Simultaneous Deposition of Human Microvascular Endothelial Cells and Biomaterials for Human Microvasculature Fabrication Using Inkjet Printing

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Abstract

The current tissue engineering paradigm is that successfully engineered thick tissues must include vasculature. As biological approaches alone such as VGEF have fallen short of their promises, one may look for an engineering approach to build microvasculature. Layer-by-layer approach for customized fabrication of cell/scaffold constructs have shown some potential in building complex 3D structures. With the advent of cell printing, one may be able to build precise human microvasculature with suitable bioink. Human Microvascular Endothelial Cells (HMEC) and fibrin were studied as bioink for microvasculature construction. Endothelial cells are the only cells to compose the human capillaries and also the major cells of blood vessel intima layer. Fibrin has been already widely recognized as tissue engineering scaffold for vasculature and other cells, including skeleton/smooth muscle cells and chondrocytes. In our study, we precisely fabricated micron-sized fibrin channels using a drop-on-demand polymerization. This printing technique uses aqueous processes that have been shown to induce little, if any, damage to cells. When printing HMEC cells in conjunction with the fibrin, we found the cells aligned themselves inside the channels and proliferated to form confluent linings. Current studies to characterize the biology and functionality of these engineered microvascular structures will be presented. The preliminary data suggests that a combined simultaneous cell and scaffold printing can promote HMEC proliferation and microvasculature formation.

Introduction

The development of microfabrication techniques has resulted in new tools to explore, *in vitro*, the interactions of anchorage-dependent cells with their environment. The ability to control the footprint of a specific cell through surface contact area constraints has been shown to dramatically affect cellular development and differentiation [1]. Spatial and temporal control of the chemistry and pattern geometry can provide new insights into fundamental aspects of cell-surface interactions. Microcontact printing [2] and laser-directed cell writing [3] are common patterning modalities; as an alternative, we have been investigating the use of a modified ink jet printer. Specifically, an ink jet printer was used in this work to print cells and hydrogel biomaterials in order to study cell-material interactions [4]. Advantages of this method include low cost, flexibility in substrate selection, high throughput, and wide range in substrate conformation output. Specifically, we have demonstrated as a proof of concept the adaptation of ink jet printer as a tool to print cells or a combination of cells and ink onto a variety of substrata [5]. We have further demonstrated that the printed cells remain viable over extended periods of time [6].

Materials and methods

Dulbecco's modified eagle medium (DMEM), fetal bovine serum, penicillin and streptomycin, sodium bicarbonate, Dulbecco's phosphate buffered saline solution (DPBS), trypsin-EDTA were from Sigma Chemicals (St. Louis, MO, USA). Rat tail Collagen I was from BD Biosciences (San Jose, CA, USA). Propidium iodide, dextran Texas Red 3000 MW, dextran Texas Red 10,000 MW, dextran Texas Red 40,000 MW and dextran Texas Red 70,000 MW were purchased from Invitrogen (Carlsbad, CA, USA). The DeskJet 500 inkjet printer and 51626A black ink cartridges were manufactured by Hewlett-Packard Company (Palo Alto, CA, USA), and the LSM 510 confocal laser scanning microscope was built by Carl Zeiss MicroImaging (Thornwood, NY, USA).

Human microvascular endothelial cells (HMVEC) cells were cultured with DMEM media and incubated at 37°C with 5% CO_2 for 14 days before passaging. Media was changed every two days. A cell pellet was collected using centrifugation at 1000rpm for 5 minutes. The supernatant was aspirated and the cell pellet was resuspended using 1 ml 1x PBS. The cell concentration was determined using a Coulter counter and PBS added to achieve the desired final concentration between 0.25 and 2 million cells/ml.

A new HP 51626A black ink cartridge was used for each experiment. Collagen was coated on microscope cover slips at 2mg/ml followed by gelling in the incubator at 37°C for 15 minutes. Cell suspensions were pipette into a cartridge as described elsewhere [7]. The cells were then imaged by fluorescence microscopy.

