Solid Freeform Fabrication 2018: Proceedings of the 29th Annual International Solid Freeform Fabrication Symposium – An Additive Manufacturing Conference
Reviewed Paper

Fabrication of aligned nanofibers along Z-axis – A novel 3D Electrospinning technique

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Abstract

This study presents a 3D fabrication technique of nanofibrous scaffold for tissue engineering. A divergence static electric field was introduced in an electrospinning system to induce a self-assembly of aligned nanofibers into a tunable 3D architecture with thickness ranging from 2-12 mm. The effects of collector configuration on polycaprolactone (PCL) nanofiber attributes were investigated. Human fibroblast cells were cultured on the nanofiber scaffold in vitro for 7 days. It was found that the width and inclination angle of the collector influenced the nanofiber density distribution. The cells proliferated on the scaffold and organized as a fibrous matrix which mimicked the microstructure of native musculoskeletal tissues.

Keywords: divergence electrospinning, 3D nanofiber scaffold, tissue engineering

Introduction

The demand for human organ substitutes and the limited availability of transplant donors has advanced the development of tissue engineering. Tissue engineering generally requires the use of artificial scaffolds to recreating the in vivo milieu which coordinates a combination of biophysical and biochemical stimuli governed by complex spatiotemporal mechanisms to control cell behavior [1, 2]. The scaffold fabrication processes can generally be classified as top-down or bottom-up methods [3]. In top-down approaches, the biomaterials may be shaped to resemble biological geometries first, and the cells then seeded homogenously in the scaffold. With bottom-up approaches, modular units of cells and biomaterials are integrated to form 2D or 3D composite architecture. Common top-down approaches include solvent casting, particulate-leaching techniques, gas foaming, phase separation, freeze drying, melt molding, electrospinning and others. Research in this area tends to focus on optimization of the scaffold design by controlling the pore characteristics and reinforcing the biological properties. Bioprinting is one of the most extensively studied bottom-up techniques for scaffold development. In bioprinting, small units of cells and biomaterials are dispensed with high precision to form tissue-like structures in a layer-by-layer fashion. The three major bioprinting techniques are inkjet, laser-assisted, and extrusion [4]. To date, no single technique enables the production of all scales and complexities of synthetic tissues [3].

Recent bioinspired approaches focus on creating a biomimetic cell microenvironment closely resembling the natural gradients in cell distribution, extracellular matrix (ECM), and tissue topology. ECM is a collection of extracellular molecules secreted by cells that provides structural and biochemical support to the surrounding cells [1]. Gels of polysaccharides and fibrous proteins fill the interstitial space and act as a compression buffer against the stress placed on the ECM [2]. Studies show that micro- and nano-topography and local environment of ECM influence trends in cell behavior by providing biochemical and biophysical stimuli to promote cell adhesion,

proliferation, morphogenesis, and motility [3, 4]. Critical physical features of ECM include its dimensionality, architecture, stiffness, ligand topography, and density [5]. Despite rapid progress in engineering relatively simple tissues, there is a research gap in integrating the tunable microarchitecture in heterogeneous scaffold development to closely resemble natural tissue structures. Especially, the dimension constraints limit the further applications of existing additive manufacturing techniques to mimic the unique structures and properties of natural tissues.

In this paper we presented a rapid fabrication technique of 3D nanofibrous scaffold for tissue engineering. A divergence static electric field was introduced in an electrospinning system to induce a self-assembly of aligned polycaprolactone (PCL) nanofibers into a 3D architecture. A cell culture test was performed to demonstrate the feasibility of providing 3D physical cues to guide cell growth by the nanofiber scaffold.

Materials and Methods

2.1. Polymer solution preparation

PCL polymer solution was prepared by dissolving 15 wt.% PCL pellets (MW = 80,000, Sigma-Aldrich®, St. Louis, MO) in organic solvent. The solvent was prepared by homogenizing N,N-Dimethylformamide (99.8%, Sigma-Aldrich®, St. Louis, MO) and acetone (Thermo Fisher Scientific, Waltham, MA) at volume ratio of 1:1. The solution was magnetically stirred for 4 hours at room temperature until there was no visible suspended particulates. To stain the nanofibers, 0.1 mg/ml of DilC12(3) perchlorate (Thermo Fisher Scientific, Waltham, MA) was added to the PCL solution. All materials were purchased from Sigma-Aldrich® (St. Louis, Missouri, United States).

