

Microrobotically Fabricated Biological Scaffolds for Tissue Engineering

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Abstract—A microrobotic method for fabricating multilayered poly(lactic acid) (PLA) biological scaffolds using micropipettes for tissue engineering applications is presented. Biological scaffolds are fabricated over several different substrates by drawing and solidification of a viscous liquid polymer solution pumped continuously through a glass micropipette. The proposed method produces highly aligned, multilayered, crisscrossed fiber scaffolds with user specified pore sizes and diameters in the range from 1 to 10 micrometer. Attachment, proliferation and differentiation of C2C12 mouse pluripotent cells seeded on individual, parallel, and intersecting fibers is successfully demonstrated. The proposed robotic methodology consistently provides parameterized biological scaffolds to aid studies in tissue engineering and to develop novel MEMS, filtration and controlled drug delivery devices.

I. INTRODUCTION

Biological scaffolds are of increasing interest in biomedical and biotechnological applications. Scaffolds are used to promote cell adhesion, differentiation, and proliferation and as a mechanism to organize and direct the growth of cells and natural extracellular matrices (ECM). A

recent review reports in detail potential applications for scaffolds: tissue engineering, controlled drug release, dressings for wound healing, medical implants, nanocomposites for dental applications, molecular separation, biosensors, and preservation of active biological compounds [1].

Numerous techniques have been employed to fabricate artificial biological scaffolds for aforementioned applications. Such processes include electrospinning [2], [3], phase separation [4], [5], self assembly [6], [7], melt-blown [8], [9] and template synthesis [10], [11]. Electrospinning, a top-down approach, is a popular technique for the fabrication of biological scaffolds using a variety of materials. In electrospinning, an external electrostatic field is established between a pendant polymer solution droplet and a collector. When the electric field overcomes surface tension effects, fibers are formed and ejected towards the collector. This process is capable of producing fibers with small diameters ranging from microns to tens of nanometers and is relatively simple and reliable. However, the fibers produced are aligned chaotically, non-woven, and non-uniform in diameter. Special techniques have been used to create aligned electrospun fiber membranes [12], [13].

Similar to the melt-blown technique, drawing of suspended polymer fibers involves extruding a solution of polymer dissolved in solvent from a micropipette. Fiber formation occurs due to solvent evaporation from the extruded solution. In the drawing process, polymer solution is pumped continuously through the glass micropipette, thus enabling continuous fabrication of individual fibers in any three dimensional configuration [14], [15]. Moreover, this method offers greater flexibility in the control of key parameters of drawing such as the waiting time before drawing, the drawing speed and the drawn upon surface topography; thus, enabling repeatability and control on the dimensions of the fabricated fibers.

The key parameters of interest in any biological scaffold are porosity, pore size, orientation, and the dimension of individual structures [1]. Pore size is an important determinant of cell mobility within a scaffold. Cell proliferation is inhibited in scaffolds with pores too small [2]. However, it is observed that cells pass through scaffolds with pores too large, making cell adhesion difficult. Small diameter fibers having high specific area best mimic ECM, due to increased aqueous solubility, biocompatibility, bio-recognition, and polymer chain exposure and are therefore

Manuscript received September 15, 2006. The work was supported by the PITA grant from the Pennsylvania Department of Community and Economic Development Program and by the NSF CAREER award (IIS-0448042).

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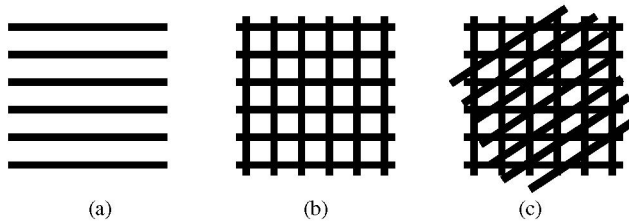


Fig. 1. Schematics of proposed biological scaffolds having high degree of alignment and controlled pore sizes: (a) single layer, (b) double layer, and (c) multiple layers.

conductive to tissue growth. It has also been shown that the degradation rate of microscale fibers directly correlates to its diameter [16]. Fiber orientation controls cell shape, physiological function, and organ architecture. Cells in vivo exhibit directional growth. Studies on the growth of smooth muscle cells on aligned, non-woven poly(L-lactid-co-ε-caprolactone) [P(LLA-CL)] fibers have shown increased proliferation, morphology, and adhesion compared to cells on polymer film [14].