Cell membrane integrity studies were performed by staining with propidium iodide. Texas Red dextran

molecules with different molecular weights (3000, 10,000, 40,000, 70,000) were also used to determine the pore sizes in the cell membranes. The crosslinker used was 50-100 u/ml thrombin augmented with 40 mMol/l calcium chloride. Human Microvascular Endothelial Cells (HMVEC) were trypsinized and re-suspended in the thrombin/PBS solution to form a bioink, and transferred into the bioink cartridge. The bioink was printed onto liquid fibrinogen solution (80 mg/ml of PBS). As a control, a small amount of the bioink was manually pipetted onto the fibrinogen containing solution. Samples were incubated at 37ºC and 5% CO_2 for varying amounts of time up to 3 weeks, then analyzed under fluorescent light using live/dead assays and lectin stains. Control samples were treated in identical manners, except for the printing, but were pipette instead.

Results and discussion

Figure 1 shows cells incubated and stained with propidium iodide. As seen in Figure 1A, only about 5% of the pipetted cells were stained by the low molecular weight dye. Since the cells were printed right after passaging, it is reasonable to have some dead cells during the passaging process.

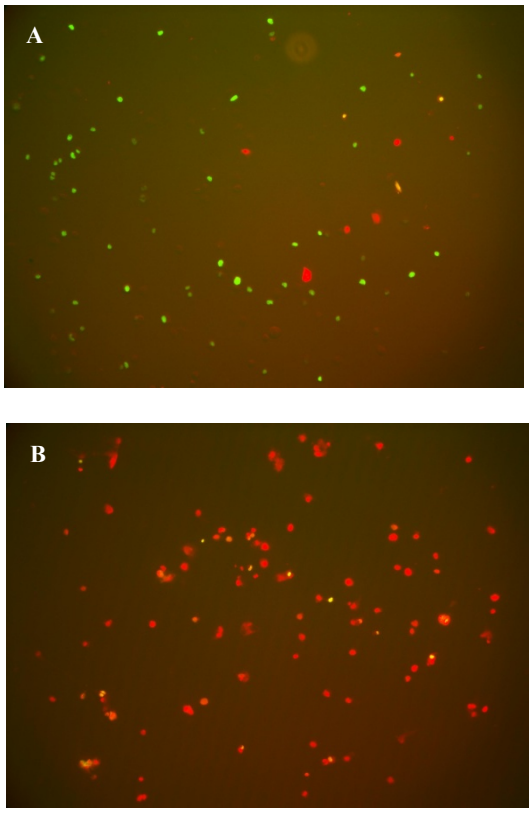

Figure 1. *Confocal microscopy of printed cells incubated with propidium iodide About 5% of control cells were stained by the dye. (A). Nearly all of the printed cells were stained by the dye.*

The 5% dead cells mixed in the bioink were further stained by propidium iodide. However, almost all of the printed cells were stained by propidium iodide (Figure 1B). This shows, that the printing process created pores in the cell membrane of the printed cells which allowed the small molecules like propidium iodide to pass through the membrane and enter the cells.

