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On-Demand Three-Dimensional Freeform Fabrication of Multi-Layered Hydrogel Scaffold With Fluidic Channels

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ABSTRACT: One of the challenges in tissue engineering is to provide adequate supplies of oxygen and nutrients to cells within the engineered tissue construct. Soft-lithographic techniques have allowed the generation of hydrogel scaffolds containing a network of fluidic channels, but at the cost of complicated and often time-consuming manufacturing steps. We report a three-dimensional (3D) direct printing technique to construct hydrogel scaffolds containing fluidic channels. Cells can also be printed on to and embedded in the scaffold with this technique. Collagen hydrogel precursor was printed and subsequently crosslinked via nebulized sodium bicarbonate solution. A heated gelatin solution, which served as a sacrificial element for the fluidic channels, was printed between the collagen layers. The process was repeated layer-by-layer to form a 3D hydrogel block. The printed hydrogel block was heated to 37°C, which allowed the gelatin to be selectively liquefied and drained, generating a hollow channel within the collagen scaffold. The dermal fibroblasts grown in a scaffold containing fluidic channels showed significantly elevated cell viability compared to the ones without any channels. The on-demand capability to print fluidic channel structures and cells in a 3D hydrogel scaffold offers flexibility in generating perfusable 3D artificial tissue composites.

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KEYWORDS: tissue engineering; 3D freeform fabrication; hydrogel scaffold; perfusion; collagen; gelatin

Introduction

Engineered tissue constructs aim to provide supplemental or total replacement of defective organs or tissue functions. To introduce the cells-of-interest into the targeted treatment areas and to support their integration into the existing physiological environment, the cells are embedded in or coated on biocompatible materials that serve as temporary "scaffolds" (Fedorovich et al., 2007; Sachlos and Czernuszka, 2003; Tsang and Bhatia, 2004). Solid materials with built-in porosity, such as biodegradable poly-L-lactide (Ma et al., 2005; Ushida et al., 2002; Yang et al., 2004) or poly(lactic-*co*-glycolic acid) (PLGA) (Huang et al., 2004; Uematsu et al., 2005), have been assembled in three-dimensions to provide a scaffold for the attachment of implanted cells and for perfusion. However, solid scaffold materials are often too rigid for application in soft tissues.

Synthetic or naturally driven hydrogels have gained popularity as candidate scaffold materials for non-skeletal tissue engineering due to their ability to facilitate the transport of oxygen through diffusion and to integrate readily into the surrounding extracellular matrix (ECM) (Drury and Mooney, 2003; Nelson and Tien, 2006). Controllable dissociation/biodegradation of hydrogel materials in physiological environments also makes hydrogels useful materials in tissue engineering (Bettinger et al., 2006). Furthermore, hydrogels can uniquely provide the necessary three-dimensional (3D) cell culture environments for chondrocytes or hepatocytes (Choi et al., 2007; Fedorovich et al., 2007; Glicklis et al., 2000). Hydrogels, either alone or combined with cells, have been deployed as in vivo implants to promote the regeneration of local tissue, ranging from peripheral nerves to skin substitutes (Belkas et al., 2005). Cells growing in a 3D hydrogel scaffold often require in vitro culture prior to implantation. The hydrogel-cell composites mimicking artificial "mini" organs or tissue have also been suggested for use in measuring pharmacological responses (Kim et al., 2007; Lee et al., 2008; Torisawa et al., 2005).

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During these processes, the composite hydrogel should be adequately perfused for the delivery of growth factors, oxygen, and other nutrients to the cells (Chrobak et al., 2006; Landers et al., 2002).

Several approaches have been developed to achieve adequate perfusion for cells in diffusion-limited environments. These include constructing poly(dimethylsiloxane) (PDMS) capillary networks (Borenstein et al., 2002; Kaihara et al., 2000; Leclerc et al., 2003), culturing cells on a permeable membrane (Vernon et al., 2005), and embedding microfluidic channels directly onto or within the hydrogel materials (Arcaute et al., 2006; Bettinger et al., 2006; Choi et al., 2007; Golden and Tien, 2007). Microfluidic channels, created within synthetic as well as naturally derived hydrogel materials, are typically produced by soft-lithographic procedures whereby the channels resembling physiological capillary networks are molded or imprinted. Golden and Tien (2007) have demonstrated that complicated channels can be constructed within hydrogels by lithographic patterning of gelatin as a sacrificial element. This method was applied to construct microfluidic channels within collagen, fibrin, and Matrigel. The channels allowed a greater rate of nutrient distribution throughout the microfluidic hydrogel scaffold, resulting in cells with higher viability and greater proliferation as compared to cells grown in scaffolds without channels.

With the emergence of on-demand 3D freeform fabrication (FF), biomaterials can be directly patterned onto substrates without using the lithographic approach. FF, also known as rapid-prototyping, has been used in creating meshed/porous scaffold materials (Zein et al., 2002). Recently, FF has been applied to generate cell–ECM composites that are constructed layer-by-layer (Landers et al., 2002) and to enable direct printing of cells along with hydrogel-based scaffold materials in an attempt to construct tissues and organs in 3D (Boland et al., 2003; Sachlos and Czernuszka, 2003). The advantage of the FF method over the lithographic approach is that it does not require the construction of layer-specific mold patterns or stamps, which simplifies experimental procedures.

