Methods 84 (2015) 99–102

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Stratified scaffold design for engineering composite tissues

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ARTICLE INFO

Article history:
Received 1 January 2015
Received in revised form 27 March 2015
Accepted 30 March 2015
Available online 3 April 2015

Keywords:
Biological fixation
Multi-phased scaffold
Co-culture
Enthesis
Anterior cruciate ligament
Interface tissue engineering

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.

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 fibrocartilagenous 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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http://dx.doi.org/10.1016/j.ymeth.2015.03.029
1046-2023/

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 μm, 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% CO_2.

3.3. Cell seeding

1. Pre-coat the wells of a 12-well plate with 250 μL/well 2% sterile agarose in order to limit cell migration out of the scaffolds (see Notes 5 and 6).
2. Load 5×10^5 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 2.5×10^5 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 5×10^5 fibroblasts/scaffold.

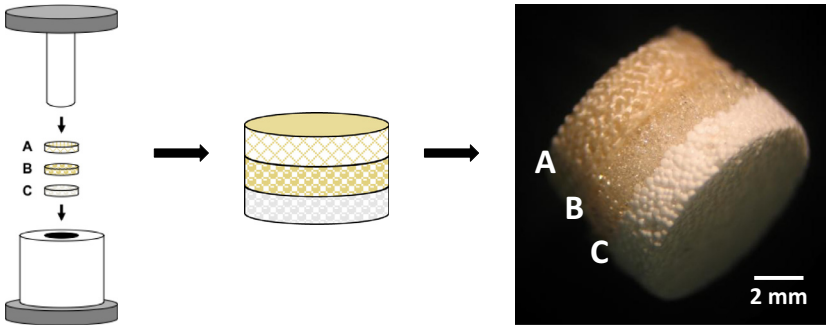

Fig. 1. Tri-phasic scaffold fabrication. A, B, and C correspond to the phases listed under Section 3.1 Scaffold phase fabrication.

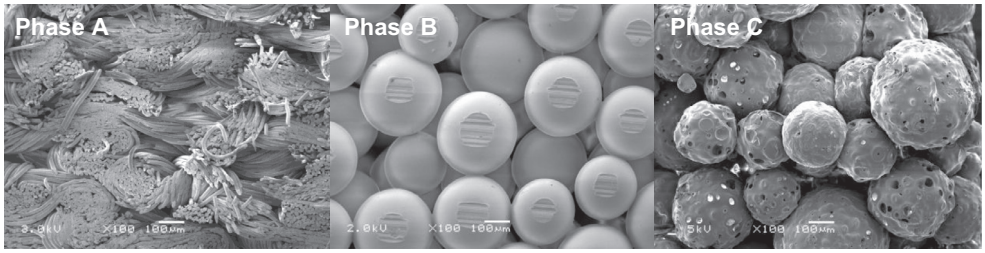

Fig. 2. Microstructure of phases A, B, and C of the tri-phasic scaffold.

7. Allow the cells in tri-culture (see Note 9) to attach for 20 min, followed by the addition of 3 mL F/S culture media in each well of the 12-well plate.
8. Incubate the samples at 37 °C under humidified conditions and 5% CO_2.

4. Results

Adherence to the proposed fabrication protocol produced a tri-phasic scaffold designed for multi-tissue integration via co- or tri-culture of cells within heterogeneous, stratified scaffold layers [34,35] (*see* Fig. 2) where each phase displayed significantly different porosity characteristics and intrusion volumes post-fabrication, summarized in Table 1. The height and diameter of each phase was similar, while porosity and the resulting intrusion volume decreased from Phase A to B to C. This demonstrates the capability of the fabrication design to produce a structurally contiguous scaffold with distinct, integrated layers that mimic the transition between multiples tissue types.

5. Discussion and conclusions

Here, tri-culture of fibroblasts, chondrocytes, and osteoblasts within distinct regions of the scaffold is suggested to recapitulate the ligament–fibrocartilage–bone interface associated with ACL repair [34,35]. The scaffold design is broadly applicable in other areas of composite tissue engineering, and provides a useful tool for multi-tissue integration and functional repair. Additionally, the scaffold's ease of fabrication and seamless transition between tissue types makes it an attractive option in this field, where stratified designs facilitate multi-tissue healing. The use of sintering in this system is especially advantageous, where the same bulk polymer is utilized in each phase and a single glass transition temperature is exceeded to achieve successful sintering. Through this method, polymer chains in each phase rearrange simultaneously and promote integration of adjacent, stratified scaffold layers. Intermingling of the polymer network during sintering prevents delamination. Moreover, composition and organization of the layers can be tailored to achieve desired local and bulk mechanical properties. Here, a functionally graded structure is formed with increasing compressive mechanical properties progressing from Phase A to Phase C. While this study demonstrates the feasibility of simultaneously engineering three types of integrated tissues, additional layers can be sintered on in order to form other composite tissues or functional tissue units. This design may be applied to a variety of biomimetic tissue engineering applications, where multi-layer scaffolds are desired for regeneration of stratified and functionally integrated tissues.

6. Notes

1. Dissolve 20× collagenase II into Dulbecco's modified eagle medium containing only antibiotics (no fetal bovine serum). Filter this with a syringe before adding to F/S culture media to achieve a final solution with 10% collagenase II.

2. Supplemented culture media should be stored at 4 °C when not in use, and may be stored for up to 1 month prior to use.

3. Filter PBS using a sterile filter with 0.22 μm pores.

4. Sterile-filtered PBS can be stored in a glass container at room temperature for up to 6 months after filtering.

Table 1
Summary of postfabrication characterization of triphasic scaffold phases, modified from Spalazzi et al., 2006 [35].

Phase	Composition	Height (mm, $n = 15$)	Diameter (mm, $n = 5$)	Porosity (%, $n = 3$)	Mode pore diameter (μm, $n = 3$)	Intrusion volume (μL, $n = 3$)
A	10:90 PLGA	2.44 ± 0.14	6.75 ± 0.12	58 ± 5	73 ± 11	41 ± 8
B	85:15 PLGA	2.2 ± 0.2	7.32 ± 0.08	34 ± 4	75 ± 7	28 ± 7
C	80% 85:15 PLGA, 20% BG	1.5 ± 0.2	6.5 ± 0.4	26.7 ± 0.4	62 ± 3	14.5 ± 0.1

5. Liquefy agarose by microwaving, and allow the solution to cool/gel for 10–20 min at room temperature. The gelling temperature of agarose is 32–45 °C.

6. Post-fabrication diameter of scaffold phases A, B, and C described for cell seeding is approximately 6.5, 6.9, and 7.1 mm, respectively, and the thickness for each phase measures 1.85, 1.90, and 1.84 mm, respectively.

7. For co-culture of osteoblasts and fibroblasts, skip steps 2 and 3.

8. The fibroblast-to-osteoblast ratio was 2:1 due to the higher surface area of Phase A as determined by mercury porosimetry.

9. For tri-culture, the primary cells used are typically between passage 2 and 5.

Acknowledgments

This work was supported by the National Institutes of Health (R01-AR055280, HHL), Presidential Early Career Award for Scientists and Engineers (PECASE, HHL), the National Science Foundation (Graduate Research Fellowship, JPS) and the New York State Stem Cell ESSC Board (NYSTEM, HHL).

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