This paper is structured as follows. In section II, we formulate the problem of drawing suspended fibers and attaching cells to them. In section III, a description of the experimental method for fabricating biological scaffolds and a strategy for cell adhesion to respective scaffolds is reported. This is followed by experimental results and discussions in section IV, and finally conclusions with future directions are presented in Section V.

II. PROBLEM STATEMENT

Initial goals of the project entail validating the proof of concept of achieving controlled cell adhesion on fabricated single, double and multiple layer fiber biological scaffolds built out of a biodegradable material. The fabricated biological scaffolds should have aligned fibers and the desired porosity and pore sizes as shown schematically in Fig. 1. Additionally, mimicking natural ECM requires fabricating micron/nanoscale diameter fibers. Cell adhesion to these biodegradable scaffolds requires making the surface of the fibers attractive for the cells to adhere and subsequently proliferate and differentiate. Scaffolds created using this approach should be able to withstand several processing steps for making the fibers attractive to cell adhesion and for subsequent imaging.

The proposed technique has not been tested and the feasibility has not been shown for controlled cell adhesion to highly aligned micro/nanoscale polymer fiber scaffolds with user defined porosities and pore sizes. Due to the very high surface-to-volume ratio of the polymer micro/nanofibers, several unknown constants such as surface chemistry of the fiber, probe tip size and its effects on desired pore sizes, polymer viscoelasticity, and robotic control strategies for fabricating scaffolds with precise shape control with high survivability rates need to be addressed.

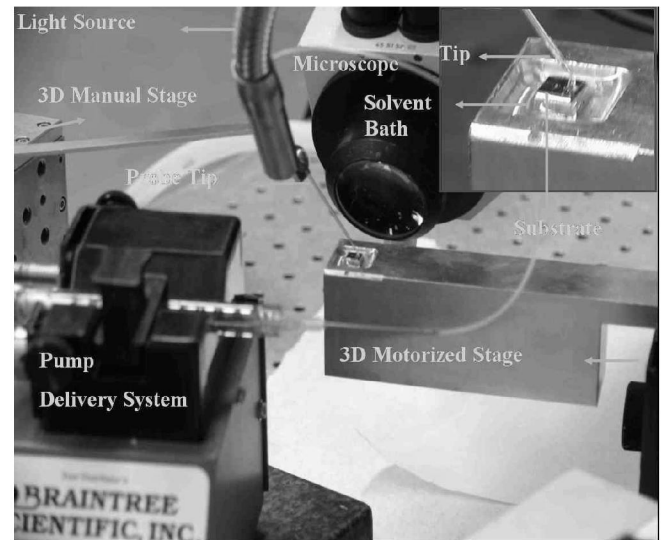


Fig. 2. Photograph of the experimental setup for glass micro-pipette based drawing. Inset shows the zoomed view of substrate and probe tip in an island of solvent bath.

III. METHODS

A. Fiber Drawing Experimental Setup

A glass micropipette is mounted to a manual XYZ stage and is stationary during the fiber drawing process as shown in Fig. 2. The polymer solution is pumped into the micropipette via capillary tube and syringe. The substrate is vacuum mounted to a computer controlled XYZ nanopositioner (VP25-XA, Newport Inc.). A vacuum pump is attached to the stage to hold the substrate in place during drawing. An optical microscope (Stemi SV 11, Carl Zeiss Inc.) is focused on the probe tip and a fiberoptic light source is used to illuminate the setup.

B. Microrobotic Fiber Drawing Strategy

In the drawing process, polymer solution is pumped continuously through the glass micropipette, which provides greater flexibility in drawing continuous fibers in any configuration. Moreover, this method offers increased flexibility in the control of key parameters of drawing such as waiting time before drawing, the drawing speed and the drawn upon surface topography; thus, enabling repeatability and control on the dimensions of the fabricated fibers.

