimetric assay has a detection limit of 2.5×10^5 cells, and it provides information on both cell metabolic activity as well as cell number when used with an accurate standard curve ($r^2 = 0.91$).

Table 1
Characterization of the braided scaffolds post fabrication

Polymer composition	Linear density (denier)	Braiding angle (°)	Surface area (cm^2)	Porosity (%)	Mode pore diameter (μm)	Median pore diameter (μm)
PGA ($n=3$)	60	25±1	55±1	63±7	177±68	103±18
PLAGA ($n=7$)	70	28±3	70±12	59±5	195±44	84±20
PLLA ($n=7$)	70	28±3	64±9	54±8	226±23	103±20

Porosimetry analysis confirmed that independent of polymer composition, all three types of circular scaffolds exhibited similar porosity and pore diameters.

2.6. Scaffold degradation before and after cell culture

Polymer degradation was determined before and after in vitro culture with ACL cells. Prior to cell culture, multi-filament yarns (30 filaments/yarn) of PGA, PLAGA, and PLLA were plied into yarn bundles (10 multi-filament yarns/bundle). These polymeric yarns were cut into 6.0 cm samples and sterilized with 70% isopropyl alcohol for 5 min followed by UV irradiation for 5 min. Degradation was assessed by immersing the yarns in 10 ml of α-MEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The centrifuge tubes were loaded into a water bath shaker (Model YB-521, American Scientific Products, OH) running at 100 strokes/min at 37 °C. The solution was changed weekly.

Polymer degradation was examined at 1, 2, 4, 6, and 8 weeks. At each time point, total weight loss ($n=4$), morphological changes, mechanical strength ($n=6$), and weight average molecular weight of PLAGA and PLLA ($n=3$) were determined for each sample. Solution pH was also measured (Model 320, Corning Inc., MA) at each time point. After incubation, the samples were rinsed with deionized water, and allowed to dry under vacuum using a lyophilizer for 24 h. The dry weight was measured over time (Model R160P, Sarorius, Data Weighing Systems Inc., IL).

At each time point, scaffolds cultured with ACL cells were first treated in triton-X (Sigma, St. Louis, MO) and subsequently solublized in tetrahydrofuran. Weight average molecular weight ($n=3$) was determined by gel permeation chromatography (GPC) (1100 series, Hewlett Packard, CA) in tetrahydrofuran (THF) calibrated with polystyrene standards. Mass retention was calculated as the ratio of the dry weight before and after solution immersion.

2.7. Statistical analyses

Data in the graphs are presented in the form of mean±standard deviation (Mean±SD). An unpaired *t*-test was used to determine significant differences between two means. In the case of multiple comparisons, one-way analyses of variance (ANOVA) were performed, and when a significant *F* ratio was obtained, the Tukey–Kramer test was used to compare between the means. Significance was attained at $p<0.05$.

3. Results

3.1. Scaffold characterization

Results from the post fabrication characterization of the polymeric scaffolds are summarized in Table 1. The porosity of the scaffolds ranged from 54% to 63% and the average mode pore diameters were between 177 and 226 μm. No statistically significant differences were detected in braiding angle, surface area, porosity and average pore diameter between the PGA, PLAGA, and PLLA scaffolds. SEM analyses revealed that fiber diameter were similar between the three types of scaffolds, ranging between 15 and 25 μm.

Scaffold tensile properties as a function of polymer composition are shown in Fig. 1. Specifically, the circular, 3×8 braid of PGA measured a maximum tensile load (Max Load) of 502±24 N, an ultimate tensile strength (UTS) of 378±18 MPa, and the PLAGA scaffold measured a Max Load of 215±23 N, an UTS of 117±12 MPa, while the PLLA scaffold measured a Max Load of 298±59 N, an UTS of 165±33 MPa. Statistically significant differences ($p<0.05$) in both Max Load and UTS were found between the three polymer groups, with PGA measuring the highest tensile properties, followed by PLLA and PLAGA.