Boland et al. (2006, 2007) demonstrated FF-based single line patterning on a substrate using sodium alginate gels; however, the feasibility of creating a multi-layered hydrogel channel structure with on-demand cell printing remained to be tested. Here, we report the generation of channels embedded in a multi-layered hydrogel scaffold using on-demand FF in combination with a sacrificial strategy where the channel cavity is filled with temporary materials that are subsequently removed (Golden and Tien, 2007). In order to achieve this goal, we developed a computer-controlled 3D bio-printer capable of dispensing hydrogel materials (collagen precursor and gelatin) and cells (fibroblasts) (Lee et al., 2009a,b). The desired spatial pattern of the channel was printed in gelatin and embedded in a collagen layer. The process was repeated layer-by-layer, generating a 3D collagen block with embedded gelatin channel structures. After the completion of printing the hydrogel scaffold, the entire hydrogel block was heated to liquefy and remove the gelatin. The space, once occupied by the gelatin, formed the channels. Primary human dermal fibroblasts (FB) were also printed during construction of the hydrogel scaffold and embedded in the collagen hydrogel. The channel was perfused with cell culture media, and cell viability was measured to demonstrate the potential utility of the technique for tissue engineering.

Materials and Methods

Overview of 3D Bio-Printer

The bio-printer system consists of a dispenser array and controlling mechanism, a two-dimensional (2D) robotic stage (X and Y axes) that moves the dispenser, and a vertical stage (Z axis), which controls the distance from the dispenser to the printing target substrate. The dispenser array consists of four independently controlled electro-mechanical microvalves (SMLD Fritz Gyger AG, Thun-Gwatt, Switzerland), which systematically gate the flow paths of four different liquid materials to be printed, enabling continuous dispensing without the need for intermittent aspiration of the materials (Fig. 1a). It also has an attachable thermo-electric device (TED), which can be heated or cooled for dispensing temperature-sensitive hydrogel materials such as gelatin (Fig. 1a[2]). Pneumatic pressurization forces the liquid materials, including cell suspensions, through a microvalve. The volume of the

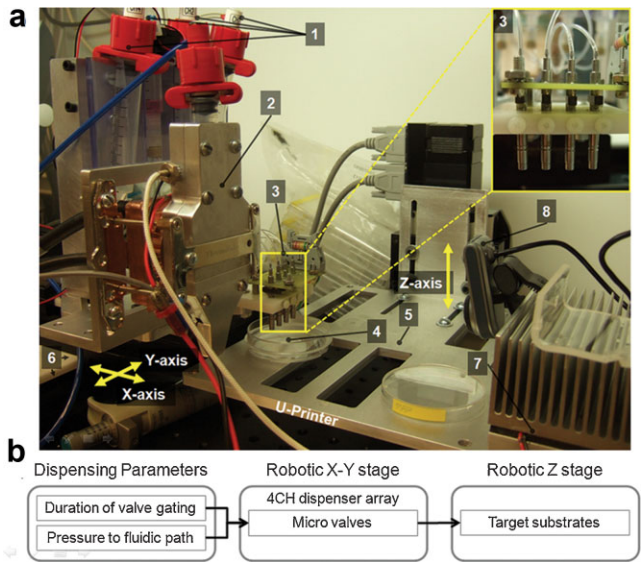

Figure 1. **a**: Picture of the 3D bio-printer platform shown with [1] a 4-channel syringe array as fluid cartridges for cells and hydrogel precursors, [2] independent heating/cooling unit for the dispenser, [3] a 4-channel dispenser array, [4] target substrate, [5] vertical stage, [6] horizontal stage, [7] vertical stage with heater/cooler, and [8] a webcam for monitoring of 3D printing process. Inset: close-up view of the 4-channel dispenser array. **b**: Schematics of dispensing operations of the 3D bio-printer. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

dispensed liquid material was controlled by either (a) regulating the time duration of the valve gating or (b) regulating the pneumatic pressure to the fluid pathway (illustrated in Fig. 1b). The *XY* movement of the dispenser array for on-demand printing was controlled by the robotic stage (Newmark Systems, Mission Viejo, CA) with 5 μm bidirectional reproducibility and 0.4 μm movement resolution.

Materials and Fibroblasts Preparation

Chemically crosslinkable (solution-phase in acidic pH and gel-phase in neutral pH) collagen hydrogel precursor (rat tail, type I, BD Bioscience, Billerica, MA) and temperature-sensitive gelatin (Porcine skin Type A, Sigma Aldrich, St. Louis, MO) were used for the construction of the hydrogel scaffold with fluidic channel. To characterize the dispensing parameters and to examine the ability to perfuse the hydrogel via the channel, FB (ScienCell Research Laboratories, Carlsbad, CA) were purchased and maintained at 37°C under 5% CO_2 in a humidified water-jacketed incubator. The confluent cell layer in the FB culture media was briefly rinsed with phosphate-buffered saline (PBS) at a pH of 7.4 (Gibco, Grand Islang, NY). The media was buffered with bicarbonate and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). The media also contained 2% fetal bovine serum (FBS), 1% fibroblast growth supplement (FGS), and 1% penicillin/streptomycin solution (P/S). Trypsin–EDTA solution (0.25% trypsin and 1 mM EDTA·4Na, Gibco) was used to harvest the cells.

