Hybrid Additive Microfabrication Scaffold Incorporated with Highly Aligned Nanofibers for Musculoskeletal Tissues

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Received: 27 June 2018 / Revised: 8 October 2018 / Accepted: 9 November 2018 / Published online: 1 December 2018

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Abstract

BACKGROUND: Latest tissue engineering strategies for musculoskeletal tissues regeneration focus on creating a biomimetic microenvironment closely resembling the natural topology of extracellular matrix. This paper presents a novel musculoskeletal tissue scaffold fabricated by hybrid additive manufacturing method.

METHODS: The skeleton of the scaffold was 3D printed by fused deposition modeling, and a layer of random or aligned polycaprolactone nanofibers were embedded between two frames. A parametric study was performed to investigate the effects of process parameters on nanofiber morphology. A compression test was performed to study the mechanical properties of the scaffold. Human fibroblast cells were cultured in the scaffold for 7 days to evaluate the effect of scaffold microstructure on cell growth.

RESULTS: The tip-to-collector distance showed a positive correlation with the fiber alignment, and the electrospinning time showed a negative correlation with the fiber density. With reinforced nanofibers, the hybrid scaffold demonstrated superior compression strength compared to conventional 3D-printed scaffold. The hybrid scaffold with aligned nanofibers led to higher cell attachment and proliferation rates, and a directional cell organization. In addition, there was a nonlinear relationship between the fiber diameter/density and the cell actinfilament density.

CONCLUSION: This hybrid biofabrication process can be established as a highly efficient and scalable platform to fabricate biomimetic scaffolds with patterned fibrous microstructure, and will facilitate future development of clinical solutions for musculoskeletal tissue regeneration.

Keywords Musculoskeletal tissues · Hybrid biofabrication · Patterned fibrous microstructure · 3D printing · Electrospinning

1 Introduction

It is reported that in the United States, about 250,000 anterior cruciate ligament (ACL) injuries occur annually [1], and between 200,000 and 300,000 rotator cuffs are surgically repaired each year [2]. Current surgical repair

techniques have high re-injury rates: up to 94% of repaired massive rotator cuff tears fail and 56% of ACL reconstruction patients experience knee pain 1 year post-surgery [3]. Halving the OA osteoarthritis after ACL injury alone could lead to \$1.1 billion annual cost savings [4]. Current treatment options generally involve removing the tear while trying to preserve as much healthy tissue as possible, and potentially replacing the missing part with a graft from a donor. However, in clinical practice it still remains challenging to fully restore the damaged tissues especially when the avascular zone is also involved. In addition, the results are often limited to short-term pain relief and slight

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improvement of joint function [5]. In recent years, tissue engineering has emerged as an alternative strategy for musculoskeletal tissue regeneration, including tendon, ligament, and meniscus, etc. Tissue engineering strategies for musculoskeletal tissue development focus on replicating the native cell environment in its complex cellular and biochemical composition, as well as the micro- and nano-structure of the extracellular matrix. The patterned microstructure, especially the fibrous structure, is important for tissue functions such as stabilization of intercellular junction positioning [6], and regulation of cell growth and communication [7].

Conventional methods of scaffold manufacturing include solvent casting, particulate leaching, gas foaming, fiber meshes, and bonding. These are limited in their capability to offer precise pore size, geometry and high mechanical strength. Therefore, engineers are looking into hybrid printing methods that incorporate two or more available manufacturing techniques to achieve a superior structure. Shim et al. [8] proposed a hybrid scaffold with synthetic biomaterials and hydrogel using solid freeform fabrication technology. Here hydrogel was intentionally infused into the space between the lines of a synthetic biomaterial-based scaffold and collagen was dispensed on the structure. The group was able to show superior effects on cell adhesion and proliferation and showed the high viability of dispensed cells. Another approach is the use of 3D bioprinting and electrospinning together to create a more porous and a mechanically stable structure, effectively surpassing the limitations of each individual process. Yan et al. [9] proposed a method where 3D printing is used to create a macro-scaled scaffold, which is followed by compositing near field electrospinning to build tissue micro-morphology with a specific extracellular matrix structure. The process was switched and repeated to obtain a composite structure. The composite structure seemed to

demonstrate an elevated level of porosity and improved mechanical properties. However, the efficacy of this scaffold in terms of cellular growth and cell proliferation is yet to be determined.

