

Leading Opinion

Ligament tissue engineering: An evolutionary materials science approach [☆]

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Abstract

The anterior cruciate ligament (ACL) is important for knee stabilization. Unfortunately, it is also the most commonly injured intra-articular ligament. Due to poor vascularization, the ACL has inferior healing capability and is usually replaced after significant damage has occurred. Currently available replacements have a host of limitations, this has prompted the search for tissue-engineered solutions for ACL repair. Presently investigated scaffolds range from twisted fiber architectures composed of silk fibers to complex three-dimensional braided structures composed of poly (L-lactic acid) fibers. The purpose of these tissue-engineered constructs is to apply approaches such as the use of porous scaffolds, use of cells, and the application of growth factors to promote ligament tissue regeneration while providing mechanical properties similar to natural ligament.

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1. Introduction

The anterior cruciate ligament (ACL) is the most commonly injured ligament of the knee with over 200,000 patients per year diagnosed with ACL disruptions [1–3]. The ACL is the major intra-articular ligament of the knee and is critical to normal kinematics and stability. The ACL controls motion by connecting the femur to the tibia and stabilizing the joint, preventing abnormal types of motion. Its main functions are to support and strengthen the knee and prevent

excessive anterior translation of the femur that could cause a dislocation and fracture of bones in the knee joint.

The ACL is a dense, highly organized, cable-like tissue composed of collagens (types I, III, and V), elastin, proteoglycans, water, and cells. The human ACL has an average length of 27–32 mm and a cross-sectional area of 44.4–57.5 mm² [4,5]. Ligaments have a hierarchical structure with different levels of organization including collagen molecules, fibrils, fibril bundles, and fascicles that run parallel to the long axis of the tissue. The collagen fibrils in ligaments display a periodic change in direction called a crimp pattern. In ACL, this crimp pattern repeats every 45–60 μm [6,7]. The fascicles contain collagen fibrils, proteoglycans, and elastin. The ligament is surrounded by a sheath of vascularized epiligament [8]. An additional level of structure exists in the ACL, the collagenous network is twisted by approximately 180° from the femoral attachment site to the tibial attachment site [6]. The ACL also contains antromedial and posterolateral bands. Knowledge about ligament structure and its

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components is extremely important when designing scaffolds for ligament regeneration because it is the interaction between these components and their arrangement in the tissue which give ligaments their unique mechanical properties.

Ligaments display triphasic behavior when exposed to strain. First there is a region where the ligament exhibits a low amount of stress per unit strain, this is called the non-linear or toe region. This region is followed by an area noted for its increase in stress per unit strain, called the linear region. The last region displays a slight decrease in stress per unit strain and marks the failure of the ligament, this is the yield and failure region [6]. The presence of this unique behavior is due to the components of the ligament and their arrangement in the tissue. When force is first applied to the tissue it is transferred to the collagen fibrils. This results in lateral contraction of fibrils, the release of water, and the straightening of the crimp pattern in the collagen fibrils. Once the crimp pattern is straightened, the force is applied directly to the collagen molecules [6,9]. The collagen triple helix is stretched and interfibrillar slippage occurs between crosslinks [6,10]. This results in an increase in stress per unit strain. Finally, the collagen fibers in the ligament fail by defibrillation causing a decrease in stress per unit strain and tissue failure [6,11].

After injury, ligament healing can be divided into three phases: inflammation, cellular proliferation and matrix repair, and remodeling [6]. Inflammation occurs within 72 h of the injury. During this stage, serous fluid accumulates in both the ligament and the surrounding tissues and the damaged area becomes fragile [6,12,13]. Monocytes, leukocytes, and macrophages migrate to the wound site. In the cellular proliferation and matrix repair stage, fibroblasts are present and vascular granulation tissue is formed. Collagen is produced with a high ratio of type III to type I collagen, forming the new extracellular matrix. This stage typically lasts for 6 weeks. The final stage, remodeling, lasts for several months. During this stage, the new extracellular matrix matures into a slightly disorganized hypercellular tissue [6,13]. Healing of the ACL is inhibited by lack of vascularization. Optimal healing is achieved when the continuity of collagen fibers is maintained. Due to the lack of tissue organization and difference in crimp pattern between the new and old extracellular matrices, there is a difference in the mechanical properties of the new scar tissue and the original ligament leading to a reduction in mechanical properties. When injury causes a full rupture of the ligament midsubstance or detachment of the ligament, an insertion point surgery is required. If the ligament is not repaired, the loading of the joint leads to abnormal stress on the articular cartilage, which can lead to early osteoarthritis [14,15].