Collagen hydrogel precursor (2.23 mg/mL; diluted with PBS) was used to construct the hydrogel block which contained the channel structure. The collagen hydrogel precursor was maintained at a pH of 4.5 and kept in an ice bath before dispensing. Sodium bicarbonate ($NaHCO_3$) in distilled water (0.8 M) was used as a crosslinking material for the collagen hydrogel precursor (Gangatirkar et al., 2007). A 7% (weight/volume; w/v in distilled water) gelatin solution was prepared at 40°C before printing and then loaded into a heated dispenser unit.

Electromechanical Dispenser Array

Commercial ink-jet-based dispensing devices, based on either bubble jet or piezoelectric elements, are pre-calibrated for dispensing ink with a fixed degree of viscosity, but do not provide flexibility in printing hydrogel materials with different viscosities. In addition, a small nozzle diameter often limits the size of the cells to be printed. Miniature electromechanical valves (Fig. 1a inset) allow for the dispensing of a wide range of low viscosity liquids less than 200 centipoise (cP). With a nozzle diameter of 150 μm, the valve accommodates the dispensing of larger cells, which are unable to be printed using commercial ink-jet printers. The advantage of the pneumatically driven continuous dispensing method includes the ability to control the volume of droplets by changing either the pressure to fluid pathway or the duration of the valve opening time (Fig. 1b).

The liquid state hydrogel precursors and suspended cells were kept in disposable 10 mL syringes (Fig. 1a[1]) and pressurized with HEPA filtered ambient air. The pneumatic pressure to the liquid was regulated by a digitally controlled pressure regulator (ITV2010, SMC, Tokyo, Japan) and the duration of the valve opening was controlled by changing duration of a standard TTL pulse (>150 μs). To dispense the gelatin as a liquid substance, one of the dispensers and syringe reservoirs (5 mL) was enclosed in an aluminum housing (Fig. 1a[2]) and heated to 40°C by a temperature regulated TED (also known as Peltier device, TE Technology, Traverse City, MI). A total of three microvalves were used for dispensing collagen, gelatin, and FB suspension.

The volume of the dispensed droplet size of 7% (w/v) gelatin solution in distilled water was characterized in terms of applied pneumatic pressure and valve gating period at 40°C. The pneumatic pressure was varied from 6 to 13 psi (with a step of 1 psi), and valve gating time was adjusted among 450, 600, and 750 μs. The size of the dispensed gelatin droplet was directly measured by imaging the droplet in midair at room temperature (20°C) by a high-speed camera (Pixelink PL-A741, Ottawa, Canada) by synchronizing the image acquisition upon dispensing (shutter speed = 20 μs).

Printing Software

We developed printing software to control the stage movement and dispensing rate, with the capability to adjust the printing resolution (minimum 50 μm between each dispensing). The software interface, based on MATLAB script (Mathworks, Natick, MA), was designed to sample bitmap images into dispensing coordinates. During this process, the coordinates that are adjacent to each other were automatically clustered to expedite the printing speed by reducing the length of robotic movement paths. The coordinates and motion dispensing parameters were translated into appropriate robotic control codes (Galil Motion Control, Rocklin, CA) and executed with the capability to repeat or pause printing (graphic user interface developed with Visual Basic, Microsoft). The spatial gradient of dispensing density and the clustering of dispensing channels were controllable by the user. The entire device was housed in a laminar flow hood (StreamLine, Fort Myers, FL) with two cameras (UBV-49, Logitech, Fremont, CA and MW200, MobiTechPlus, Inc., Seoul, Korea) for real-time monitoring of horizontal and vertical robot movements and visual inspection of the hydrogel constructs (Fig. 1a[8]).

Construction of Multi-Layered Hydrogel Containing Fluidic Channels

The schematic shown in Figure 2 illustrates the method of constructing five layers of collagen with patterned sacrificial gelatin channels. First, the surface of a tissue culture dish was

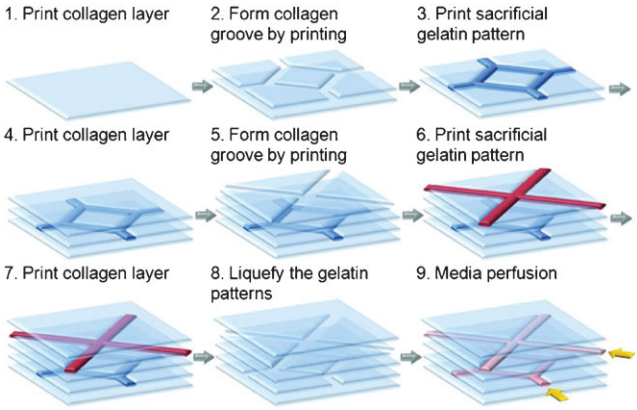

Figure 2. Schematic procedure of the construction of the multi-layered, $10 \times 10\,mm^2$ collagen scaffold with embedding and removal of sacrificial gelatin patterns using the 3D bio-printer. After planar layers (1 and 4) and grooves (2 and 5) of collagen hydrogel were printed and gelated by a crosslinking agent ($NaHCO_3$), the sacrificial gelatin pattern was printed into the collagen groove and cooled under 20°C for 1 min to be gelated (3 and 6). One more planar collagen layer was printed and gelated to cover the 2nd gelatin pattern (7). The constructed 3D collagen–gelatin hydrogel structure was kept in an incubator (37°C, 20 min) and then the gelatin was selectively liquefied (8). The liquefied gelatin was further removed by the perfusion of warm (~40°C) phosphate-buffered saline (PBS) and fibroblasts culture media (9). [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

coated with a nebulized $NaHCO_3$ solution (each droplet less than 2 μm in diameter) using an ultrasonic transducer (piezoelectric element, 14 mm in diameter; SU-1051W, Sunpentown, City of Industry, CA). The coating was crucial to bind the collagen precursors to the dish surface and maintain the shape of the initial collagen layer. The small diameter of the droplets of nebulized crosslinking solution allowed the gelation of the much larger collagen precursors printed on the coated surface. Each collagen droplet was immobilized by the crosslinking agent, allowing the formation of the hydrogel structure with minimal effects from surface tension. Excessive coating results in droplet formation, which causes the distortion of the printed pattern by surface tension, which attracts adjacent droplets towards each other.