Fiber attachment to the different substrates is highly dependent upon the surface chemistry. To increase the survivability of the fibers for cell adhesion, a novel ‘double attachment’ of the fibers to the substrate was developed to enhance the adhesion between the fiber scaffolds and substrate. The attachment scheme and drawing strategy is schematically represented in Fig. 3. The pipette is fixed almost perpendicular to a given substrate mounted on the motorized XYZ nanopositioner. At first, the substrate is raised until it comes into contact with the polymer droplet at the end of the glass micropipette tip (Step A, Fig. 3). Next, the stage is moved up and horizontally forming a double attachment for the fiber (shown by a star; Step B). Here, the

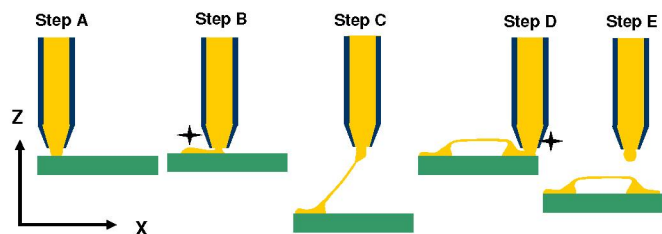


Fig. 3. Schematic of fiber drawing process: Step A: contact with substrate; Step B: move substrate up and horizontally forming 'double attachment'; Step C: retract substrate and move horizontally forming fiber by solvent evaporation; Step D: move substrate up and horizontally making contact with probe and dragging horizontally to form 'double attachment'; Step E: retract substrate and breaking contact with suspended fiber.

waiting time after stopping, i.e. before drawing a fiber, offers control on the viscosity of the polymer solution. The stage is then subsequently moved along a predetermined XYZ trajectory with tunable respective speeds, thus forming the solid polymer fiber by the evaporation of the solvent (Step C). After drawing the fiber, the substrate is brought in contact with the glass micropipette forming a suspended fiber (Step D). At this point, the stage is moved horizontally forming the second double attachment for the fiber (shown by a star; Step D). Finally, the substrate is retracted quickly, which breaks the contact with the droplet (Step E) and the probe tip is subsequently immersed in the solvent bath to remove extraneous polymer build up on the probe tip. The probe tip is then indexed to the next starting point and biological scaffolds are built sequentially with the desired pore size between consecutive fibers. The desired porosity is achieved by forming double and multiple layer scaffolds in user defined orientations.

C. Cell Attachment Strategy

Drawn polylactic-acid (PLA) scaffolds were fabricated over different substrates. The scaffolds consisted of one to four layers of fibers, 1-10 μm in diameter and spaced 8-40 μm apart, forming a 1 mm^2 bed of fibers onto which cells were seeded. Prior to cell seeding, the scaffolds were sterilized by submersion in 100% ethanol for ten minutes. Excess ethanol was gently aspirated and the scaffolds were allowed to air dry for five minutes. To remove air and ensure that no ethanol remained beneath the sample, scaffolds were slid over a 2 μl bead of basal cell culture medium (Dulbecco's Modified Essential Medium, 10% fetal calf serum and 1% penicillin/streptomycin (all from Life Technologies)). To minimize the effects of capillary forces and increase the survivability of scaffolds, the samples were immobilized to tissue culture wells by contacting with a drop of vacuum grease at the side. All scaffolds were seeded with C2C12 mouse pluripotent cells which have been genetically modified to stably express α -tubulin fused to green fluorescent protein (GFP) [17]. Cells were seeded on scaffolds in basal growth medium (as described above) and were incubated at 37°C, 5% CO_2 with humidity. Cells were allowed to attach for 4-5 hours and then imaged using fluorescence microscopy with excitation at 395 nm and emission at 540 nm. Fluorescence images were taken from

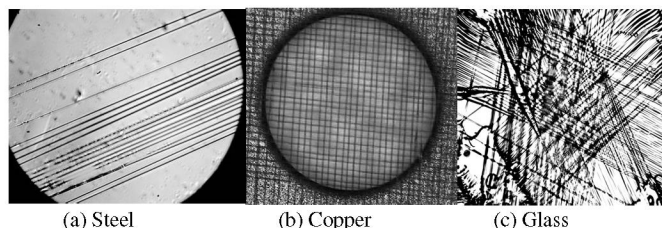


Fig. 4. Optical images of scaffolds on various substrates: (a) single layered steel, (b) double layered on copper, and (c) quadruple layered on glass.

above the scaffolds using water immersion lenses on a Zeiss AxioPlan 2 microscope employing Axiovision Software.