2.2. Electrospinning configuration

The divergence electrospinning was configured based on a high voltage direct current power source and a specially-designed polylactic acid (PLA) collector. The design of the collector was demonstrated in **Figure 1a**. The collector was comprised of two tilted surfaces, which had an inclination angle of 115 degrees to the collector bottom. This double-bevel collector was 3D printed by the Ultimaker3 system (Ultimaker®, Netherlands). Aluminum foil was glued on the inner surface of each bevel using PCL solution, and was connected to the aluminon wire through a small central hole on the bevel.

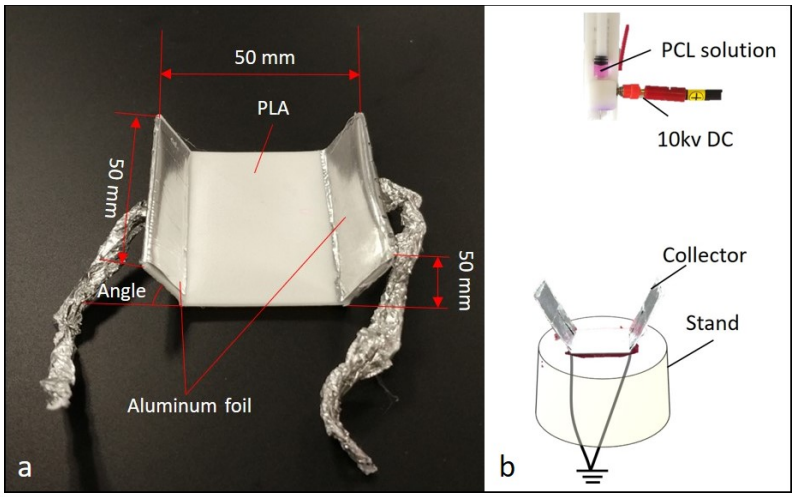

Figure 1. (a) Collector design. (b) Divergence electrospinning configuration

The collector was placed on an insulating stand in the electrospinning chamber. The aluminum foils on collectors were grounded separately using electrical wires passing through the inner cavity of the stand (**Figure 1b**). The PCL solution syringe was set to be 10 cm above the bottom center of the collector, and was pumped with a rate of 1 mL/hr. The voltage was set to be 10 kV, and the electrospinning time was approximately 2 minutes. The double-bevel induced a divergent electric field so that nanofibers were deposited onto both bevels of the collector while formed connecting fibers in between.

2.3. Scaffold characterization

To characterize the internal nanofiber distribution, the scaffold was vertically sectioned by glass slides as illustrated in **Figure 2**. The top and bottom sectioning was performed at the 2 mm from the top and bottom surface, respectively. The nanofiber morphology at each section was examined by scanning electron microscopy (SEM, Hitachi S-4300). Fiber diameter, density and alignment were analyzed by ImageJ after the SEM images was segmented and processed. Fiber density was characterized by counting the number of fibers that intersected a line drawn across the middle of a binary SEM image. Fiber alignment were characterized by convergence degree of the fiber orientation histogram.