An initial layer (layer #1) of collagen was printed to fill the $10 \times 10\,mm^2$ square area with a printing resolution (i.e., inter-dispensing distance) of 400 μm, a pressure of 1.6 psi, and a microvalve opening time of 600 μs. Nebulized $NaHCO_3$ solution was applied on the top surface to crosslink the remainder of the printed collagen bed. The coating of $NaHCO_3$ also served as the binder and crosslinker for the subsequent collagen layer. In the next layer (layer #2), collagen was patterned as a wall while leaving space for the gelatin patterns. After crosslinking the collagen pattern, gelatin was printed on the groove with a printing resolution of 200 μm under an operating pressure of 6.7 psi and a valve opening time of 750 μs, and subsequently cooled to room temperature (20°C). An additional layer of collagen (layer #3) was printed on top of the hydrogel layer containing the gelatin patterns to seal the channel space. The process was repeated again to print different shapes of the collagen–gelatin patterns. To demonstrate the versatility of the method, fluidic channels with "rotary" patterns (Fig. 2[3]) and "cross" patterns (Fig. 2[6]) were constructed within the 3D collagen hydrogel scaffold. The top layer was sealed with collagen. The printed scaffold was subsequently heated to 37°C (via TED in the target substrates or placing the structure in the incubator) for approximately 10 min. The liquefied gelatin in the channels was carefully removed by flushing the channel with warm FB media that were injected through a 30½-gauge syringe needle. After removing the sacrificial gelatin channels, the channel was injected with colored polystyrene microspheres with a diameter of 0.47 μm (DS02B, DS02R; Bangs Laboratory, Fishers, IN) (Fig. 5c), and the entire hydrogel structure was photographed. The channel-containing hydrogel structure was too large ($10 \times 10\,mm^2$ square area with thickness of >1 mm) to be practically imaged using confocal microscopy (requiring significantly long imaging time to cover the entire volume). We estimated the dimension of the channel by adjusting the focal depth difference of top and bottom of the formed channels using bright-field microscopy.

Testing of Perfusion Capability of Printed Collagen Scaffold Through the Fluidic Channel

In order to examine the potential utility of the created fluidic channels in collagen for applications in tissue engineering, mechanical integrity of a created channel structure under hydrostatic pressure was tested. First, a 12 layered collagen hydrogel block (2.23 mg/mL collagen precursor) containing a single straight channel in the 3rd layer (from the bottom) was prepared. The channel was perfused with normal saline. One end of the channel was sealed with crosslinked collagen while the other end was connected to the outlet of a pressure regulator and subjected to different hydrostatic pressures. The pressure was increased from 0 to 2 psi (=1,406 mmH_2O at 4°C) with a step of 0.2 psi, while inspecting for the presence of any leaks or cracks in the collagen block. A separate collagen block containing a single channel was prepared and placed on the planar silicone substrate (Sylgard 184 silicone elastomer kit; Dow Corning, Midland, MI). The construct was then submerged in the FB media for 7 days in a 37°C incubator. The collagen block was stained with Trypan Blue, fixed using cooled methanol (99%; at 4°C), and blade-cut to examine the cross-section of the channel structure via microscope.

Upon testing the mechanical integrity of the channel-containing hydrogel block, the cell viability after the printing and subsequent hydrogel crosslinking (the each layer of printed hydrogel precursor must be crosslinked upon cell printing to achieve 3D structure) was examined. The FBs (prepared in 10^6 cell/mL concentration) were printed under an operating pressure of 1 psi and a valve opening time of 450 μs. They were embedded in the multi-layered collagen hydrogel according to the method described in Lee et al.

(2009a). The cell viability was tested using live/dead viability/cytotoxicity assay (L-3224, Invitrogen Calcein-AM and ethidium homodimer-1) 3 h after printing and compared to the ones that has been manually plated onto a culture dish (TPP, MidSci, St. Louis, MO).

Upon evaluation of the direct effect of printing on cell viability, we also examined the viability of the FB printed inside the scaffold with media-perfusion through the channel. To do so, a 60 mm tissue culture dish with a hole (for infusion) was prepared whereby the bottom of the dish was penetrated by a 30½-gauge syringe needle. The needle outlet (made blunt by cutting and polishing the end) was connected to a loaded syringe through a Tygon tube (see the schemes in Fig. 6 middle, right). The assembled tissue culture dish was UV sterilized for at least 30 min, and then a 17 layered collagen hydrogel block containing a single straight channel was printed on $10 \times 10\,mm^2$ square area. The straight line of gelatin (for channel creation, ~400 μm in width and 110 μm in height) was printed in the midline of 2nd collagen layer from bottom and aligned to intersect the infusion inlet (see schemes of Fig. 6 top, right and middle, right). During the construction, collagen was printed with a resolution of 400 μm at a pressure of 2 psi and a valve opening time of 600 μs. Gelatin was printed on the collagen groove twice at a printing resolution of 150 μm under an operating pressure of 6.7 psi and a valve opening time of 750 μs. FB (1×10^6 cells/mL) were embedded in the scaffold by printing the cells in each of the two layers, starting with the 2nd collagen layer from bottom according to the method described in Lee et al. (2009a). The cells were printed on to the uncrosslinked hydrogel layer and subsequently cross-linked by the nebulized $NaHCO_3$ solution on the top surface. Thick FB–laden collagen composites were necessary to examine the effects of perfused channel on the cells located beyond passive diffusion limitation on the order of 1,000 μm according to the Ling et al. (2007).