IV. EXPERIMENTAL RESULTS

A. Fabricating Biological Scaffolds

Fibers are formed when the substrate is moved from steps C and D (Fig. 3) and its success and properties are determined by the operator-defined two-dimensional trajectory. The path has three parameters: velocity, acceleration, and position of the stage in the X- and Z-directions. It is observed that the velocity and acceleration determine the thickness and uniformity of the fibers formed. Because of solvent evaporation from ejected solution, slow draw speeds form non-uniform diameters due to increased viscosity of the ejected volume. On the other hand at high drawing speeds, fibers are smaller in diameter and more uniform along fiber length, but fiber formation is more likely to fail due to increased tensile forces.

Pore size is limited to a certain extent by the probe tip diameter. Smaller tip diameters lead to closer packed scaffolds and larger diameter probe tips lead to adjoining fibers in the scaffold attached to each other. Polymer solution solidification inside the probe tips limits the duration of fiber drawing experiments, with the smaller probe tips having a short experimental life. Scaffolds with 8 μm pore size have been successfully fabricated using this approach. Pore size is further reduced and porosity is

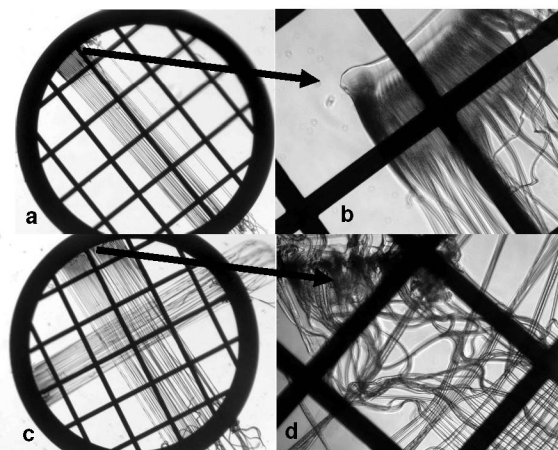


Fig. 5. Optical images of scaffolds over gold substrates before and after treatment for cell adhesion showing the detachment and fiber breakage: (a-b) single layer, (c-d) double layer.



Fig. 6. Optical image of double layered scaffold with adhesives applied to the ends.

increased by depositing multiple layers at different orientations to previously deposited layers. Some examples of scaffolds built over different substrates are shown in Fig. 4.

Fiber adhesion to the substrate is very important for successfully attaching cells to the scaffolds. The severity of the processing steps for preparing the scaffolds for cell adhesion often damages the scaffolds by detaching the fibers from the substrates as shown in Fig. 5.

The 'double attachment' scheme described before for enhancing the fiber adhesion provides enough surface for applying adhesives at the ends of the scaffolds, thus eliminating the problem of scaffold detaching from the substrate as shown in Fig. 6.