3.2. Effects of scaffold composition on cell attachment and morphology

Results from SEM evaluation of ACL cell attachment and growth morphology as a function of culturing time are presented in Fig. 2 for 1 day, Fig. 3 for 7 days, and Fig. 4 for 14 days. In general, primary ACL cells exhibited semi-ovoid, fibroblast-like morphology (Fig. 2) and when confluent, formed extended cultures with specific growth orientations (Fig. 4). Cell spreading was observed on all scaffolds examined independent of polymer composition, while cell morphology and growth patterns were found to be dependent on the

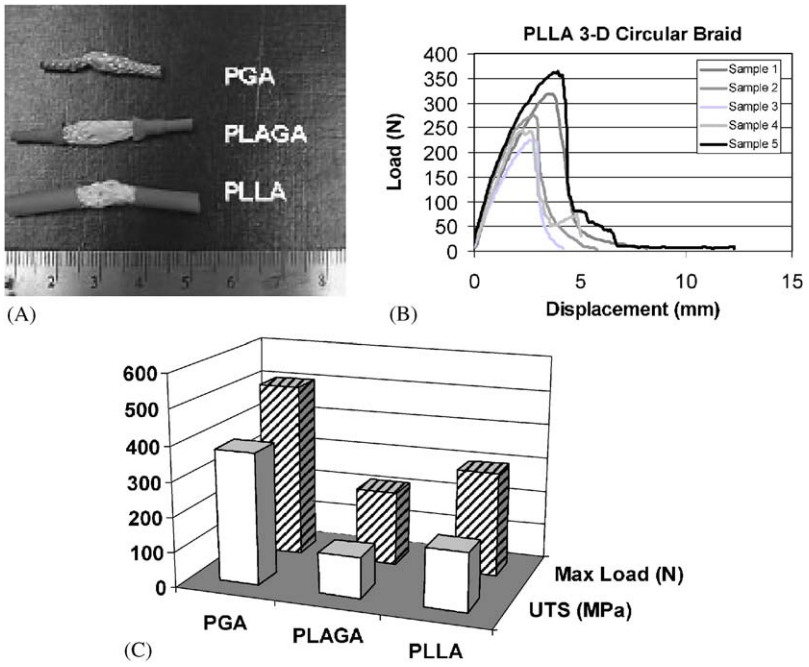

Fig. 1. Mechanical properties of the braided polymer scaffolds. The three types of scaffolds shown in (A) were tested under tension ($n = 6$, 2%/s). An example of a representative load deformation curve (B) is shown. Scaffolds based on PGA fiber consistently exhibited significantly higher UTS, followed by PLLA and PLAGA scaffolds. Statistical significance ($p < 0.05$) was found between all groups tested (C).

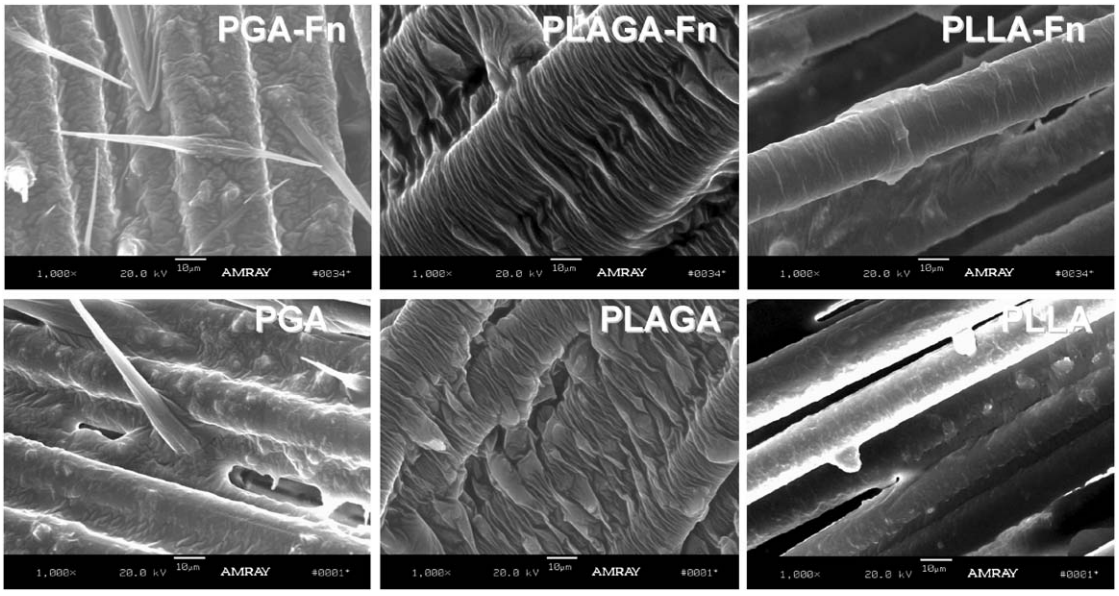

Fig. 2. ACL fibroblast attachment on braided scaffolds and effects of fibronectin (Fn) adsorption after 1 day of culture. Cell spreading was observed on all scaffolds examined, and it was independent of composition and Fn coating. ACL fibroblasts conformed to scaffold geometry on PLAGA and PLA scaffolds. Bridging between fibers and rod-like cell projection were observed only on PGA scaffolds. A higher number of cells were found on PLLA-Fn scaffolds, likely due to the improved cell attachment efficiency with Fn.

type of polymer. ACL fibroblasts conformed to scaffold geometry on PLAGA and PLA scaffolds, but not on PGA scaffolds. Bridging between fibers and rod-like cell projection was found only when cells are seeded on PGA scaffolds. While extensive sheets of cells were observed on all three types of polymers, the morphology and degree of cell spreading differed between PLAGA and PLLA scaffolds. ACL cells attached on PGA and PLLA scaffolds were smoother and had fewer cellular bundles compared to cells on PLAGA braids.