Once printing was completed, the collagen–gelatin structure was kept in an incubator to liquefy and remove the gelatin. Then, warm FB culture media was perfused into the fluidic channel inside collagen scaffold at a rate of 4.0 μL/min using a syringe pump (NE-1000, New Era Pump Systems, Wantagh, NY). Both ends of the channels were not plugged, which allowed for free flow of the media through the channel. As a comparison with the perfusable collagen scaffold, an identical FB–laden collagen scaffold, without the channel, was prepared. These two FB–laden collagen scaffolds were submerged in 5 mL of FB culture media, and cultured in 5% CO_2 at 37°C. A live/dead viability/cytotoxicity assay (L-3224, Invitrogen Calcein-AM and ethidium homodimer-1) was conducted after 1 week of culture (day 8).

Results

Measurement of Gelatin Droplet Volume and Channel Width

The relationship between dispensed droplet volume of 7% (w/v) gelatin (at 40°C) and valve opening time and applied pneumatic pressure is shown Figure 3. The minimum droplet volume was estimated to be on the order of 25 nL with a valve opening time of 450 μs and a pressure of 6 psi. A longer valve opening time and an increase in the applied pressure resulted in dispensing larger droplet volumes. The printing resolution for a given droplet size can influence the width and homogeneity of the printed gelatin pattern since each droplet will conglomerate after landing on the substrate surface. Figure 4a–c demonstrates an example of such influence. When we examined the shape of printed gelatin line by changing the printing resolution (300, 400, and 500 μm) at 4 psi, larger printing distances (>500 μm in a given dispensing condition) generated uneven line shape (Fig. 4a).

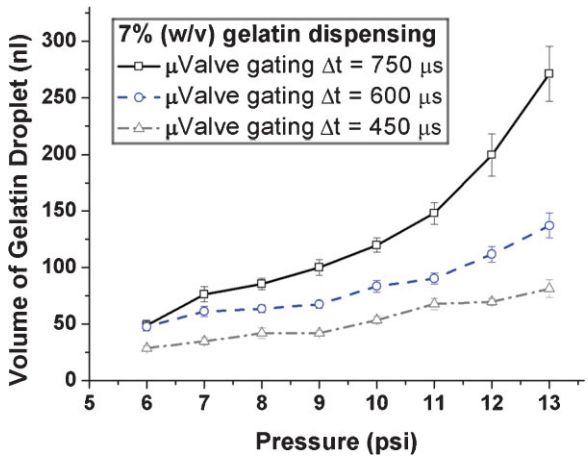

Figure 3. Mean droplet volume with standard error of 7% (w/v) gelatin solution ($n = 8$) with increasing pneumatic pressure to microvalve when the valve opening duration were 750, 600, and 450 μs. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Figure 4d and e shows the printed straight gelatin line and the gelatin-removed fluidic channel in multi-layered collagen blocks, respectively. At the dispensing condition of the gelatin (pressure: 6 psi; valve opening duration: 450 μs; printing resolution: 700 μm), a channel width of approximately 400 μm was achieved. Since the patterns were created based on the sequential dispensing of the gelatin droplets, the line width was slightly inhomogeneous (typically less than 20 μm). After removing the gelatin from the collagen scaffold, air bubbles were intentionally injected using a 30½-gauge needle into the formed channel to visually confirm the channel construction (Fig. 4e). We also loaded colored microbeads inside the channel for the visualization to measure the channel height (~110 μm through the adjustment of the focal depth of a microscope). After 1 week of construction, the cross-sectional areas of the fluidic channel at the center (Fig. 4f) and the edge (Fig. 4g) of the collagen block showed that channels remained open, although a degree of shrinkage occurred at the edge of the hydrogel (Fig. 4g; channel border marked with arrows). To better visualize the 3D structure of scaffolds and