B. Cell Adhesion

C2C12 mouse pluripotent cells were seeded on the scaffolds and imaged using fluorescence microscopy and scanning electron microscopy (SEM) at different intervals. For SEM imaging scaffolds were fixed in 2% glutaraldehyde buffered with phosphate buffer solution (PBS) for at least one hour. Critical point CO₂ drying dissolved PLA and was unsuitable for preparation of the scaffolds for SEM. Instead, a series of osmium tetroxide (OsO₄) and thiocarbohydrazide treatments was used to strengthen the scaffolds before air drying. After 3 washes of PBS, the scaffolds were fixed for one hour in 1% OsO₄ buffered with PBS. Excess OsO₄ was removed with three, five minute washes of distilled water (dH₂O). Scaffolds were then exposed to a 1% aqueous thiocarbohydrazide solution for five minutes. The scaffolds were washed three times for five minutes with dH₂O, followed by a five minute exposure to aqueous 1% OsO₄. This four step regimen was repeated. The scaffold was then washed three times with dH₂O followed by dehydration in an ascending series of ethanol baths (50%, 70%, 80%, 90%, and three changes of 100%) and then air dried. Dried scaffolds were attached to SEM stubs using double stick tape, and coated with gold using a Pelco SC-6 sputter coater. Scaffolds were examined using a Hitachi 2460N Scanning Electron Microscope and digital images were obtained using Quartz PCI Image management system software.

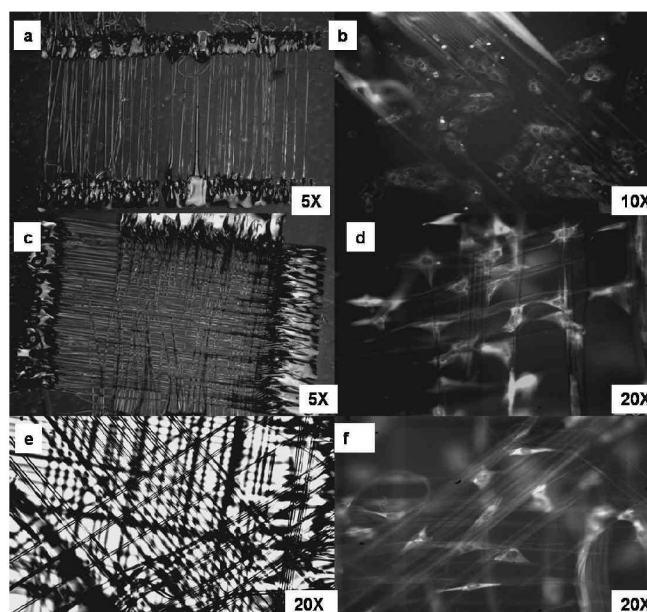


Fig. 7. Phase contrast (a-c) and bright field (e) images of PLA scaffolds seeded with cells and the corresponding (b,d,f) fluorescence images demonstrating cell adhesion.

Cell adhesion to the various scaffolds is demonstrated in Fig. 7. The phase contrast and bright field images were taken before seeding the scaffolds with cells and the fluorescence images were taken after three days. Single layered scaffolds (Fig. 7a) show good attachment initially, but after three days only a few cells are attached to the fibers, and most of them have proliferated on the underlying glass substrate (Fig. 7b). Decreasing the pore sizes by having multiple layers at different orientations greatly enhances cell adhesion to scaffolds as shown in Fig. 7(c-d) for two layered, and Fig. 7(e-f) for quadruple layered scaffolds.

Adhesion of cells to single layer scaffolds is demonstrated in Fig. 8. It was observed that when cells perform amoeboid movement through the fibers, they have enough force to either separate fibers (Fig. 8a) or clump them together (Fig. 8b). Of particular interest is the study of the dynamic nature of amoeboid movement of cells in the environment of solitary fibers as shown in Fig. 8c, where, one end of the cell is attached to the fiber and the other end to the underlying substrate, while the main body of the cell is attached to another fiber. Uniquely, in the absence of a neighboring fiber the cell attaches to a single fiber by completely wrapping around it (Fig. 8d) and a cell attached to the substrate and not proliferating can be seen directly underneath it.

Surface compatibility and attachment of cells to the fibers represent only a subset of tissue engineering, as an environment for increased attachment, growth and migration of cells in the form of three-dimensional matrix needs to be provided. The environment should provide for appropriate porosity, pore size, fiber diameter, and orientation for biocompatibility with the cells. In such an environment, the cells should be able to maintain their biological activity, be able to proliferate along the fiber structure, possess the ability to migrate and cross link with the other fibers in the