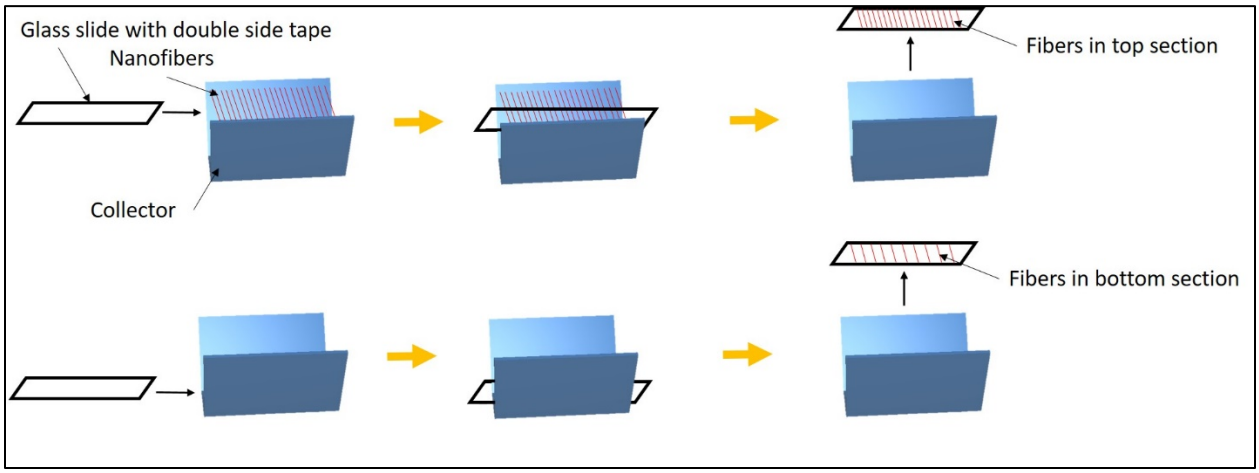

Figure 2. Sectioning of the nanofiber scaffold.

2.4. Cell Culture

We fabricated a scaled-down scaffold (20 mm × 10 mm, width × length) to fit the tissue culture plate. The collector was immersed in 70% ethanol for 30 minutes prior to electrospinning. The electrospinning chamber was sprayed and wiped with 70% ethanol and maintained sterile during electrospinning process. After the nanofiber scaffold was obtained, the collector and the scaffold were sterilized by UV light in the biosafety cabinet for 30 minutes. The scaffold in the collector was then placed in a sterile 50 ml beaker. Human fibroblast cells (ATCC®, MRC-5) were suspended in complete growth medium. The medium was slowly added into the beaker to cover the scaffold. The cell-seeded microfiber was incubated for 7 days. Cells were fixed with 4% formaldehyde after 7 days. For microscope imaging, cells were stained with CruzFluor Phalloidin fluorescence conjugate and 4',6-diamidino-2-phenylindole (DAPI) for cell cytoplasm and nucleus, respectively.

Results

The divergent electric field induced a nanofiber deposition on the surfaces of both bevels, meanwhile the whipping process led to a self-assembly of nanofiber between the two bevels. As a results, an aligned fiber bundle structure gradually formed between the two bevels from the bottom to the top (**Figure 3**). The thickness of the obtained nanofiber scaffold was approximately 20 mm. Fluorescent nanofibers at the top and bottom sections were presented in **Figure 4**. It was observed that the fiber density was higher at the top layers than at the bottom layers.

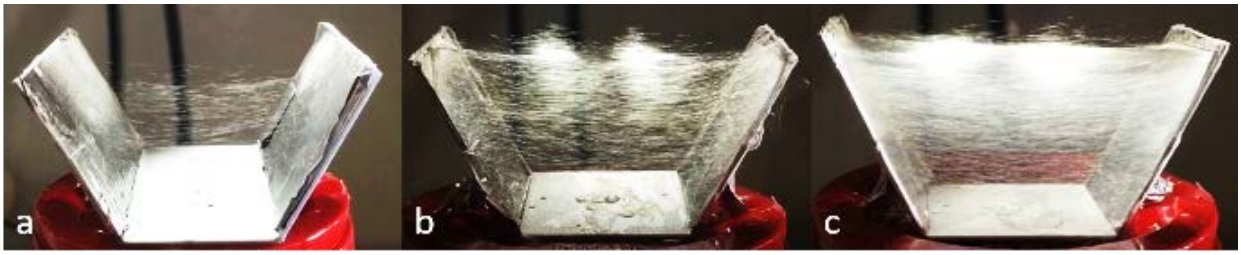

Figure 3. Formation of the 3D nanofiber scaffold. (a)-(c) represent 10 secs, 30 secs, and 60 secs after electrospinning, respectively.

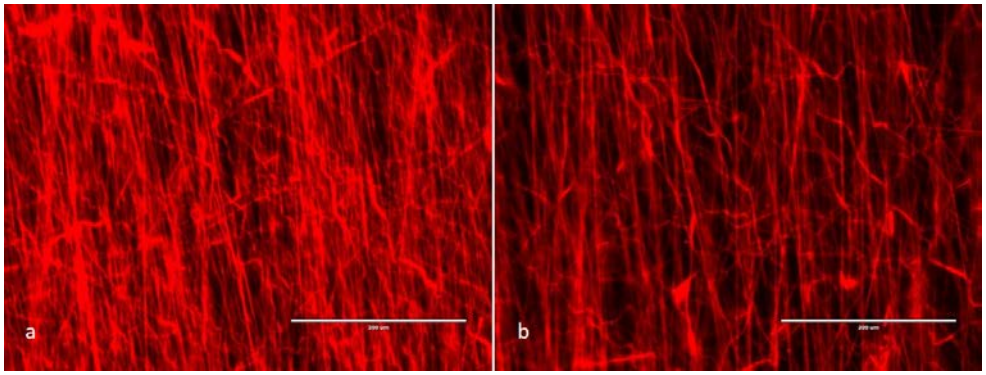

Figure 4. Fluorescent nanofibers at (a) the top section, and (b) the bottom section of the scaffold. Scale bar = 200 µm.