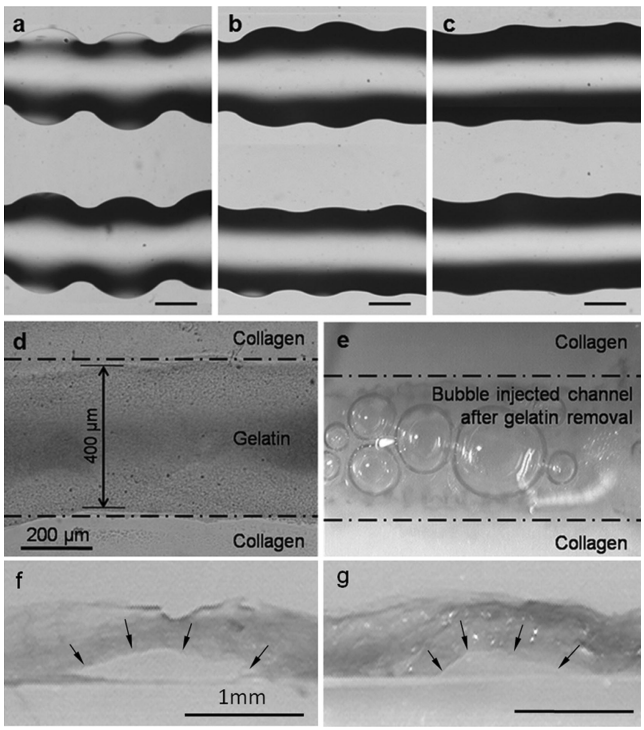

Figure 4. Image of the printed line patterns of gelatin (**a**–**c**) printed gelatin channels on tissue culture dish with different conditions of pressure, valve opening time, and printing resolution of (a) 4 psi, 400 μs, 500 μm, (b) 4 psi, 400 μs, 400 μm, and (c) 4 psi, 400 μs, 300 μm, respectively (scale bar = 250 μm). **d**: The printed gelatin line pattern (between the dotted lines) in the collagen groove. **e**: The printed gelatin line pattern was embedded in multi-layered collagen scaffold and selectively removed. Air bubbles were injected for inspections of selective gelatin removal under stereo-microscopy. The cross-section images of (**f**) center and (**g**) the edge of the channel-containing hydrogel scaffold obtained after one week of incubation (arrows indicating the channel margin).

channels, adoption of magnetic resonance (MR) microscopy (Mitsouras et al., 2009), a type of magnetic resonance imaging (MRI), may provide a practical solution (imaging will take less than an hour) of the entire scaffold cross-section. Scanning electron microscopy (SEM) at low magnification may also be conducive to visualize the cross-section of the constructed channel structure.

Constructing Multi-Layered Collagen Scaffold With Embedding and Removal of Printed Gelatin Patterns

To demonstrate the versatility of the method in creating multi-layered collagen–gelatin scaffolds, three different 3D configurations of collagen scaffolds were also designed and constructed (shown in Fig. 5). Both "cross" (Fig. 5a) and "rotary" patterns (Fig. 5b) of the sacrificial gelatin were printed in the 2nd layer of a three-layer collagen block. The gelatin was then selectively removed and visualized with perfusion of warm PBS containing blue-colored microspheres. We also demonstrated that these patterns could be

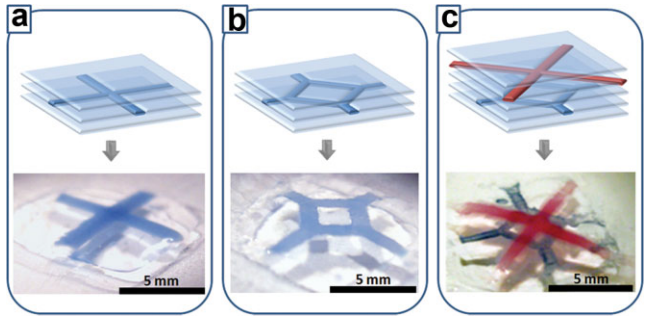

Figure 5. Three different designs of multi-layered collagen scaffold with fluidic channels (the upper row) and the actual constructions of those after gelatin removal using the bio-printer (the lower row). **a**: The printed "cross" pattern of the sacrificial gelatin was embedded in 2nd layer of three multi-layered collagen scaffold and selectively removed by warming and perfusion. PBS with blue-colored microspheres was injected into the gelatin removed fluidic channel (visualized in blue). **b**: The printed complicated "rotary" pattern of sacrificial gelatin was formed in 2nd layer of a three-layered collagen scaffold and selectively removed. Visualized in blue as in (a). **c**: Five multi-layered collagen scaffold with embedding and removal of sacrificial gelatin patterns was constructed as in Figure 2. Gelatin removed fluidic channel of rotary (blue, in 2nd collagen layer from bottom) and cross (red, in 4th collagen layer from bottom) patterns in collagen scaffold were visualized using PBS with colored microspheres. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

overlaid to form more complicated channel structures, as separated by a layer of collagen (Fig. 5c).

Testing of Perfusion Capability of Printed Collagen Scaffold Through Gelatin Removed Cavities

The printed collagen that contained a straight channel resisted over 103.4 mmHg (=2 psi = 13.79 kPa) of hydrostatic pressure without any leaks and cracks of the collagen scaffold. The collagen scaffolds (consist of 17 layers of cell/hydrogel) with embedded FB had a thickness of approximately 1,450 μm as measured by adjusting the focal distance of the microscope between top and bottom of the scaffolds.

The printed FB cells were initially well-attached and uniformly spread in both collagen scaffolds, and no contamination was observed after 1 week of culture. We confirmed that the ends of channels stayed open throughout the culture period. Evidence of closure or collapse of channel structure, which tend to distort the morphology/structure of collagen scaffold by perfusion, was not found during our inspection (using bright field microscopy). The post-printing viability of the cells was $95.0 \pm 2.3\%$ while the viability of manually plated cells was $96.6 \pm 3.9\%$ ($n = 30$; no significant difference in viability with Student's t-test, $P > 0.05$).