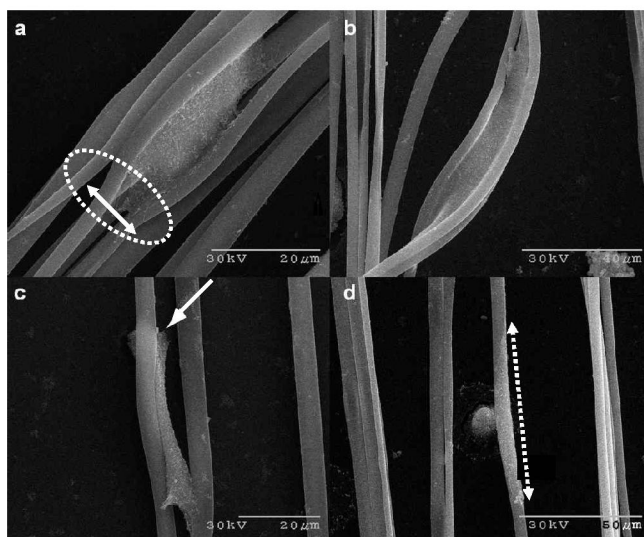


Fig. 8. SEM images of cell attachment to single layered scaffolds: (a) deformation of two fibers shown by arrow, (b) clumping together two fibers, (c) cell attached to the two fibers and migrating towards the substrate, shown by arrow, and (d) single cell wraparound a unitary fiber, shown by arrow.

three-dimensional matrix and finally differentiate into two healthy cells. In Fig. 9, we demonstrate the attachment (Fig. 9a,c) and cross-linking (Fig. 9b,d) of cells in multi-layered scaffolds. The spreading and linking of cells is indicative of the biocompatibility of the scaffolds with the cells.

Proliferation of cells into two healthy cells is demonstrated in Fig. 10. The mitotic cells were found to be attached to parallel fibers and the exact mechanics of proliferation and differentiation is currently under investigation.

The process of sterilizing, increasing surface compatibility and seeding of scaffolds fabricated using the microrobotic strategy is harsh on the structural integrity and orientation of the scaffolds. As evident from the images, the orientation order is greatly reduced before and after the processing steps and often leads to breakage of individual fibers. However, the proposed microrobotically assisted strategy for fabricating biological scaffolds sheds direct evidence on the

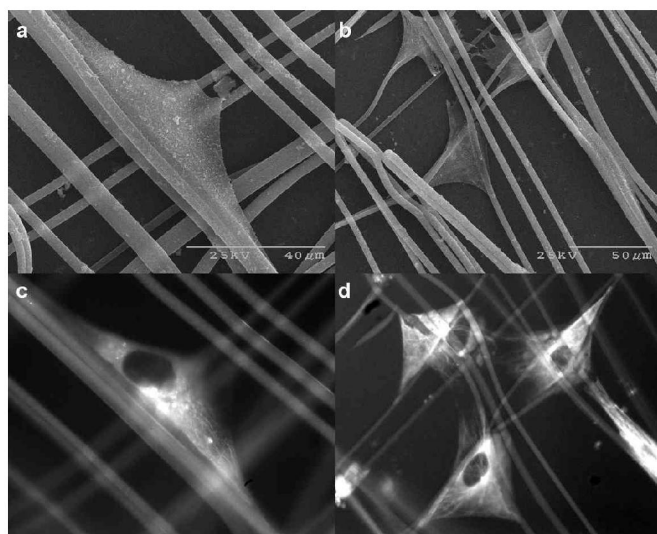


Fig. 9. SEM and fluorescence images of cells attached to the multiple layered scaffolds.

