



Stratified scaffold design for engineering composite tissues



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ABSTRACT

A significant challenge to orthopaedic soft tissue repair is the biological fixation of autologous or allogeneic grafts with bone, whereby the lack of functional integration between such grafts and host bone has limited the clinical success of anterior cruciate ligament (ACL) and other common soft tissue-based reconstructive grafts. The inability of current surgical reconstruction to restore the native fibrocartilaginous insertion between the ACL and the femur or tibia, which minimizes stress concentration and facilitates load transfer between the soft and hard tissues, compromises the long-term clinical functionality of these grafts. To enable integration, a stratified scaffold design that mimics the multiple tissue regions of the ACL interface (ligament–fibrocartilage–bone) represents a promising strategy for composite tissue formation. Moreover, distinct cellular organization and phase-specific matrix heterogeneity achieved through co- or tri-culture within the scaffold system can promote biomimetic multi-tissue regeneration. Here, we describe the methods for fabricating a tri-phasic scaffold intended for ligament–bone integration, as well as the tri-culture of fibroblasts, chondrocytes, and osteoblasts on the stratified scaffold for the formation of structurally contiguous and compositionally distinct regions of ligament, fibrocartilage and bone. The primary advantage of the tri-phasic scaffold is the recapitulation of the multi-tissue organization across the native interface through the layered design. Moreover, in addition to ease of fabrication, each scaffold phase is similar in polymer composition and therefore can be joined together by sintering, enabling the seamless integration of each region and avoiding delamination between scaffold layers.

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1. Introduction

The anterior cruciate ligament (ACL) is the most commonly injured knee ligament [1], with approximately 100,000 reconstructive surgeries performed annually in the United States [1–5] to restore the ligament's function as the primary knee joint stabilizer. The ACL's lack of vascularity and poor healing potential [6] make surgical intervention necessary, where autologous bone–patellar tendon–bone (BPTB) and hamstring tendon grafts are often used to rebuild the ligament. Hamstring tendon-based grafts are increasingly used as a replacement for BPTB grafts, which have been reported to result in severe donor site morbidity [7,8].

The long-term performance of hamstring tendon grafts is a measure of the structural and material properties of the graft, intra-articular positioning [9,10], initial graft tension [11–16], and post-operative rehabilitation. Here, we focus on the graft's potential as a function of its fixation [17,18] in the joint space. Although mechanical graft fixation has been improved clinically

through the use of transfemoral pins and screws, biological fixation with subchondral bone remains a limiting factor to clinical success [17–19]. With current ACL reconstruction methods, the native fibrocartilage insertion site fails to regenerate [20] and without this functional interface, biological graft–bone fixation cannot be achieved [17–19,21–23].

The ACL, and other soft tissues with direct insertions into bone, exhibit three distinct yet continuous regions with controlled variation in cell type and matrix heterogeneity: ligament, fibrocartilage, and bone [24–32]. The ligament proper is composed of fibroblasts embedded in a type I and II collagen matrix. Within the fibrocartilaginous region, nonmineralized (ovoid chondrocytes, type II collagen, and proteoglycan-rich matrix) and mineralized (hypertrophic chondrocytes in a calcified matrix [30] containing type X collagen [28]) fibrocartilage zones are observed. Lastly, the subchondral bone consists of osteocytes, osteoblasts and osteoclasts within a mineralized type I collagen matrix. The method for tri-phasic scaffold fabrication outlined below aims to mimic this control over matrix heterogeneity in order to recapitulate the multi-tissue ligament–bone transition. The scaffold is thus designed to support interface regeneration that will facilitate load transfer between soft and hard tissues, minimizing stress

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concentration [25,33] and enabling graft–bone integration. Moreover, seeding of the scaffold layers with fibroblasts, osteoblasts, and chondrocytes has demonstrated spatial control over cell and matrix distribution [34,35], which may be translated to other interface tissue engineering or composite tissue regeneration strategies.

2. Materials

2.1. Scaffold preparation

2.1.1. Scaffold Phase A

Polyglactin 10:90 Knitted Mesh Sheets (Vicryl VKML, Ethicon, Somerville, NJ), 5 × 5 mm squares.

2.1.2. Scaffold Phase B

Poly(D-L-lactide-co-glycolide) 85:15 copolymer (PLGA, $M_w \approx 123.6$ kDa, Alkermes, Cambridge, MA).
Dichloromethane (DCM, EM Science, Gibbstown, NJ).
Polyvinyl alcohol (PVA, $M_w \approx 89$ kDa, Sigma, St. Louis, MO).

2.1.3. Scaffold Phase C

Poly(D-L-lactide-co-glycolide) 85:15 copolymer (PLGA, $M_w \approx 123.6$ kDa, Alkermes, Cambridge, MA).
45S5 bioactive glass (BG, 20 μ m, Mo-Sci Corp., Rolla, MD).
Dichloromethane (DCM, EM Science, Gibbstown, NJ).