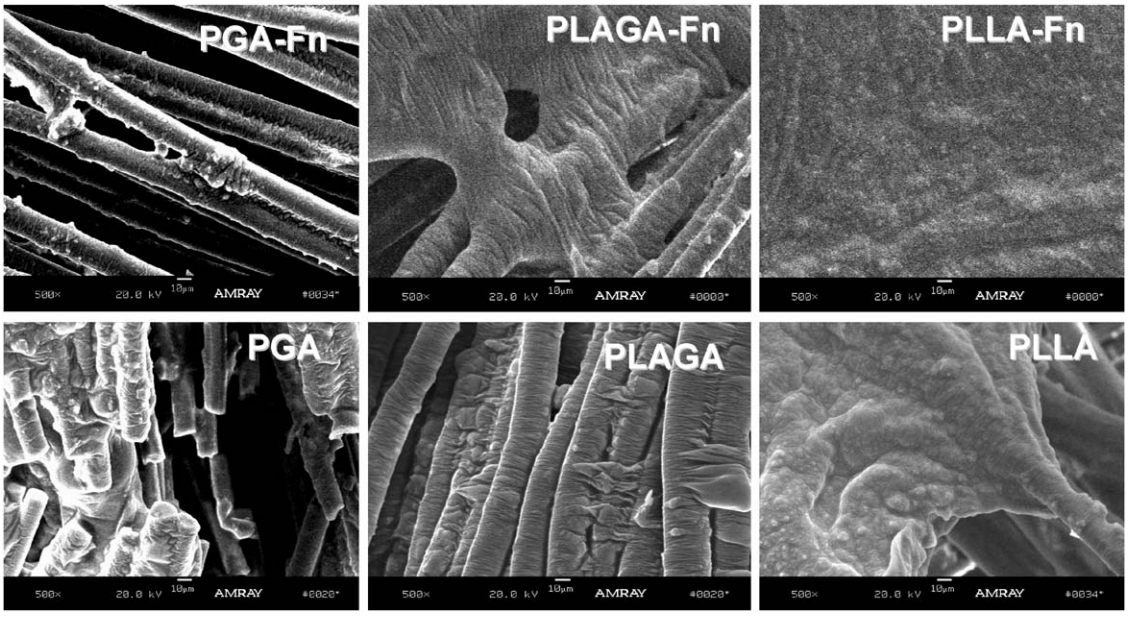

Fig. 3. ACL fibroblast growth on braided scaffolds after 7 days of culture. Extended matrix elaboration was observed on all scaffolds examined. Cells grown on PLAGA or PLLA scaffolds pre-coated with fibronectin (Fn) produced more matrix compared to scaffolds without Fn. However, more extensive matrix elaboration was found on the PLLA-Fn scaffold. Rapid degradation of the PGA scaffold in the presence of cells and culture medium was detrimental to matrix formation, as shown in the micrograph on the left (PGA-Fn and PGA). Modification of the PGA surface with Fn also appears to have delayed scaffold degradation.