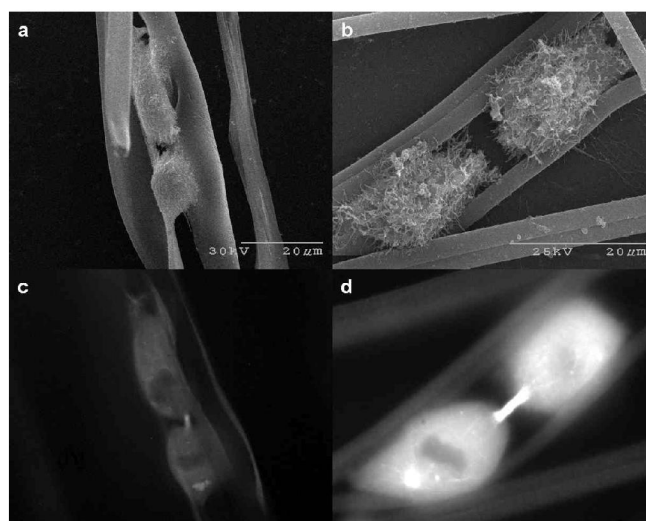


Fig. 10. SEM (a, b) and corresponding fluorescence images (c, d) of mitotic cells.

possible mechanics of cell attachment, proliferation and differentiation on individual and cross-linked fibrillar structures, which have not been studied in detail due to the limitations of other fabrication techniques.

V. CONCLUSIONS AND FUTURE DIRECTIONS

The proposed microrobotic assisted strategy for fabricating novel biological scaffolds possessing high alignment, controlled pore size and three dimensional structures serves as a unique platform for enhancing our knowledge in tissue engineering. The current technique has been shown to favor cell attachment, proliferation and differentiation. However, it is limited by the serial production method and harsh processing steps required for seeding cells.

We envision that improving upon this technology requires fabricating scaffolds in a parallel manufacturing environment with multiple probes and advanced programming routines, which account for the material behavior and solvent evaporation rates. The harsh environment of seeding cells can be minimized, not eliminated, as suspended fibrillar structures having high alignment and controlled pore sizes are extremely sensitive to external influences. The individual fibers have demonstrated structural integrity to the harsh conditions. The microrobotic strategy of providing 'double attachment' points with the substrate coupled with the use of adhesives for clamping down the scaffolds, has greatly improved the survivability of the scaffolds. Reducing the capillary and turbulent flow forces by actively controlling the flow rates during processing will further increase the survivability of scaffolds.

The fiber diameters presented in this study are at best in the micrometer range. Fabricating scaffolds at higher operating speeds and appropriate solution viscosities should scale the diameter down to sub-micrometer ranges. Furthermore, this technology uniquely permits fabricating custom scaffolds over different substrates of different geometries, which can be geared towards specific applications.

The proposed technique has been used to demonstrate the feasibility of fabricating biological scaffolds using PLA and can be easily extended to fabricate scaffolds made of other similar biodegradable materials. To increase cell attachment, the polymer solution can be mixed with cell-binding proteins, which can eliminate some of the processing steps involved in seeding cells. Furthermore, having multiple probe tips with different materials in the same set up can lead to fabrication of exotic biological scaffolds tailored towards seeding different cell lines.

Future studies would be to improve the ability to fabricate custom biological scaffolds of different polymeric solutions having high alignment, desired porosity, user defined pore size, and high survivability rates. These are envisioned to be geared towards carrying out fundamental studies in tissue engineering, specifically looking at cell functions on the fibers: proliferation and differentiation towards myogenic or osteogenic lineage, cell attachment properties, and response to growth factors as a function of scaffold parameters: material, alignment, fiber diameter, pore size and porosity. Another set of studies will be application driven, where special biological scaffolds will be fabricated on implantable chips, MEMS and filtration devices, custom bandages and controlled drug delivery devices.

In conclusion, a microrobotically assisted method for fabricating biological scaffolds using PLA has been successfully demonstrated. Fabricated scaffolds have fiber diameters in the micrometer range and controlled pore size as low as 8 μm . Biological scaffolds having high porosities were fabricated by depositing multiple layers. Mouse C2C12 cells were seeded on the fabricated scaffolds and cell attachment, proliferation, and differentiation demonstrated. New frontiers in tissue engineering can be explored by the proposed technique, which offers, great versatility in fabricating parameterized and custom biological scaffolds of different materials over various substrates.

ACKNOWLEDGMENT

The authors are thankful to Joseph Suhan for the valuable suggestions on cell culture and imaging techniques, to Ed Wojciechowski for the help provided in fabricating the experimental setup and to Kevin P. Pham for helping in setting up the preliminary experiments and proof of concept.

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