2.1.4. Sintering and sterilization of scaffold phases

Ethylene oxide (Sigma, St. Louis, MO).

2.2. Cell culture and seeding

Bovine Knee Joints (Green Village Packing Co., Green Village, NJ).

#22 scalpel blade (Thermo Fisher Scientific Inc., Waltham, MA).
Phosphate-buffered saline (PBS, Sigma Chemicals, St. Louis, MO): one packet PBS powder into 1 L deionized water.

Fully supplemented (F/S) culture media: Dulbecco's modified Eagle medium with 10% fetal bovine serum, 1% non-essential amino acids, and 1% penicillin/streptomycin (Mediatech, Herndon, VA).

Digestion media: F/S culture media with 10% penicillin/streptomycin instead of 1%, and the addition of 1% ampicillin B, 1% gentamicin, and 10% collagenase II solution (Worthington Biochemical Corp., Lakewood, NJ) (see Note 1).

Cell culture grade agarose (Sigma Chemical, St. Louis, MO).

3. Methods

Carry out the following procedures at room temperature (non-sterile).

3.1. Scaffold phase fabrication

1. Phase A (see Fig. 2A):

- Sinter square segments of Vicryl VKML sheets into cylindrical molds at 150 °C for 20 h under a 1.6 N load.

2. Phase B (see Fig. 2B):

- Form PLGA microspheres via water/oil/water emulsion [36]. First, dissolve PLGA in 10% w/v DCM.
- Slowly pour this solution into 1% PVA mixing at 200 RPM to form microspheres. Allow the microspheres to harden for 20 h, then recover by filtration, rinse with deionized water, and air dry.
- A microsphere yield of ~90% the original PLGA mass should be achieved.

- Sinter the resulting microspheres above the polymer glass transition temperature at 55 °C in an oven for 5 h in a cylindrical mold under a 1.1 N load to form Phase B of the scaffold.

3. Phase C (see Fig. 2C):

- Fabricate composite 4:1 PLGA:BG microspheres.
- Dissolve 1 g PLGA in 15 mL DCM by vortexing for 1 h.
- Suspend 0.25 g BG in the solution by vortexing for 10 min. Let the solution sit at room temperature for 20 min.
- Slowly pour into 1% PVA while mixing at 225 RPM. Mix for 4 h and rinse with 4 L deionized water.
- Dry the microspheres at room temperature for 24 h in a fume hood. A yield of ~70% the original PLGA and BG total mass should be achieved.
- Sinter the resulting microspheres at 55 °C in an oven for 5 h in a cylindrical mold under a 1.1 N load to form Phase C of the scaffold.

4. Integrate the three scaffold phases by sequentially inserting each phase into a cylindrical mold followed by a mesh disk, and sintering the 5 layers for 10 h at 55 °C under a 1.1 N load, above the glass transition temperature of PLGA (see Fig. 1).

5. Sterilize the resulting tri-phasic scaffold with ethylene oxide and vacuum desiccate in a sterile environment for at least 3–5 days prior to cell seeding.

Carry out all remaining procedures at room temperature under sterile conditions, unless otherwise specified.

3.2. Cell culture

1. Wash neonatal bovine knee joints in soapy water for 20 min, followed by a 40 min wash in 70% ethanol to sterilize.
2. Excise the patella and meniscus to expose the cruciate ligaments, and harvest the mid-section of the ACL. Mince ligament tissue with a #22 scalpel blade and submerge the pieces in F/S culture media (see Note 2). Monitor cell migration from the explant, and only use cells derived from the second migration to ensure a homogenous fibroblast population [37,38].
3. Harvest articular cartilage from the distal femur and proximal tibia with a scalpel blade, taking care not to violate the subchondral bone. Isolate primary bovine chondrocytes from the cartilage through collagenase digestion in digestion culture media.
4. Isolate cortical bone chips from bovine tibiae using a bone rongeur. Rinse the bone chips thoroughly with PBS (see Notes 3 and 4) to remove bone marrow, and submerge them in F/S culture media. Only use osteoblasts from the second migration for tri-culture on the scaffold.
5. Expand fibroblasts and osteoblasts in F/S culture media, and passage them at least once. Plate chondrocytes in F/S culture media 3 days prior to seeding onto scaffolds. Incubate all the cells at 37 °C under humidified conditions and 5% CO₂.

3.3. Cell seeding

1. Pre-coat the wells of a 12-well plate with 250 μ L/well 2% sterile agarose in order to limit cell migration out of the scaffolds (see Notes 5 and 6).
2. Load 5 × 10⁵ chondrocytes/scaffold, in a suspension of 0.5% agarose, onto Phase B of the scaffolds (see Note 7).
3. Allow the agarose to gel for 15 min.
4. Add osteoblasts to Phase C of the scaffolds at a density of 2.5 × 10⁵ osteoblasts/scaffold (see Note 8).
5. Allow the cells to adhere to the scaffold for 20 min.
6. Add fibroblasts to Phase A of the scaffolds at a density of 5 × 10⁵ fibroblasts/scaffold.

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