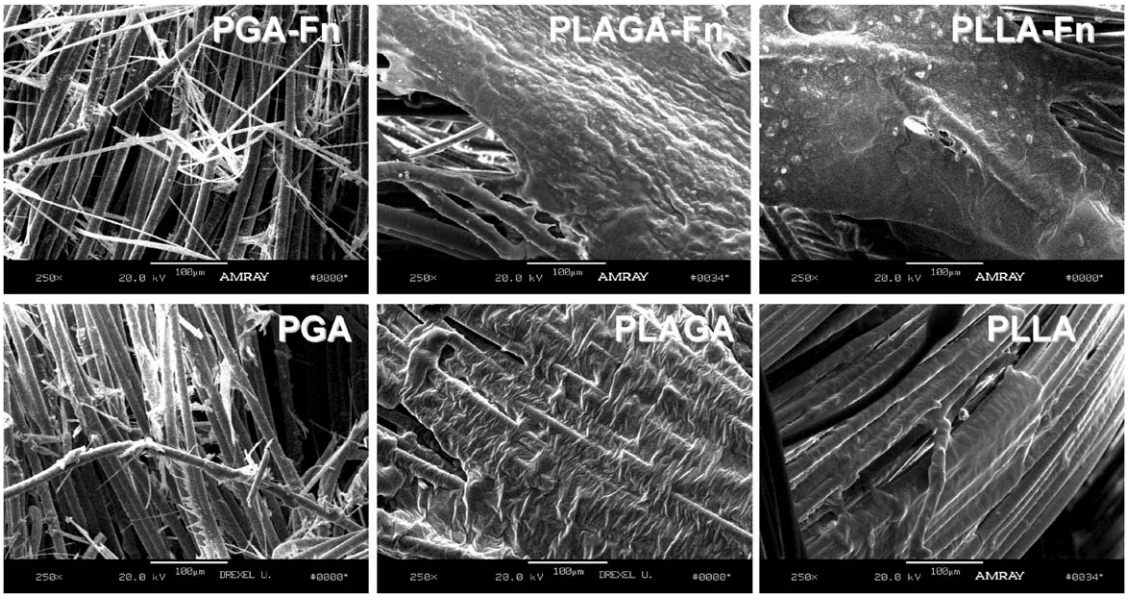

Fig. 4. ACL fibroblast growth on braided scaffolds after 14 days of culture. Cells grown on braided scaffolds pre-coated with fibronectin (Fn) continue to elaborate a greater amount of matrix compared to PLAGA or PLLA scaffolds without Fn. Degradation of the PGA scaffold after two weeks of culture resulted in extensive cell loss and matrix depletion.

At day 1, depending on the scaffold type, the primary rabbit ACL cells exhibited a varied morphology (sheath, spherical, sharp projections, and spindle-shaped) along the long axis of the fibers (Figs. 2–4). In addition, the PGA scaffolds showed that ACL cells produced sharp projection in the form of spiked rods (Fig. 2), which were absent on PLAGA and PLLA scaffolds (Fig. 2). After 3 days, these cells produced extensive sheath that enveloped the fibers, with cells bridging and growing in the grooves between fibers on all three types of

polymers. Fewer pointed cell extensions were observed on the PGA scaffold, accompanied by an increase in the number of spindle-shaped cells and the production of the extracellular matrix sheath. Cells grown on PLAGA exhibited a different morphology than those found on PGA, forming large globular aggregates accompanied by cells with ruffled membranes. Cell attachment was directed along the striated filament surfaces. Cells grown on PLLA were observed to exhibit both spindle and sheath-like morphologies.

After a week of culture, extensive extracellular matrix was observed along the long axes of the fibers in all three types of polymeric scaffolds studied, with matrix bridging found between fibers (Fig. 3). Due to degradation, the PGA scaffolds began to show filament fragmentation (Fig. 3), whereas the PLAGA and PLLA scaffolds remained intact and confluent cell sheaths were observed. Qualitatively, a larger area of the PLLA scaffold was covered by extracellular matrix compared to the PLAGA and PGA scaffolds. At 14 days, SEM photomicrographs of PGA scaffolds revealed a lower cell density on these scaffolds, and a large network of spiked cell extensions was again observed throughout the scaffold (Fig. 4). Large voids were observed in the PGA scaffolds due to extensive degradation. In contrast, the PLAGA and PLLA scaffolds retained scaffold integrity and continued to sustain large areas of confluent matrix sheath, with cells bridging and growing in the grooves between the fibers (Fig. 4).

3.3. Effects of surface modification with fibronectin on cell morphology and growth

Figs. 2–4 also compare cell response on PGA, PLAGA, and PLLA to those of cells grown on PGA-Fn, PLAGA-Fn, and PLLA-Fn scaffolds. As shown in Fig. 2, after initial cell seeding, there was little difference in cell attachment morphology between the Fn-coated and uncoated samples, and no difference was observed between the three different polymers. Cell spreading was observed on all scaffolds, and it was independent of composition and Fn coating. At day 7, cells grown on PLAGA or PLLA scaffolds pre-coated with Fn produced a greater amount of matrix than scaffolds without Fn. However, more extensive matrix elaboration was found on the PLLA-Fn scaffold (Fig. 3). Rapid degradation of the PGA scaffold in the presence of cells and in the culture medium was detrimental to matrix formation, as evident in the micrographs for the PGA-Fn and PGA scaffolds. At day 14, cells grown on braided scaffolds pre-coated with Fn continue to elaborate larger amount of matrix compared to PLAGA or PLLA scaffolds without Fn. The difference between the surface-modified scaffold and untreated surface became more pronounced at this time point. Photomicrographs of control PGA-Fn, PLAGA-Fn and PLLA-Fn scaffolds not seeded with cells showed smooth filament surfaces after 14 days of immersion in α-MEM with 10% serum protein (Fig. 6).