2. Traditional implantable solutions

Traditionally, ACL injuries have been treated with biological grafts, autografts, or allografts [16]. Autograft material for ACL repair is usually taken from the patellar tendon, hamstring tendon, or quadriceps tendon of the patient. The patella and hamstring tendons have been the autografts of choice for most surgeons. The patellar tendon graft material is usually removed with a piece of bone from the patella and from the insertion point at the tibia. The “bone–patellar–bone” graft is then fed through a tunnel drilled through the tibia, drawn across the knee, and anchored into a tunnel drilled through the femur. Allografts are tissues obtained from cadavers such as patellar tendon, hamstring tendon, and achilles tendon [16,17]. The benefits of using allografts include the lack of a second surgery for tissue harvest. There is also no limit to the supply of graft tissue as experienced with autografts. Both autografts and allografts possess good initial mechanical strength and promote cell proliferation and new tissue growth.

However, both of these options suffer from a number of disadvantages. For example, autografts have limited availability and require additional surgery for tissue harvest, which may cause donor site morbidity. Allografts could potentially transmit disease, cause bacterial infection, elicit an unfavorable immunogenic response from the host, and cannot be sterilized without altering the mechanical properties of the tissue [18,19].

In the past, attempts have been made to use synthetic materials in ligament replacements. Non-degradable synthetic materials used for ACL repair include polyethylene terephthalate (Leeds-Keio ligament), polypropylene (Kennedy Ligament Augmentation Device), and poly (tetrafluoroethylene) (Gore-Tex) [20–25]. These synthetic ligament replacements have been conditionally approved by the FDA for augmentation but are not recommended for primary ACL repair [23]. The Leeds-Keio ligament is a woven porous tube composed of polyethylene terephthalate and attached to woven tapes [6,26]. This device is designed to allow the ingrowth of new tissue. The Kennedy Ligament Augmentation Device is a cylindrical prosthesis with a diamond-braided construction. This device is implanted in conjunction with biological grafts such as the patella tendon [6]. The Gore-Tex prosthesis is composed of an expanded poly (tetrafluoroethylene) fiber that is wound into loops, which are joined together to form a braid [27,28].

Although these synthetic devices initially supply the function of the ligaments that they replace or protect the ligament that they augment, these devices fail over time because they cannot duplicate the mechanical behavior of the ligament. Repeated elongation of these devices leads to permanent deformation at the points of stress. Contact with sharp edges of the bone tunnel causes

abrasions, which weaken the implant and create debris that can cause synovitis in the joint. Woven prostheses face the additional problems of axial splitting, low tissue infiltration, low extensibility, and abrasive wear. Eventually, these implants fail due to fragmentation, stress shielding of new tissue, fatigue, creep, and production of wear debris [22,23,29].

3. Tissue-engineered constructs

The aforementioned limitations associated with both biological and synthetic grafts have initiated a growing interest in tissue-engineered solutions for ACL reconstruction. The ideal ACL replacement scaffold should be biodegradable, cause a minimal inflammatory response, and be porous. It should exhibit sufficient mechanical strength, demonstrate mechanical behavior similar to natural ACL, and promote the formation of ligamentous tissue. The scaffold should degrade at a rate that allows new tissue to receive the appropriate level of load without danger of rupture.

The advantage of a tissue-engineered scaffold over other treatment methods lies in its interdisciplinary approach to tissue repair. Tissue engineering is the application of biological, chemical, and engineering principles toward the development of substitutes for the repair or restoration of tissue function [30]. There are three approaches to tissue engineering: the use of three-dimensional, porous matrices that promote tissue regeneration, the use of isolated cells that have been expanded *in vitro*, and the use of growth factors. A combination of any of these approaches may also be used.