A summary of fiber diameter distribution and scaffold porosity was shown in **Figure 5**. The diameter of the nanofibers ranged from 200 nm to 780 nm. The mean values were was 468 nm at the top section, and 514 nm at the bottom section. No substantial difference in fiber diameter was found. On the other hand, a gradient in scaffold porosity along the z-axis of the collector was observed. The collector saw the densest fibers on both top and peripheral areas, resulting in a low porosity, while less fibers were formed at the bottom and central areas, resulting in a high porosity.

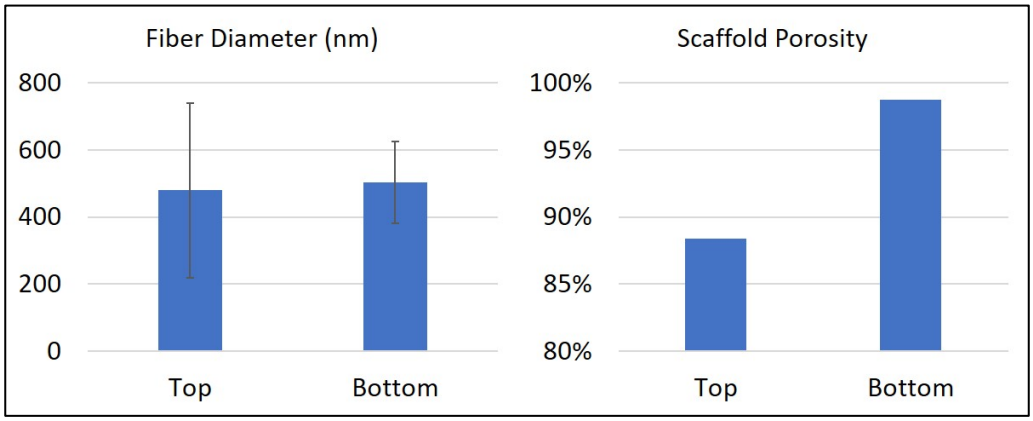

Figure 5. A summary of nanofiber diameter and scaffold porosity.

Representative images of cell culture were shown in **Figure 6**. The cells stretched along the nanofibers after 24 hours, and proliferated in the scaffold during the 7 days. The florescent image (**Figure 7**) showed the cell skeleton and organization, proving that the nanofiber scaffold guided the cell alignment and growth.