3.4. Effects of scaffold composition and Fn coating on cell proliferation

The growth of primary rabbit ACL cells on the three types of 3-D circular braids are shown in Fig. 4. The number of cells on the Fn-coated tissue culture plastic control plates increased significantly over the incubation period ($p<0.001$, $n=3$), and a higher number of cells were consistently measured on TCPS-Fn compared to TCPS only wells (data not shown). Thus the data presented in Fig. 5 are relevant for substrates pre-coated with Fn (PGA-Fn, PLAGA-Fn, PLLA-Fn, and TCPS-Fn). When comparing between the polymeric scaffolds, cell number after 1 day was found to be the highest on the PLLA-Fn scaffold, followed by the PLAGA-Fn and PGA-Fn groups, although this difference was not statistically significant. While the same trend was observed between substrates, cell proliferation did not increase significantly at day 7. At day 14, a significant increase ($p<0.01$) in cell number over time was observed for the PLAGA-Fn and PLLA-Fn groups. Moreover, the PLLA-Fn supported significantly higher ($p<0.05$) number of cells compared to PLAGA-Fn. Cell attachment efficiency and proliferation remained consistently

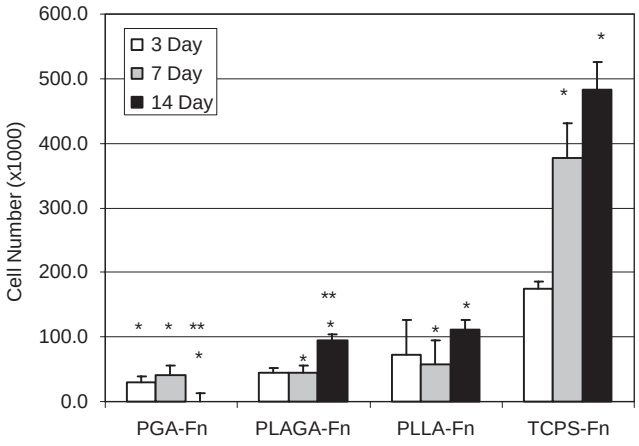

Fig. 5. Proliferation of primary rabbit ACL cells on braided scaffolds pre-coated with fibronectin (Fn). Cells grew on all scaffolds examined after 7 days of culture, and no significant differences were detected between the three polymer substrates. Tissue culture polystyrene pre-coated with Fn (TCPS-Fn) served as controls. At day 14, a significant higher number of cells were measured compared to PLAGA-Fn and PGA-Fn substrates at day 14 ($p<0.05$, $n=3$). Cell number on the PGA scaffolds was significantly lower due to polymer degradation ($p<0.05$, $n=3$). These results suggest that PLLA-Fn is an optimal material for ACL cell attachment and growth. *Note*: *denotes significant differences in time within the polymer group ($p<0.05$); **significant difference between PLLA and other polymer time points ($p<0.05$).

higher on PLLA scaffolds compared to PLAGA over time. In contrast, while the number of cells on the PGA scaffolds increased at day 7 ($p<0.05$), it dropped significantly at day 14 ($p<0.001$) and was the lowest among all substrates tested.

3.5. Effects of polymer composition and in vitro culture on scaffold degradation

As expected, greater degradation was found with PGA compared to PLAGA and PLLA fibers when immersion in α-MEM + 10% FBS. PGA degraded significantly faster compared to PLAGA and PLLA, and lost integrity after 2 weeks of immersion. Thus no quantitative data on PGA were generated. SEM analysis revealed that irregular surface pores were present on PGA surfaces (Fig. 6A), and large PGA debris or yarn fragments were found in the medium. In contrast, the surface of PLLA fibers remained pore-free and relatively smooth, while micro-cracks and small amount of debris were observed for the PLAGA samples after 2 weeks of immersion in protein-containing α-MEM (Fig. 6A). Solution pH decreased initially, but returned to physiological levels after 1 week of culture. In the absence of cells, the weight average molecular weight of PLLA and PLAGA decreased over time, with a faster degradation rate observed for the PLAGA group.