This paper presents a novel hybrid fabrication method for musculoskeletal tissue scaffold. The scaffold has a 3D-printed frame integrated with aligned nanofibers to mimic the microstructure of the native meniscus extracellular matrix. The scaffold is composed of four 3D-printed polylactic acid (PLA) layers with an electrospun poly(ϵ -caprolactone) (PCL) nanofiber mat between each layer. We hypothesize that the nanofiber filler will not only enhance the cell attachment rate, but also provide a micro topological cue for cells to organize into a patterned structure. The objective of this paper is to investigate the mechanical and biological performance of the hybrid scaffold.

2 Material and methodology

2.1 Fabrication of the hybrid scaffolds

The skeleton of the scaffold was 3D-printed by fused deposition modeling (Ultimaker[®] 3, Geldermalsen, Netherlands). The design of the PLA frames is shown in Fig. 1. The scaffold consists in four square-shaped layers clipped on top of each other. The 4×4 grid design in the middle allows proper cell attachment and proliferation. The fabrication process of the hybrid scaffold was demonstrated in Fig. 2. A layer of electrospun PCL nanofibers were embedded between two frames. The polymer solution was prepared by dissolving PCL pellets in DMF/chloroform (1:1 volume ratio). To fabricate random fibers, the 3D-printed samples were laid on a 5×5 cm aluminum sheet connected to a negative potential of -2.83 V. The needle voltage was 10.83 V for every sample. To fabricate the

Fig. 1 **A** Design of the 3D-printed scaffold frame. **B** Dimension summaries of four layers of frames

Fig. 2 Schematic illustration of the fabrication process for one layer of the hybrid scaffold

aligned fibers, the samples were placed on a PLA support between two ground-connected metal rods separated by 4 cm.

In Phase-1, two process factors were investigated to alter the nanofiber attributes, namely tip-to-collector distance and electrospinning time. In order to achieve the same level of fiber density, the electrospinning time for random and aligned fibers were set differently. The two levels of electrospinning time were 45 s and 90 s for random fibers, and 150 s and 360 s for aligned fibers. The tip-to-collector distance was set to be 12.5 cm or 16 cm. In Phase-2, the

interactive effects of PCL concentrations and tip-to-collector distance were investigated. The PCL concentrations were 10%, 15% and 20% (weight/volume). A complete factorial design resulted in a total of six groups. Electrospinning chamber was maintained at an approximate temperature of 38 °C and 40% relative humidity for all experiments. The morphology of the nanofibers was characterized by Scanning Electron Microscopy (SEM) (Phenom Pro, Phenom-World B.V., Eindhoven, Netherlands) and analyzed by ImageJ.

Fig. 3 Compression test results of the hybrid scaffolds

Fig. 4 SEM images of A, B, the aligned fibers and C random fibers on the 3D-printed scaffold

Fig. 5 The effects of tip-to-collector distance and electrospinning time on nanofiber attributes: **A–D** summaries of fiber alignment; **E** summaries of fiber diameter, and **F, G** summaries of fiber density

2.2 Mechanical properties study

We conducted a compression test on the scaffolds with and without PCL nanofibers (electrospun by 15% PCL solution) to study the effects of nanofiber alignment on the mechanical properties of the scaffold. We stacked three sets of scaffolds on top of each other achieving a test object of twelve PLA 3D-printed scaffold stacked on top of each other. The test was conducted on a Shimadzu Tensile tester (Shimadzu Corporation, model AGS—50 kNXD, Kyoto, Japan) with a test speed of 2.5 mm/min with a full scale of 50,000 N. Test data was used to plot a stress–strain curve and calculated the compression modulus in the end.