Viabilities of FB in collagen scaffolds after a week of culture with and without perfusion are shown in bottom of Figure 6. The regions-of-interest where the viability of FB was measured (middle of Fig. 6) were positioned on cross-sectional area at M–M′ (Fig. 6) and radial from the perfusion channel. In region-by-region comparisons between the two

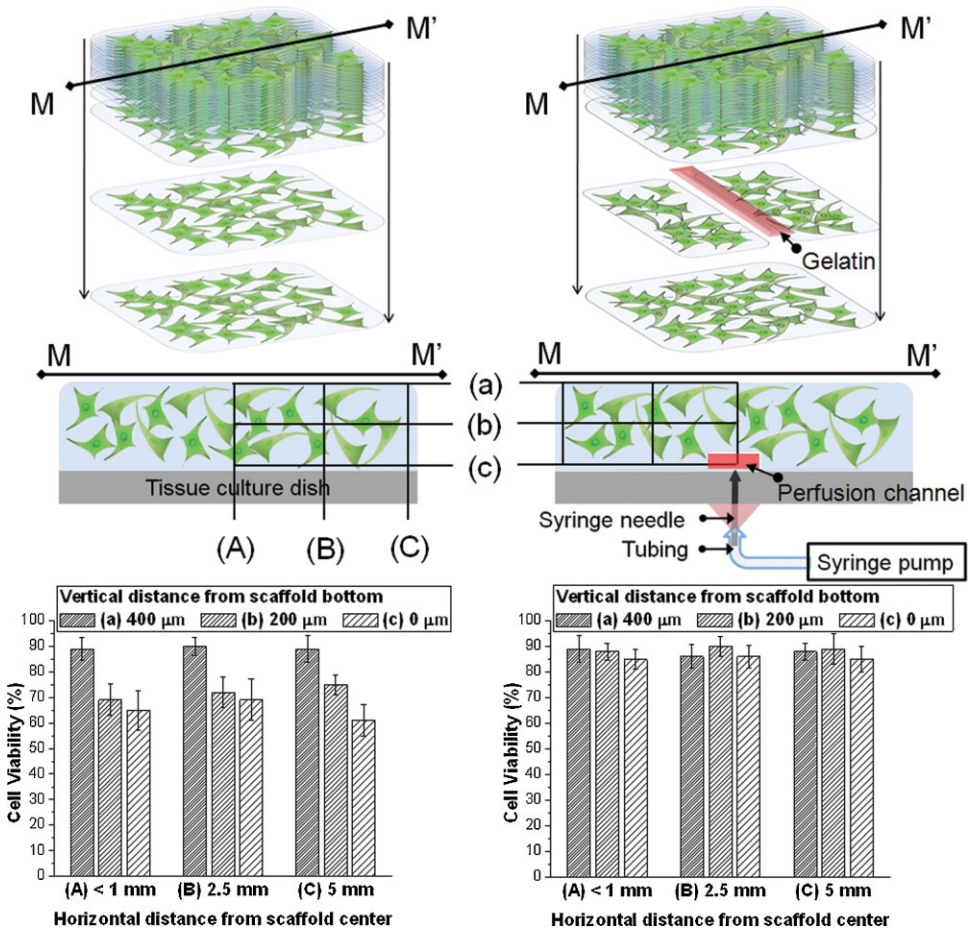

Figure 6. **Top**: Schematics of FB–laden collagen scaffold construction without (top, left) and with (top, right) embedding and removal of printed sacrificial gelatin channel. The figure is not drawn in scale. **Middle**: FB viability inspected locations (a vertical section at M–M′) in the collagen scaffolds without (middle, left) and with (middle, right) inside media perfusion. Capital letters of (A), (B), and (C) indicate the horizontal distances of <1, 2.5, and 5 mm, respectively from scaffold center. Lower case letters of (a), (b), and (c) indicate the vertical distances of 400, 200, and 0 μm, respectively from scaffold bottom. **Bottom**: Measured FB viability with standard error bar ($n = 12$, with respect to the total FB cells) at the inspected locations after 1 week of culture without and with media perfusion. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

scaffolds with and without the perfusion, the FB located in the top layer (Fig. 6 layer (a)) of the both collagen scaffolds showed high cell viability (greater than 85%). However, the viability of FB in layers (b) and (c) in the middle of the non-perfused collagen block was reduced significantly (on the order of 70% viability) compared to the ones measured from the collagen block perfused using the channel. We also observed that the collagen block with the channel showed a uniform distribution of high cell viability (Fig. 6).

Discussion

Fabrication of a cell–laden hydrogel scaffold with micro-fluidic channels is important to provide adequate perfusion to the cells in the context of tissue engineering (Ling et al., 2007). Construction of 3D biodegradable structures with microchannels has relied on soft lithographic techniques. For example, PDMS molds with micro-patterns have been prepared to construct microchannels into biodegradable thermoplastic materials such as PLGA (King et al., 2004) or elastomers such as poly(glycerol sebacate) (Bettinger et al., 2006). Microfluidic channels have also been generated by patterning temperature-sensitive gelatin as a sacrificial element within hydrogel scaffold (Golden and Tien, 2007). These lithography-based methods are extremely accurate, claiming to have control of features at the cellular scale; however, it requires multiple fabrication processes such as the production of templates, stamping steps for patterning, and bonding processes between layers to successfully generate a multi-layered construct. This complicated fabrication process prohibits rapid and high-throughput construction of 3D hydrogel scaffolds with cells embedded.