After Fn adsorption and in vitro culture with primary rabbit ACL cells, PLAGA-Fn continued to degrade at a faster rate compared to PLLA-Fn (Fig. 6B). While degradation was observed on PGA-Fn, modification of the PGA surface with Fn appeared to have delayed the degradation of the braided scaffold. A significant decrease in weight average molecular weight was

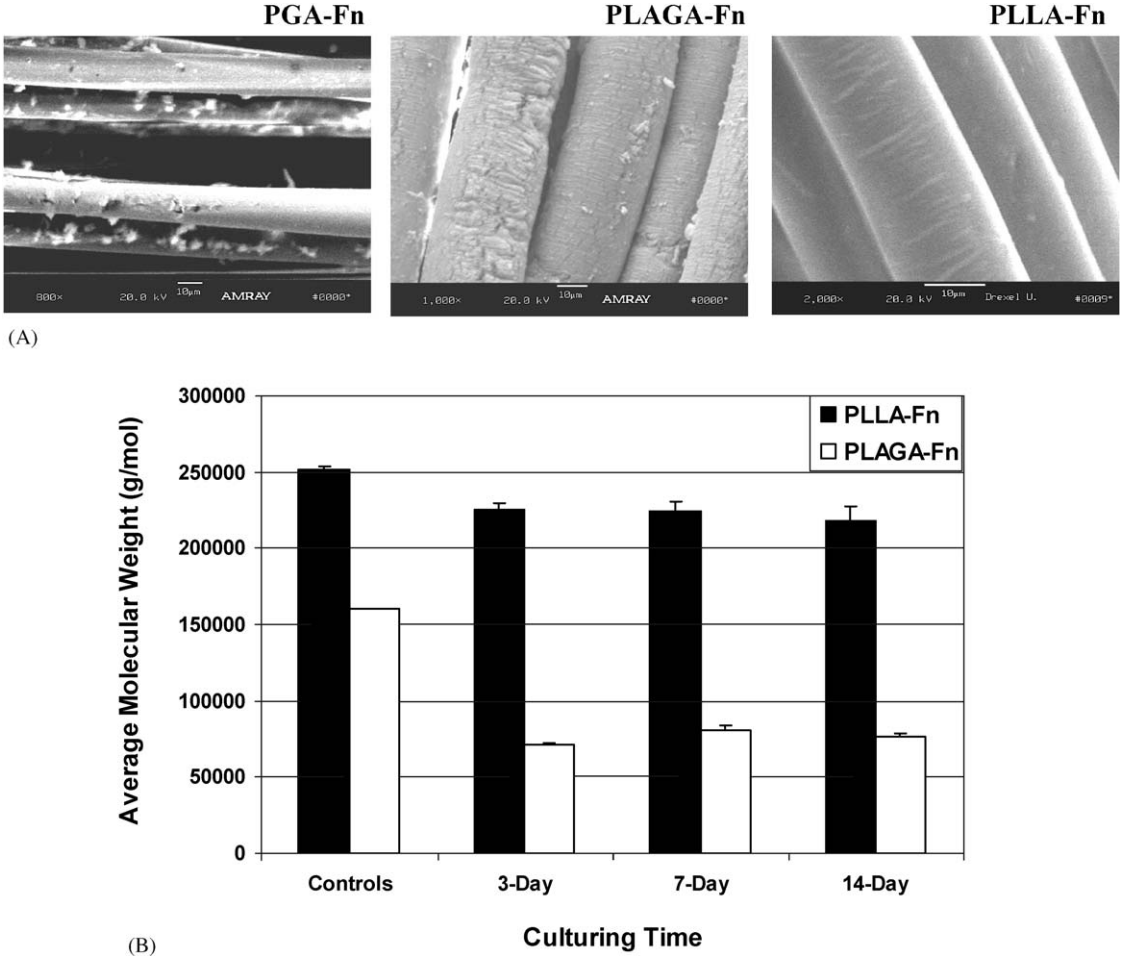

Fig. 6. Effects of polymer composition and cell culture on scaffold degradation. (A) Electron micrographs of PGA, PLAGA, and PLLA scaffolds coated with Fibronectin (Fn) after 14 days of immersion in tissue culture medium without cells. Extended scaffold degradation was observed on PGA scaffolds as indicated by the presence of surface pore and polymer debris. In contrast, the surface of PLLA scaffolds remained pore-free and relatively smooth compared to PLAGA and PGA. (B) Effects of polymer composition and *in vitro* culture on scaffold degradation. The temporal changes in the weight average molecular weight (M_w) of the braid scaffolds after culturing with primary ACL cells were determined. The PLLA scaffold degraded significantly slower compared to the PLAGA scaffold ($p<0.05$, $n=3$). For both polymer types, scaffold degradation plateaued after 3 days of culture. It is likely that cell adhesion and matrix production may have a protective effect on polymer degradation. PGA scaffolds lost integrity after 7 days of culture (data not shown).

observed over the incubation time (PLAGA, $p<0.001$, and PLLA, $p<0.001$). As seen in Fig. 6B, the percent change in weight average molecular weight was approximately 12% for PLLA-Fn and about 56% for the PLAGA-Fn scaffolds. For both polymer types, the largest change in M_w was found after 3 days, and the M_w value remained relatively constant over 14 days of culture. There was no significant difference in polymer M_w between scaffolds cultured in a protein-containing solution or in the presence of cells.