In recent years, a variety of different materials and structures have been investigated for use in tissue-engineered ligament replacements. Polyurethane urea has been used as a material for ligament replacements [31]. A poly desaminotyrosyl-tyrosine ethyl carbonate (poly (DTE carbonate)) scaffold developed by the groups of Dunn and Kohn have shown the ability to support fibroblast growth and display the necessary strength for use as an ACL graft [31]. The use of polydioxanone (PDS) has also been investigated as a potential material for scaffold construction [32]. The rapid loss of its mechanical strength due to degradation makes PDS a poor choice for use in ligament tissue engineering. Dunn has also studied the use of type I collagen fibers in potential ACL scaffolds [33,34]. The grafts showed excellent biocompatibility and enhanced cell attachment, proliferation, and production of extracellular matrix.

The potential flaw in all of these matrices lies in their architecture. The (poly (DTE carbonate)), polyurethane urea, and collagen grafts are composed of polymeric structures arranged in parallel. The poly (DTE carbonate) matrix is made of parallel fibers, the polyurethane urea structure is a woven band, and the collagen scaffold

is made from type I collagen fibers arranged in parallel (some of the scaffolds are also coated with a collagen solution). The lack of structural reinforcement and arrangement of the fibers in parallel with the direction of stress may cause long-term failure due to fatigue, creep, and abrasive wear.

Other tissue-engineered structures account for some of these possible problems by adopting a more complex fiber arrangement. One recent tissue-engineered structure developed by Altman et al. is a matrix composed of twisted silk fibers [4,35,36]. This structure combines two of the tissue engineering approaches to regenerate lost or damaged tissue: the use of a three-dimensional, porous matrix and the use of isolated cells that have been expanded *in vitro*.

The silk matrix is a hierarchical structure. Bundles of silk fibers are wound into strands that are wound into cords and arranged to form the matrix. This is similar to the arrangement of collagen fibers in the ligament and tendon, which has collagen fibers arranged to form fascicles and the fascicles join to form the ligament [37]. This silk matrix is not cytotoxic in proliferation tests with bone marrow stromal cells [4,35,36]. The twisted fiber architecture gives the scaffold mechanical properties similar to ACL. The matrices have a maximum load of 2337 ± 72 N, an elastic modulus of 354 ± 26 N/mm and a strain at failure of $38.6 \pm 2.4\%$. Both of these values are similar to ACL. The scaffolds also demonstrate the three-phase mechanical behavior seen in ligament and tendon. The scaffolds demonstrate a toe region (low stress per unit strain) followed by a linear region (high stress per unit strain). This characteristic is important for the prevention of damage due to fatigue and creep.

In other studies, Altman et al. have increased the biocompatibility and the ability of this matrix to elicit new tissue growth by coating the surface with RGD sequences [38]. This step gives the matrix all three of the approaches used in tissue engineering. The addition of these sequences has been shown to greatly increase cellular attachment and proliferation [36]. The presence of the RGD sequences on the silk fiber surfaces also increased the production of extracellular matrix by bone marrow stromal cells creating the possibility of a quicker and more complete tissue regeneration.

More recently, Laurencin and his colleagues have developed a tissue-engineered solution based on a cell-seeded, degradable, three-dimensional (3-D) braided poly L-lactic acid (PLLA) scaffold [38,39]. There have been other ligament prostheses made of flexible composites consisting of fibers that have been woven or braided into structures [21,24]. These scaffolds performed well for a short period after implantation, but the long-term outcomes of these prostheses have been poor [21,24]. These structures were limited by poor tissue integration, poor abrasion resistance, and fatigue [21,24]. The scaffold of Laurencin and colleagues uses

3-D braiding techniques to create a scaffold with controlled pore size, integrated pores, resistance to wear and rupture, and mechanical properties comparable to natural ACL. The control of pore size and integration of pores in the scaffold are important for the movement of nutrients throughout the scaffold and removal of cellular waste from the scaffold. This enhances cell proliferation and tissue ingrowth. This braided scaffold also has a hierarchical structure similar to the ligament. It is composed of fibers (similar in diameter to collagen fibers) which are arranged into bundles and wound throughout the thickness of the scaffold.