2.3 Cell culture

Human fibroblast cells (ATCC MRC-5 CCL-171TM) were used for the cell culture study. The cells were quickly thawed, centrifuged, and resuspended in Eagle's Minimum Essential Medium (EMEM, ATCC[®] 30-2003TM) with 10% fetal bovine serum (FBS, ATCC[®] 30-2020TM) and 100 µg/mL ampicillin (Santa Cruz Biotechnology, Dallas, TX, USA). The cells were cultured in 75-cm² flasks for a week to reach 80% confluency for the study. Each electrospun scaffold was soaked in 70% isopropyl alcohol prior to electrospinning. Once ready they were washed in a phosphate buffer solution (PBS) and then sterilized under UV light for 90 min. Scaffolds without nanofibers served as the control group. The tests were conducted in triplicate. An approximate number of 300,000 fibroblast cells per each well was cultured in a 2 mm of total medium containing 90% Eagles Minimum Essential Medium (EMEM, ATCC, Manassas, VA, USA) with 10% Fetal Bovine Serum (FBS, ATCC, Manassas, VA, USA). The cultured setup was incubated in an incubator with 5% CO₂ at 37 °C for 7 days. The culture medium was changed after day 1 and then every 3 days. Cell viability, attachment, proliferation and morphology studies were carried out on the 7th day.

On 7th day scaffolds were transferred to a new well plate. AlamarBlue assay quantified the cell viability. Here the presence of healthy viable cells causes the reduction in AlamarBlue (Thermo Fisher Scientific, Waltham, MA, USA) concentration which allows us to quantify the cell viability. We added equivalent amount of assay to 10% of total volume in the well and analyzed via Spectrometer as per supplier's protocol. The cell morphology was evaluated with the aid of fluorescent microscopy. DAPI (Santa Cruz Biotechnology, Dallas, TX, USA) with a concentration

300 nM and Phalloidin stock solution (Santa Cruz Biotechnology, Dallas, TX, USA) with 1% concentration were used to stain cell nuclei and filamentous actin respectively.

In Phase-1, we investigated the effects of fiber alignment on cell growth. Cells were cultured in scaffolds with random or aligned fibers, and scaffolds without nanofibers served as the control group. In Phase-2, we investigated the effects of fiber diameter and density on the cell growth. Aligned nanofibers were electrospun from different PCL concentrations with different tip-to-collector distances as described in Sect. 2.1.

3 Results

Figure 3 presents the compression test results. The compression modulus of the scaffold without nanofibers was approximately 380 MPa. Incorporated with random fibers, the compression modulus of the scaffold increased to 400 MPa. The scaffold with aligned fibers showed the highest compression modulus of 490 MPa, which was a 29% increase from the bare scaffold. Interestingly, addition of random nanofibers did not increase the compression strength of the scaffold, while the aligned nanofibers increased the strength by approximately 46%. It indicates that a uniaxially organized structure would lead to a stronger and stiffer scaffold.

Figure 4 displays the SEM morphology of both aligned and random electrospun fibers. Through parallel electrospinning, highly aligned electrospun fibers are well embedded in the 3D-printed construct. The fiber density, porosity, and diameter data for Phase-1 study were summarized in Fig. 5. The analysis of the electrospun scaffolds revealed that all electrospun fibers were within 250–550 nm. The diameter of random fibers was at the same level as the aligned fibers. Increasing the tip-to-collector distance enhanced the fiber alignment and decreased the fiber diameter. The porosity calculation of each structure showed a porosity of 56.44% with a standard deviation of 12.8% for aligned fibers, and a porosity of 76.64% with a standard deviation of 4.7% for random fibers. For both random and aligned fibers, increasing the electrospinning times reduced the porosity because more fibers were deposited.

The cell proliferation of Phase-1 study was summarized in Fig. 6. The alamarBlue test revealed that all hybrid scaffolds were biocompatible and led to a higher cell proliferation rate than scaffolds without nanofibers. This was because the nanofibers provided more areas for cells to attach, especially in the pore areas of the 3D-printed scaffolds. Scaffolds with random nanofibers showed a marginally higher cell proliferation than those with aligned

Fig. 6 AlamarBlue test results on the 7th day of culturing

fibers. This was consistent with the porosity results that a lower porosity permitted a higher cell attachment rate. It should be noted that the porosity should be kept in an optimal range for both cell attachment and cell infiltration. Stained cells were observed under fluorescent microscopy to determine the cell attachment and organization. Figure 7 shows that aligned fibers induced an alignment of cell

organization which mimics the tissue pattern widely observed in native musculoskeletal tissues. Without nanofibers, cells only attached on the struts of the 3D-printed scaffold. A higher cell density was observed in scaffolds with random nanofibers, however, there was no predefined pattern in cell organization.