4. Discussion

The objectives of this study were to optimize the 3-D braided ACL tissue-engineering scaffold and to determine an appropriate polymer with respect to cellular response, construct degradation, and associated mechanical properties. To this end, this study examined the effects of polymer composition and surface modification on construct degradation, mechanical properties, and the attachment and proliferation of primary rabbit ACL fibroblasts. It was found that both polymer composition and surface modification had significant effects on the mechanical properties and degradation rate of the scaffolds. These changes resulted in consequences for cell attachment morphology and growth rate. The findings of this study underscore the importance of material selection in tissue-engineering applications and its potential impact on the subsequent cellular response.

Our findings indicate that polymer composition had an effect on the mechanical properties of the braided scaffold. A compositional dependence was found between PGA, PLAGA, and PLLA scaffolds, with the strongest scaffold being PGA, followed by PLLA and PLAGA. The PGA scaffold measured the highest tensile modulus post fabrication, thus if based solely on initial mechanical properties, PGA may be more suitable as a scaffold material for ACL tissue engineering. It is important to note that the mechanical properties of these fibers are dependent on the molecular weight of the polymers. The braided scaffolds described here are currently being designed for testing in a rabbit model. Thus the targeted mechanical properties will be based on the rabbit ACL, which has a maximum tensile load of 314 N [35] and an elastic modulus of 514 MPa [36]. Since in functional tissue engineering the objective is to match the mechanical properties of the native tissue, the PLLA scaffold was thus most similar to the rabbit ACL. While the PGA matrix was stronger compared to the rabbit ACL, it may be important to compensate for scaffold degradation in vivo. It was observed here that the initial mechanical advantage of PGA was not sustained in vitro, with the scaffold losing scaffold integrity after just 1 week of culture. In contrast, the M_w of the PLLA scaffold decreased only by 12% as opposed to approximately 50% for PLAGA after 3 days of culture. It follows that the tensile strength of the PLLA will be higher than that of PLAGA, making it a more functional material for ACL reconstruction. It is notable that scaffold porosity and pore diameter were independent of composition, confirming the reproducibility of the novel braiding technique. This is critical since scaffold architecture is an important design factor modulating cellular response and long-term outcome. A minimum pore diameter of 150 μm is suggested for bone and 200–250 μm for soft tissue ingrowth [3,37,38].

The attachment morphology of cells derived from connective tissue such as ACL usually progresses from spherical, spindle, sheath to confluent cell sheath [39–41]. Spherical cells are about 6–10 μm in diameter and attach to fiber surfaces with microvilli [40,41]. Spindle-shaped cells are approximately 20–50 μm in length; they have ruffled membranes and attach to filaments with small pointed or filipodia-like projections [40,41]. On striated fibers, the spindle cells proliferate over grooves and attach to the crests. Sheath cells are flat, multipolar and have irregular shapes with few surface microvilli [40,41]. These cells are usually 80 μm in length and cover large areas of fibers with attachments similar to those of spindle cells [40,41]. The confluent cell sheath extends over an area of 100–200 μm^2, and the size of the sheath depends on the scaffold geometry. In this study, it was observed that primary ACL cells exhibited semi-ovoid, fibroblast-like morphology and when confluent, formed multinucleated cultures with specific growth orientations. Cell growth and morphology were dependent on polymer composition. Extensive cell sheaths were observed on all three types of polymers, but the morphology and cell spreading were different between PLAGA and PLLA scaffolds. Cell spreading observed on PGA and PLLA was smooth with fewer cell bundles compared to PLAGA, upon which rough, wrinkled membranes were seen during growth. This difference may be a function of increased surface roughness and striations observed on the PLAGA polymer filaments after UV sterilization. The observed initial cell attachment morphology and its progression are similar to those reported for normal tendon or ligament development [42]. The PLAGA and PLLA scaffolds supported physiologic cell morphology, based on this parameter alone these materials are better suited for ACL tissue engineering than PGA.