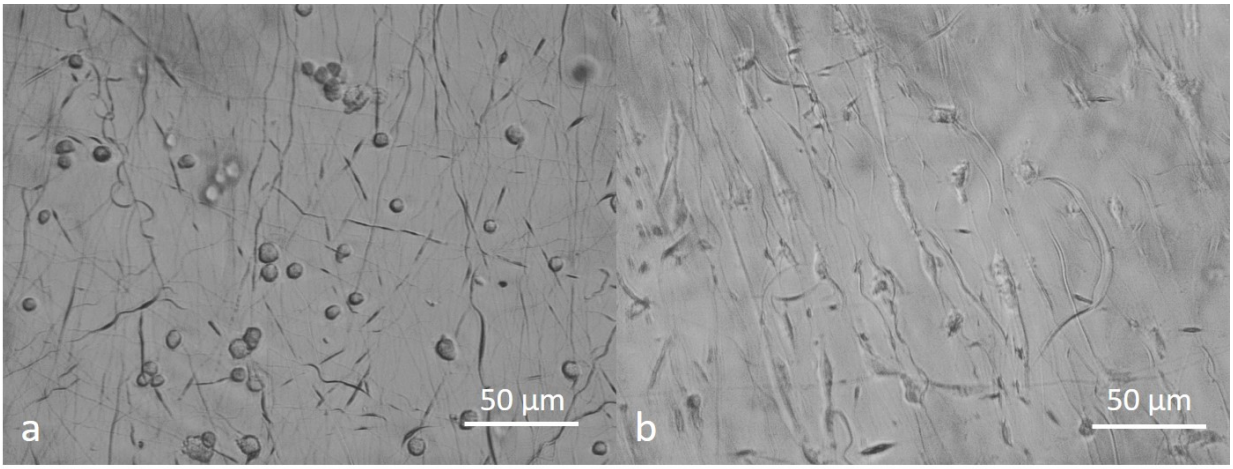

Figure 6. Fibroblast cells seeded on the scaffold after (a) 2 hours and (b) 24 hours.

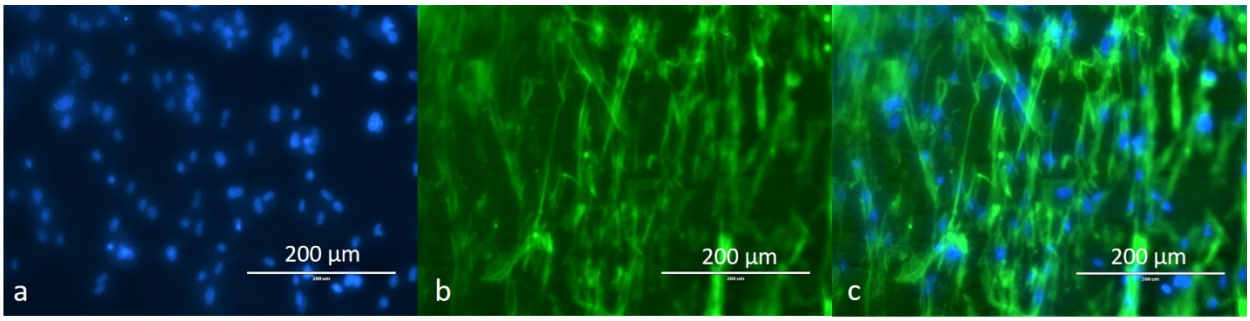

Figure 7. Florescent cells after 7 days. (a) nucleus stained by DAPI, (b) cytoplasm by Phalloidin, and (c) an overlap.

Discussion

As the ECM is a composite structure combining fibrous proteins held within gelatinous ground substance, the integration of micro-/nano-fibers and hydrogels may offer potential for development of superior composite scaffolds resembling the natural 3D environments with enhanced mechanical properties and cell penetration [6]. This becomes especially critical in applications involving musculoskeletal soft tissues such as tendons, ligaments, knee menisci, and the annulus fibrosus (AF) of the intervertebral disc, which have a more pronounced fibrous cytoskeletal organization [7]. Although the concept of combining hydrogel and microfiber is in its infancy, a number of pioneering works have been explored to create biomimic scaffold for different applications.

One of the most straightforward methods of creating fiber-embedded hydrogel is to mix cell-embedded medium with microfibers and then crosslink the hydrogel. Narayana et al. 3D-printed alginate hydrogel containing polylactic acid (PLA) nanofibers and human adipose-derived stem cells [7]. The electrospun PLA fibers were bended in alginate by vortex and sonication. The composite hydrogel was 3D printed into a shape of human medial knee meniscus. Results showed that the nanofiber-reinforced bioink allowed higher levels of cell proliferation within bioprinted strands. Fiber-hydrogel composites can also be achieved by encapsulating pre-determined fiber architecture within a hydrogel. Bas et al. combined the ECM-like structure of hydrogels with highly oriented PCL fibers fabricated by Melt Electrospinning Writing (MEW). Stacked fibers with lay-down patterns were prepared by MEW, and then infiltrated with hydrogels in custom-made molds and crosslinked by a reduction–oxidation initiating system. The fiber reinforced hydrogels achieved an outstanding increase in the mechanical properties at high strain rates. However, the thickness of the scaffold was limited to 1 mm.

This paper presents a novel divergence electrospinning strategy to rapid assemble aligned nanofibers along Z-axis by two axisymmetric and separately grounded bevels. This phenomenon was attributed to the whipping of the continuous electrospun jet in a symmetrically diverged electric field. While most of the nanofibers attached on either bevel of the collector, some remained between the two bevels due to the dragging effect of the whipping. As the fiber density increased, the electrostatic repulsive forces resulted from the residual charges on the fibers enhanced, driving the

subsequent electrospun fibers away from the existing bundles, resulting in a fast build-up along the z-axis.