The braids are comprised of three regions: femoral tunnel attachment site (bony attachment end), ligament region (intra-articular zone), and tibial tunnel attachment site (bony attachment end) (Fig. 1). The attachment sites have a high-angle fiber orientation and the intra-articular zone has a lower-angle fiber orientation. These differences in fiber orientation cause changes in pore size between the three regions. It has been reported that calcified tissue ingrowth can occur at a minimum pore size of 100 μm and a minimum pore diameter of 150 μm is suggested for bone and 200–250 μm for soft tissue ingrowth [40,41]. The different regions of the scaffold contain pore sizes within these listed ranges in order to encourage tissue (ligament and bone) ingrowth and capillary supply. The higher braiding angle (and smaller pore size) at the insertion points also provide resistance to wear within bone tunnels, and improve the quality of anchorage in bone tunnels through the integration of

bone tissue. Along with pore size, overall scaffold porosity can alter the response of cells to the implant. The presence of pore interconnectivity extending through an implant increases the overall surface area for cell attachment, which in turn can enhance the regenerative properties of the implant by allowing tissue ingrowth into the interior of the matrix.

The structure is also resistant to sudden rupture [39]. The 3-D braiding scheme allows fibers to be woven throughout the entire thickness of the braid. This gives the braid toughness and reinforces the structure preventing total scaffold failure if some of the fibers become damaged. The material used in this matrix, PLLA, has been approved by the FDA for a variety of clinical applications. PLLA has been researched for use in tissue-engineering applications, as it does not elicit a permanent foreign body reaction and is gradually resorbed and replaced by natural tissue. Unlike autografts, there is no limit to the supply of these polymeric matrices. They have a minimal risk of disease transmission and can be easily sterilized without significant alteration of mechanical properties (unlike allografts). In the long term, the fatigue properties of PLLA are not a concern as the scaffold is eventually replaced by natural tissue. In a degradation study, PLLA fibers displayed very little change in mechanical properties over an 8-week period in media (Table 1).

In a study designed as a precursor to an in vivo rabbit study, the matrices were designed to possess mechanical properties (ultimate tensile stress $298 \pm 59 \text{ N}$) comparable