Subsequently we performed the Phase-2 study to determine the effects of PCL concentration and fiber diameter on cell adhesion and proliferation. Figure 8 summarizes the fiber characteristics. There was a clear positive correlation between fiber diameter and viscosity of the solution. When the PCL concentration increased to 20%, the average fiber diameter exceeded 1 micron. As the result, the overall porosity decreased accordingly. All constructs showed an approximate porosity within 40–60%. This study shows that the fiber diameter and density can be controlled by both solution viscosity and electrospinning distance.

Figure 9 shows the cell attachment and morphology from the Phase-2 study. The aligned nanofiber array influenced the organization of the cells in all groups. However, nanofibers electrospun from 15% PCL with an

Fig. 7 Fluorescence images of fibroblasts **A1–C1** on the struts of the scaffold without nanofibers, **A2–C2** on the random nanofibers of the hybrid scaffold, and **A3–C3** on the aligned nanofibers of the

hybrid scaffold. Scale bar = 200 μ m. Blue stain: cell nucleus; green stain: cell actin filaments

Fig. 8 Characterization of fiber diameter of nanofibers by parallel electrospinning

electrospinning distance of 12.5 cm resulted in the highest actin filament density, indicating a better cell attachment and proliferation. 20% PCL increased the fiber diameter to over one micron, which reduced the total surface area for cellular attachment. 10% PCL, on the other hand, caused a higher porosity thus also decreased the directional growth of cells. This result indicates nonlinear relationships between the fiber diameter, density and cell growth. Quantitative studies will be needed in future to understand the interaction mechanisms and to determine the optimal designs of the hybrid scaffolds.

4 Discussion

Extra-cellular matrix (ECM) plays a profound role in the fate of tissue formation *in vivo*. Typically, four structurally distinct zones can be identified within entheses: tendon/ligament, non-mineralized fibrocartilage, mineralized fibrocartilage, and bone. The soft tissue region contains fibrous connective tissue characterized by the presence of parallel collagen fibers with interspaced elongated fibroblasts organized in arrays. Fibrocartilage tissue is avascular. Fibrochondrocytes neither express connexins and nor form gap junctions [10]. This often results in poor healing response observed at and near attachment sites. Thus, intercellular communication needs to take place indirectly via cell–matrix interactions or soluble factors [11]. In addition to tendon/ligament, the meniscus also has a unique anisotropic collagen fiber and extracellular matrix (ECM) organization, which confers its mechanical function. Creation of this patterned fibrous structure and zonal organization is thus critical to the success of meniscus regeneration at large scale.

3D printing and electrospinning are two popular scaffold fabrication techniques for tissue engineering applications [12, 13]. Efforts have been made to combine

electrospinning and 3D printing for hybrid scaffolds [14–16], in which random electrospun nanofibers were integrated within the 3D-printed scaffold. In a recent study, Baek et al. [17] examined meniscus tissue generation by encapsulating cell-seeded aligned nanofiber scaffolds into hydrogels. In this study, a novel hybrid fabrication system has been developed to combine both electrospinning and fused deposition modeling for directed cellular growth and enhanced mechanical properties. PCL was used for electrospinning due to its biodegradability and built in cell binding sites. Highly aligned electro spun fibers were used to produce extremely organized porous structures that encourage cellular infiltration and proliferation to mimic the native structure of tendon, ligament and cartilage. It was suggested that a larger porosity enhances the natural cell attachment compared to structures with smaller porosity [9, 10]. Correspondingly, achieving a porosity of 50–60% increases cell attachment due to increased surface area and binding sites compared to a high-resolution 3D printed scaffold with no fibers.