The nanofibers were uniaxial aligned in the 3D scaffold. The results from fiber density analysis revealed a gradient of fiber density from top to bottom layers: a decrease of fiber density from top to bottom, as well as from periphery to the center. We hypothesized that this phenomenon was due to the repulsive effects attributed to the residual charges on the electrospun nanofibers. For regular electrospinning, the nanofibers are deposited on a grounded surface, where the repulsive forces (FR) from the accumulated residual charges are fractional compared to the static electric force (FE) until the fiber density and thickness reach a very high level (**Figure 8a**). In our case, the bridging nanofibers were hanged between two grounded bevels. The static electric forces exerted on the nanofiber were diverged to two directions, led to a weaker resultant force downward (**Figure 8b**). When nanofibers accumulated, the repulsive forces between the fibers might soon exceed the static electric forces and thus pushed the later electrospun fibers to the peripheral space. Once the nanofibers at the peripheral areas became denser, it would be more difficult for the electrospun jet reach the central area. Therefore the gradient of fiber density would only increase as electrospinning continued.

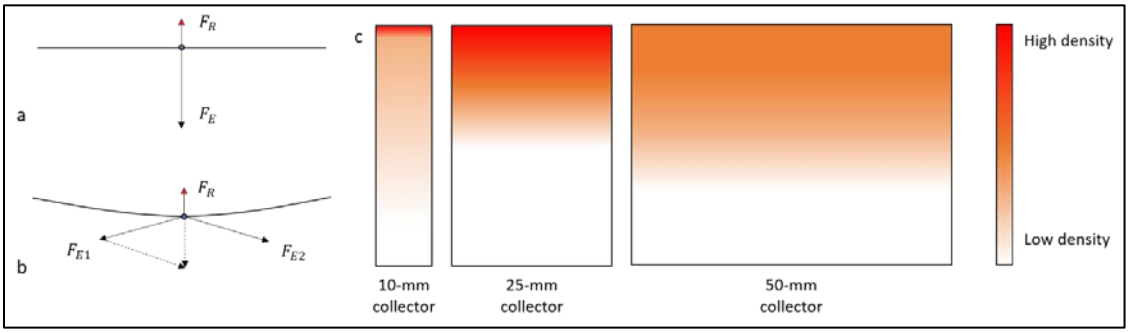

Figure 8. Force analysis of a single electrospun fiber in (a). a regular electric field, and (b). a divergent electric field. (c). A simulation of the fiber density along the z-axis of the collector bevel.

Cells were attached on the microfibers and grew along the fibers. This result is consistent with many previous studies on aligned fiber scaffold. It was reported that aligned micro- and nanofibers are favorable in replicating the ECM for specific tissues which is composed of perpendicularly interwoven collagen strips. An aligned electrospun fibrous scaffold can provide contact guidance to migration and extension, resulting in an elongation and alignment of cytoskeleton and nuclei along the axes of the fibers [8]. The effect of contact guidance of aligned electrospun fibers on cell morphological changes was evident in other many cell types [9, 10]. The scaffold developed in this study can be directly embedded in cell-laden hydrogel. This technique can be applied in engineering of musculoskeletal soft tissues such as tendons, ligaments, knee menisci, etc.[11], where fibrous cytoskeletal organization is critical for tissue formation and functions.

Future work will focus on how does the electrical and rheological properties of polymer solution determine the microfiber resolution during the dynamic stretching induced by the divergence

electric field. Most of the existing models for electrospinning focus on the rudimental configuration in which the microfibers are randomly electrospun onto a grounded surface driven by an ideal point-to-plane electric field. It is critical to establish the relationships between material properties, the finite extensibility of the polymer chains, and the microfiber diameter.

Conclusion

This paper presented a novel divergence electrospinning strategy with double-bevel collectors for 3D nanofiber scaffold fabrication. A highly aligned and porous nanofiber scaffold with a size in centimeter scale was rapidly fabricated through this technique. A porosity (fiber density) gradient was observed along the z-axis of the scaffold. The aligned fibrous structure induced an aligned cell attachment and growth. This study provided an innovative rapid fabrication approach for biomimetic microstructures and showed a great potential as a future additive microfabrication platform for tissue engineering.

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