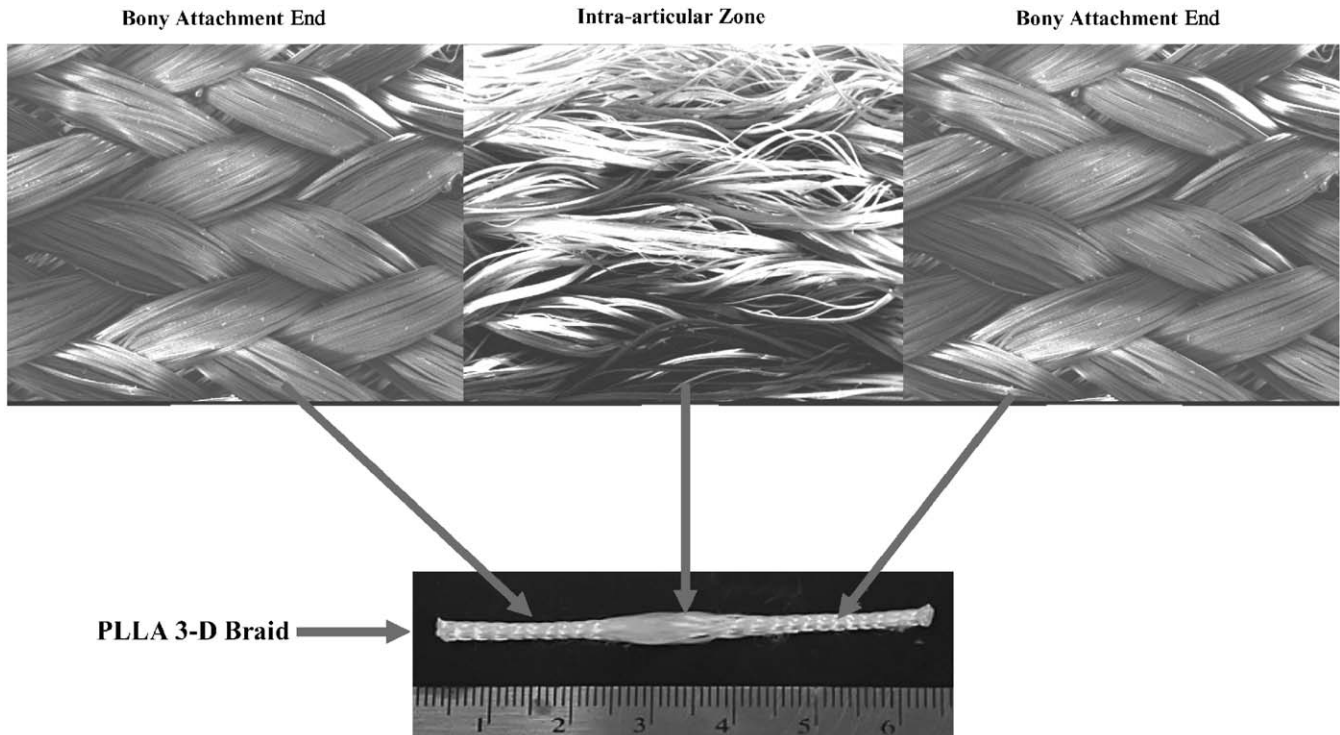


Fig. 1. Design of 3-D braided ligament scaffolds showing the macrostructure of the bony attachment and intra-articular zone.

to those of rabbit ACL (ultimate tensile stress 251 ± 47 N). The design parameters of the braid and the composition of the braid fibers promote ACL cell attachment and proliferation. When submerged in phosphate-buffered saline (PBS) for up to 12 weeks, the mass of the scaffold did not decrease (Fig. 2). The average molecular weight of the scaffold showed a linear decrease over the degradation period and there was no significant decrease in mechanical properties over a 12-week degradation period (Fig. 2). This is to be expected because PLLA can take up to 2 yrs for full resorption [42,43].

In *in vitro* studies, ACL fibroblasts conformed to the geometry of the PLLA scaffolds and extensive sheets of cells were observed on the scaffolds (Fig. 3). The 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 with matrix bridging found between fibers (Fig. 4). Cellular proliferation and tissue growth on the scaffold has been enhanced with the presence of fibronectin [40]. By employing all three of the tissue-engineering approaches (the use of a porous matrix, cells, and growth factor), cellular attachment, cell proliferation, and tissue generation have been enhanced on the 3-D braided scaffold. Fibronectin was absorbed onto the surfaces of the PLLA fibers used in the 3-D braids. Cell proliferation measurements and scanning electron microscopy (SEM) images confirm the increase in cell growth with the addition of fibronectin to the scaffolds. Western blot analysis showed an increase in type I collagen production in cells seeded onto scaffolds with fibronectin when compared to cells without fibronectin. Thus, the addition of the growth factor fibronectin to the 3-D braided scaffold has been shown to improve cell attachment efficiency, cell proliferation, and long-term matrix production by ACL cells on the 3-D braided matrix (Fig. 5).

4. Conclusions

As the number of incidents of ACL injury grows, new options for ligament repair are necessary to overcome the limitations of current treatments. Scaffolds con-

structed using tissue engineering techniques are becoming a viable option for ACL repair. These scaffolds can be optimized to provide the correct amount of mechanical support, a necessary property for this load-bearing tissue. Tissue-engineered scaffolds have also been shown to promote cell adhesion, cell proliferation, and the

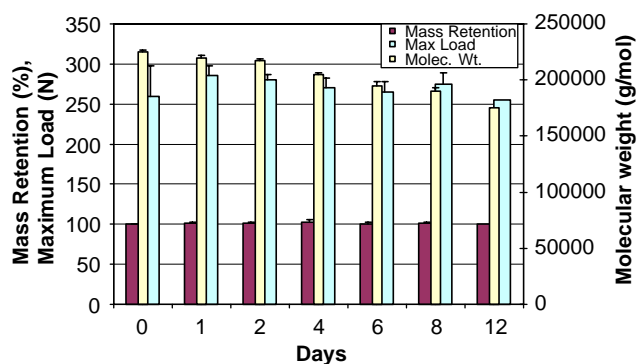


Fig. 2. Mass retention, average molecular weight, and maximum load of PLLA 5×5 3-D square braids immersed for 12 weeks in PBS (pH 7.3). There is no statistically significant change in mass retention between groups ($p < 0.05$). There is a statistically significant decrease in molecular weight over time ($p < 0.05$) and a statistically significant difference in maximum load between 8 and 12 weeks ($p < 0.05$).