The scaffold with aligned fibers showed the highest compression modulus of 490 MPa, which was a 22.5% increase from the scaffold with Random fibers. Our results agree with the study conducted by Stylianopoulos et al. [19] which showed the elastic modulus of electrospun fibers increase as the angular standard deviation to the fiber orientation decreases. It was also suggested that the fiber orientation was positive correlated with the mechanical strength of scaffolds [20]. Structural analysis using SEM showed the highly organized porous structure as shown in Figs. 4 and 5. Subsequent measurements showed an average fiber diameter in sub-micron level and an average porosity up to 70%. Electrospinning process was carefully monitored and calibrated to produce a fibrous structure. However due to viscosity and surface tension of the solution it may form beads and clusters instead of continuous fibers.

Fig. 9 Fluorescence images of fibroblasts on the aligned nanofibers generated by different PCL concentrations and tip-to-collector distances. **A1–A3** 10% PCL with 12.5 cm, **B1–B3** 10% PCL with 16 cm, **C1–C3** 15% PCL with 12.5 cm, **D1–D3** 15% PCL with 16 cm, **E1–E3** 20% PCL with 12.5 cm, and **F1–F3** 20% PCL with 16 cm. Scale bar = 200 μ m. Blue stain: cell nucleus; green stain: cell actin filaments

Printed hybrid constructs with fibroblast cells were analyzed using the alamarblue assay. All constructs showed a high reduction rate verifying that the combined electrospinning/printing process did not affect the viability of the cells. In order to study cell attachment and proliferation fibroblast cells were stained by DAPI and Phalloidin solutions and observed under a fluorescent microscope. Cells were firmly attached and spread out along the direction of the fiber alignment. This proves our initial hypothesis that well-organized nanofiber structure can be used for directing cell growth. This is important to engineering tissues with patterned microstructures, such as the radial and circumfixal fibrous structure in meniscus.

The architecture of electrospun scaffolds can be controlled by the polymer flow rate, needle size, voltage, and electric field and the collector geometry [18]. The current system we used to fabricate the constructs require going from one machine to another exposing it to contaminants. Thus, development of a system that allows both 3D printing and electrospinning to be done in a sterile environment is required. A general shape and a construct were used in this experiment, which could be improved by using MRI images of a defect in the knee meniscus as an outline to print a matching construct. Careful selection of scaffold shape for each patient's requirement would allow the implant to withstand mechanical forces while encouraging new cells to organize and replace the damaged area. We believe that this technique can also be extended to other tissue regenerative purposes, including cartilage [19] and muscle tissue engineering [20], where fibrous ECM plays a critical role in tissue functionality. Our proposed system was able to overcome the major obstacles associated with conventional bioprinting and electrospinning on their own and expanded the efficacy of biofabrication technology.

The major advantage of our technique over previous works is that we developed a new fabrication strategy of hybrid biomimetic scaffolds which incorporate aligned fibrous structure (sub-micron scale) and grid structure (minimeter scale) in a sandwich architecture. The aligned nanofibers not only enhanced the mechanical properties but also provided a topological cue to direct cell growth. This research will lead to several future works in soft tissue-to-bone interface engineering. First, the study of the cell culture strategies, culture of mesenchymal stem cells

(MSCs) alone, and coculture of MSCs together with differentiated cells in the proposed gradient scaffold. Second, the optimization of scaffold design and biomaterials, microfiber composition, morphology, mechanical properties for different applications. Finally, development of multi-cell culture system incorporated with growth factors, such as biomolecular and functional nanoparticles, to promote tissue formation.

This study demonstrated an effective approach for creating hybrid scaffold by combining 3D printing and electrospinning techniques. The consequential scaffold exhibited micrometer scale porous structure with increased cell binding sites and mechanical properties. Incorporation of highly aligned fibers resulted in well directed and organized cell growth and morphology. This hybrid biofabrication process can be established as a highly efficient and scalable platform to fabricate biomimetic scaffolds with patterned fibrous microstructure. It will facilitate future development of clinical solutions for musculoskeletal tissue regeneration.

Acknowledgements This paper was financially supported by the Foundation of the Whitacre College of Engineering and the Office of Vice President for Research at Texas Tech University.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Ethical statement The protocol of cell culture study was approved by the Texas Tech University Institutional Biosafety Committee (IBC#: 1705B1).

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