Cell proliferation can be controlled by many external factors such as surface geometry, biomaterial composition, and the presence of relevant proteins [21,39–41,43]. Surface modification of the PGA, PLAGA and PLLA scaffolds with Fn resulted in further differences in cell proliferation and extracellular matrix production between these scaffolds. Pre-coating the control and scaffold surfaces lead to an increase in cell attachment efficiency and overall cell proliferation. The PLLA

scaffold after Fn adsorption supported the highest level of cell proliferation. Thus it may be most appropriate for ACL tissue engineering than PLAGA or PGA. RGD peptide-mediated cell proliferation has also been reported in the literature. Kantlehner et al. found that surface modified with RGD peptides increased osteoblast adhesion and proliferation [26]. In addition to modulating cell adhesion, migration and proliferation, Fn is believed to play a role in ligament healing and the maintenance of soft tissues [28,31,44]. It is found primarily in the synovial sheath surrounding the ligaments [28], and post injury, the level of Fn becomes elevated during neoligament formation [28,31]. AbiEzzi et al. found that stress deprivation in ligament fibroblasts resulted in the degradation of Fn [45], and a correlation between Fn concentration and adhesion strength of ACL cells has been reported [46]. Fn-modified biomaterial surfaces can result in a biomimetic surface, which may stimulate the reparative potential of primary ACL cells.

It is interesting to note that a similar amount of fibronectin was measured on all three substrates tested, and the observed difference between groups with and without Fn only became marked after 7 days of culture. These results suggest that the chemistry of the underlying polymer substrate may play a role in the initial Fn adsorption process. Surface charge and chemistry can have a direct effect on Fn adsorption to biomaterial surfaces [47]. The conformation of the adsorbed proteins may vary as a function of substrate chemistry, which in turn would affect the integrin binding and cell-signaling pathways. Since the porosity and pore sizes of the scaffolds were not significantly different from each other, the observed differences in cellular response are independent of geometry and influenced by other factors such as polymer composition and Fn adsorption.

Based on the current literature, it was not unexpected that PGA scaffolds degraded faster than PLAGA or PLLA scaffolds [48–50]. However, the majority of these degradation studies were conducted in solutions such as deionized water or phosphate buffer saline which does not take into account the effects of serum protein adsorption and cell culture. In this study, degradation was examined in physiologically relevant solutions, such as the serum supplemented cell culture medium, and in the presence of cells. It is interesting to note that while an initial decrease in pH was measured in α-MEM, the solution later returned to control values. This is most likely due to protein adsorption and the higher buffering potential of α-MEM, rendering it a more realistic solution in which to model polymer degradation in vivo. Electron microscopy revealed that degradation may be delayed in the presence of serum proteins and during cell culture, and M_w remained relatively constant after 3 days of cell culture and up to 4 weeks in a protein-containing solution. It is possible that in the race for the surface, protein adsorption as well as the elaboration of an extensive extracellular matrix by ACL cells may act as a protective shield against polymer hydrolysis.

In summary, the PGA scaffolds, in spite of their higher initial mechanical properties, supported the lowest level of cell attachment and growth compared to PLAGA and PLLA. In addition, ACL cells were less responsive to the underlying scaffold geometry when cultured on PGA. While Fn adsorption may have increased overall cell attachment and delayed PGA degradation, the scaffold disintegrated during long-term culture and resulted in suboptimal cellular response. The PLAGA and PLLA scaffolds tested in this study sustained a similar level of cellular response during the culturing period. PLLA scaffolds consistently supported greater cell adhesion, spreading and a higher level of cell proliferation compared to PLAGA, and the overall cellular response was augmented with the adsorption of Fn. Moreover, due to its slower degradation rate, the PLLA scaffolds maintained high mechanical properties over the same culturing period compared to PLAGA or PGA scaffolds. These results collectively demonstrate that the Fn-coated PLLA scaffold may be a more appropriate system for ACL tissue engineering.

5. Conclusions

In this study, ACL cellular response on a novel braided scaffold for ACL tissue engineering was found to be dependent on polymer composition. The adsorption of Fn on polymer scaffolds improved attachment efficiency and had a positive effect on matrix production by ACL cells grown on PLLA and PLAGA scaffolds. The primary ACL cells displayed spherical, spindle, and sheath morphology and in time formed a confluent cell matrix on the scaffolds. Based on cell attachment morphology, cell proliferation, and its overall mechanical and degradation properties, the PLLA scaffold may be a more suitable material for ACL tissue engineering compared to PLAGA or PGA. Future studies will focus on the differentiation of ACL cells on the braided scaffold and the optimization of the PLLA scaffold for ACL reconstruction in vivo.

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