Instead of the lithographic approach, a FF technique based on ink-jet bio-printing was proposed to build perfusable channels in engineered hydrogel scaffolds. As reported by Boland et al. (2006), multiple parallel lines of

sodium alginate solution were printed to form channel structures and subsequently "dipped" into the crosslinking solution (i.e., calcium chloride). In the absence of sacrificial hydrogel elements to sustain the 3D shapes with surrounding structure, the concentration of hydrogel precursor and crosslinker served as a determinant factor to form a channel-like morphology. Yet, this method has only been demonstrated to construct a single hydrogel layer, perhaps due to the lack of mechanical support for a true 3D multi-layered formation. We adopted an approach whereby printed gelatin patterns served as sacrificial elements within a multi-layered collagen scaffold. We further demonstrated that FB embedded in perfused collagen scaffold showed sustained high viability, whereby the cells in the scaffold without fluidic channels, especially at the bottom of the structure, had shown reduced viability (Fig. 6). This indicates that flow of the media through the channel provided adequate perfusion to the cells. We have not demonstrated interlinking networks of channels located in the different layers to perfuse a thick and larger hydrogel scaffold; however, it is conceivable to create such structure by creating planar channel networks and interlinking them in 3D. It would thus enable the flow of culture media throughout the constructed hydrogel scaffold, which may be difficult to be achieved by lithographic approaches.

In contrast to the work of Chang et al. (2008) in which they used microfluidic channels (made using the soft lithographic method) to perfuse a biological tissue construct printed on top of the channels, a novelty of our approach is that single session of 3D bio-printing produced not only a cell-containing scaffold but also channels (made of gelatin) which can be used for the tissue perfusion. In addition, since no solid pattern templates are required (as in lithographic approach), modification to the channel patterns can be made rapidly. This reduction in design time, as well as the lack of manual assembly procedures, allowed for the construction of the hydrogel scaffold containing sacrificial elements in approximately 15 min with the aid of the computer-guided printing software.

Coating of each printed collagen precursor layer with nebulized crosslinking agents was crucial for constructing the multi-layered collagen hydrogel. Temperature-regulation of the one of the electromechanical dispensers (Fig. 1[2]) allowed the dispensing of gelatin in liquid form. The ability to print two different types of hydrogels suggests that multiple scaffold materials are available to create a construct that resembles inhomogeneous tissues (e.g., collagen for osteocytes and hyaluronic acid for chondrocytes culture in joints). This capability will also be conducive to compose spatially varying concentration of scaffold materials, for example, to induce drug/cell delivery in the context of tissue engineering.

The use of electromechanical valves for dispensing the hydrogel allowed for the adjustment of dispensing volumes. The low pneumatic pressure (<5 psi) and passive gating of the fluid path was also conducive to high cell viability if the cells need to be embedded simultaneously. Our printing platform was capable of successfully storing and dispensing both chemically crosslinkable (collagen) and temperature-sensitive hydrogels (gelatin) for the required 3D FF process. However, it is inevitable that the minimum volume of dispensed droplet is limited by viscosity of materials and high hydraulic resistance derived from the size of the micro-nozzle. Therefore, the spatial dimension of the printed channels was typically greater than that available through the soft-lithography methods. In addition, variations in width of the created straight hydrogel channels (Fig. 4a–c) were observed due to sequential non-continuous dispensing of the droplets. The use of dispensers with the capability to print picoliter-range droplets will potentially generate a smaller channel width. Since the printing resolution, viscosity of gelatin, and printed droplet volume all influence the printed channel morphology, more study is required to characterize the channel dimension and shapes in relation to these printing parameters. Such characterization is commonly employed for calibrating dispensers used in commercial ink-jet printers.

In terms of the mechanical properties of the printed hydrogel, closure of hydrogel channels may occur at the edge of scaffold structure due to the compression or swelling effect of (soft) hydrogel. We also confirmed such possibility based on the observation whereby the channel at the edge of the collagen block become narrower compared to the center after the 1 week of culture (Fig. 4f,g). Although the degree of distortion in channel morphology during long culture period seemed inevitable, we confirmed that the ends of channels stayed open throughout the culture period. Our finding is in agreement with the previous investigation on lithographed micro-channels in collagen (Golden and Tien, 2007) whereby the defined channel structures in collagen scaffold ($\sim$6 μm in dimension) did not show significant changes in its dimensions.

We have constructed up to 17 layers of collagen hydrogel (a thickness exceeding 1 mm) without structural cracks or collapse. Nonetheless, it is reasonable to predict that the construction of much thicker hydrogel materials, which resemble the size of organ subunits (few centimeters in dimension), will eventually require additional mechanical supports, either stronger hydrogel materials or even rigid solid-state scaffolds, within or outside of the scaffolds to promote their mechanical integrity. Detailed mechanical properties of these materials and fabrication methods in the context of 3D FF would warrant further investigation.

Conclusions

We presented an on-demand capability to print fluidic channel structures and cells in a 3D hydrogel scaffold to generate perfusable 3D artificial tissue composites without complicated lithographic approaches. This work ultimately aims to simultaneously handle and print different phase-transition materials such as thermo-sensitive, pH-sensitive, photocurable, or chemically crosslinkable hydrogels along with multiple cell types, which is crucial for the construction

of a complicated tissue-like hydrogel scaffold. Furthermore, printing appropriate growth factors or cell signaling molecules in on-demand locations can be utilized to derive the spatial morphology of cells and cell-to-cell and cell-to-ECM interactions, which can be applied to differentiation studies of stem cells. Integration of the printed tissue composites with a bioreactor circuit will also help to establish a stand-alone mini organ system composed of multiple cell lines.

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