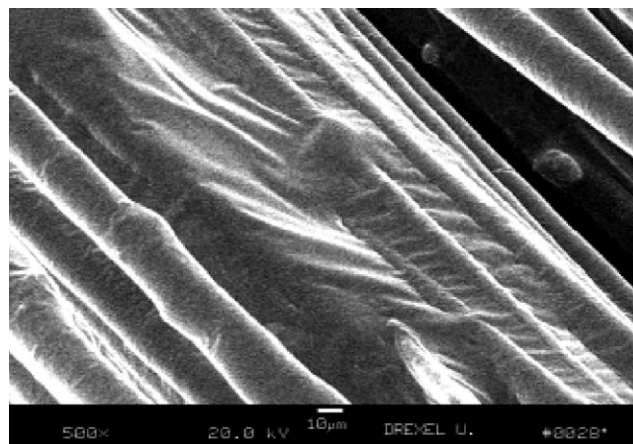


Fig. 3. ACL cell growth after 4 days on PLLA scaffolds. Extensive cell sheets have formed over the scaffolds.

Table 1
Summary of PLLA yarn bundle (30 filaments/yarn) mechanical properties

Yarn sample (weeks)	Immersion solution	Maximum load (N)	Maximum displacement (mm)	Strain at maximum (%)	Young's Modulus (N/tex)	Linear density (denier)
2	α -MEM	12.3 ± 3.7	10.3 ± 5.8	34.24 ± 19	0.82 ± 0.45	700
4	α -MEM	16.6 ± 4.6	6.22 ± 1.2	20.75 ± 4.1	1.26 ± 0.21	700
6	α -MEM	18 ± 3.0	$6.75 \pm .86$	22.5 ± 2.9	2.05 ± 0.53	700
8	α -MEM	16 ± 4.0	$6.32 \pm .90$	21.06 ± 3.0	2.09 ± 0.51	700

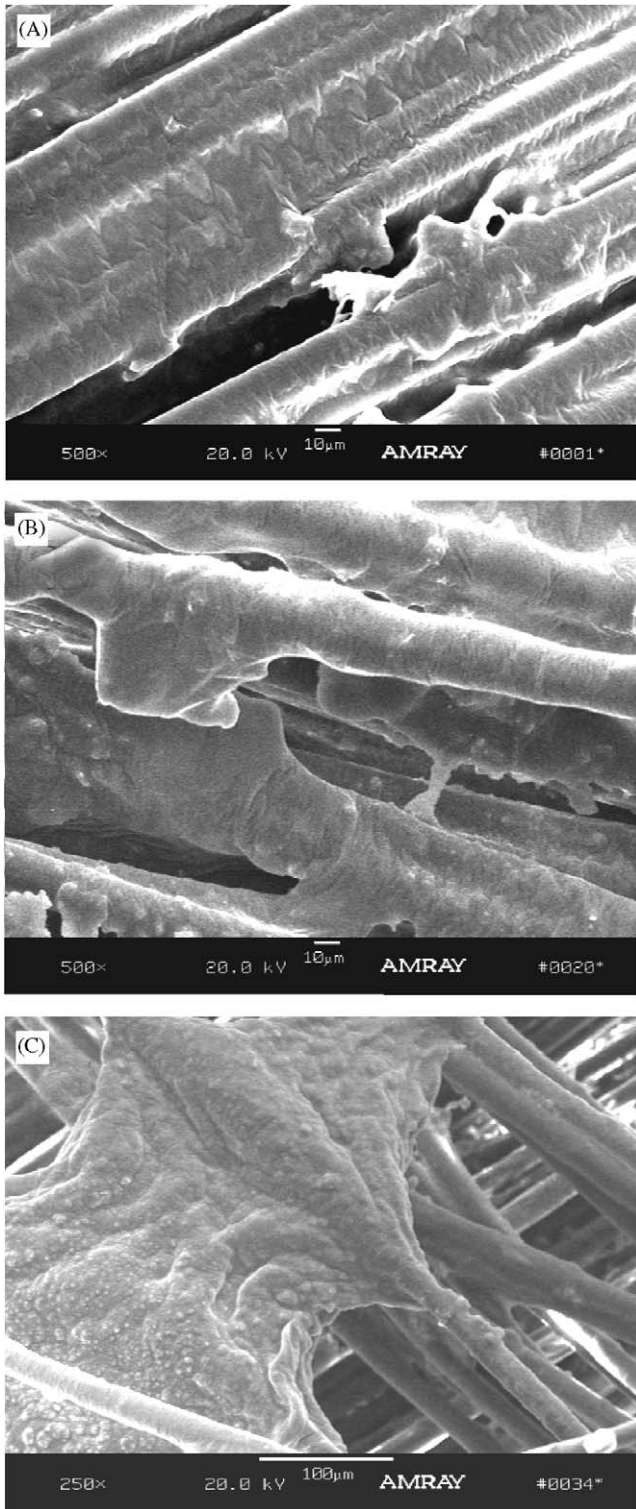


Fig. 4. ACL fibroblasts on PLLA scaffolds after 1 day (A), 3 days (B), and 7 days (C) in culture.

growth of new tissue. Recently developed scaffolds by Laurencin and colleagues display both the initial and long-term mechanical properties necessary for successful ACL replacement and regeneration. The successes of

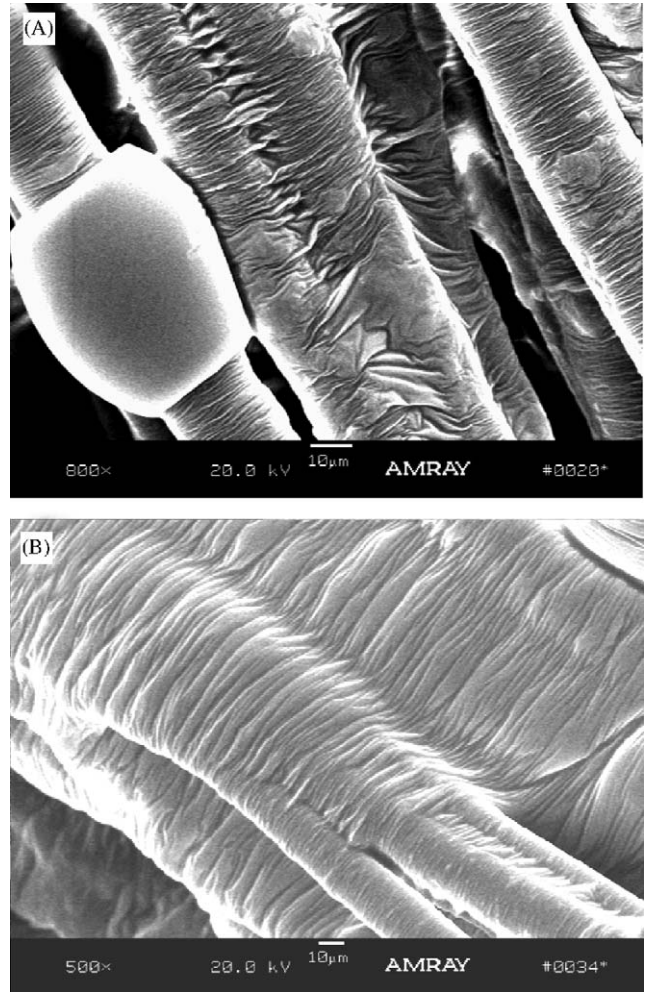


Fig. 5. PLAGA 3-D braid without fibronectin coating (A) and with fibronectin coating (B) after 3 days of growth. Note the confluent cells across filaments.

these structures can be further enhanced with the addition of growth factors such as fibronectin, or RGD modification.

The use of tissue-engineering approaches in their design allows these scaffolds to degrade while promoting tissue growth, enabling the body to fully regenerate lost or damaged tissue without the risk of scaffold or neoligament rupture or stress shielding of the new tissue. As research continues, it is expected that tissue-engineering techniques will lead to the design, production, and testing of next generation scaffolds that will mimic the mechanics of natural ligament and lead to the quick and complete regeneration of a new, mechanically sound, natural tissue.

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