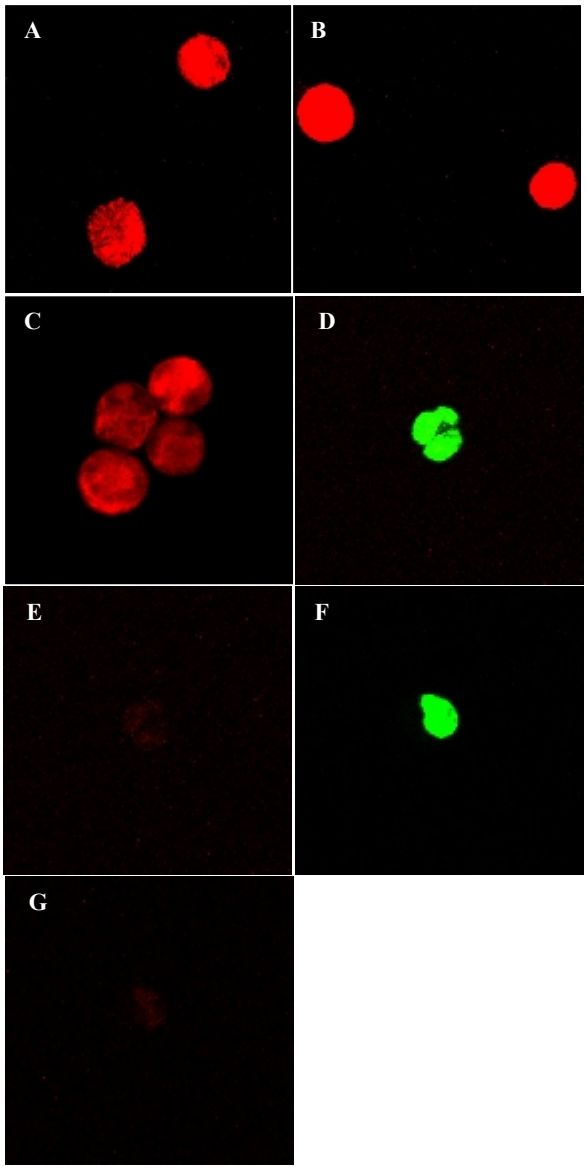

Figure 2. *Cell membrane pore sizes study using red fluorescent dextran molecular dyes. (A) Dextran 3,000; (B) Dextran 10,000; (C) Dextran 40,000 (D) GFP expression of printed cells incubated with Dextran 70,000 (E) Dextran 70,000 (F) GFP expression of pipette cells incubated with dextran 3,000 (G) Pipetted control samples incubated with Dextran 3,000.*

In the further study we wanted to estimate the pore sizes in the membranes of the printed cells. Under confocal microscopy, we could clearly see the red fluorescence from the dextran dye with molecular

weights below 70,000 (Figure 2). For the printed cells incubated with 70,000 MW dextran molecules (Figure 2E), we did not see significant fluorescence, indicating the limited penetration of the dye. Control samples showed no significant fluorescence for any dextran dye employed as shown in Figure 2G for the lowest molecular weight dextran dye. We found almost all the printed cells incubated with 3000, 10,000, and 40,000 MW dextran molecules showed solid fluorescence indicating good dye penetration. We found only weak fluorescence in the cells incubated with 70000 MW dextran molecules, which we attribute to lower MW impurities in the dye. Thus, the pore size on the cell membrane of the printed cells may range from 40000 MW to 70000 MW.

We investigated the combined printing of cells with crosslinkers in an attempt to construct vasculature. Here, we report the implementation of a fabrication method using fibrin, a gel that has potential as a vascular tissue engineering material. Fibrin is a natural material generated from cleavage product of fibrinogen by thrombin (a major component of every blood clot). Given the fast-gelling property of the precursors of the polymeric fibrin, fibrinogen and thrombin, the fibrin gel is a good candidate for printing applications. Fibrin gel has several other obvious advantages. For example, fibrinogen and thrombin are blood-borne proteins and can easily be purified from blood, offering the opportunity of an autologous source for the scaffold and eliminating immunological concerns related to human or cross-species donor incompatibility [8]. As described in a recent paper [9], fibrin structures with sufficient mechanical properties can be constructed using our printing methods. When the crosslinker is combined with cells, precise confinement of the cells is observed. Figure 3 shows a channel printed with ink containing HMVEC cells after ten days of culture.

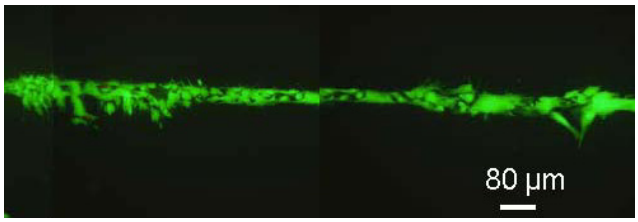

Figure 3. *Live/dead assay of HMVECs in a single channel after ten days of culture.*

Conclusions

We have shown that during thermal printing temporary pores open in the cell membranes allowing molecules with molecular weight up to 70,000 to pass. This may have promising applications for plasmid transfection for protein expression or drug delivery. Modified inkjet printers are capable of simultaneous deposition of cells and biomaterials, with minimal detrimental impact to the printed cells. Proliferation of Human microvascular endothelial cells in our fibrin channel systems demonstrated fibrin as a cell friendly substrate with great potential for human vascular fabrication.

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Author Biography

Thomas Boland is an Associate Professor in the Department of Bioengineering at Clemson University. He received his B.S. in Chemical Engineering from the Ecole Nationale Supérieure d'Ingénieurs de Genie Chimique in Toulouse, France in 1990, and his Ph.D. in Chemical Engineering from the University of Washington, Seattle, WA in 1995. In 1994, he was a finalist for the Materials Research Society Graduate Student Award. Following his Ph.D., he was a Postdoctoral Fellow at Department of Materials Science at the Pennsylvania State University from 1995-1997, and at the Naval Research Laboratory from 1997-1999. In 1999, he joined Clemson University as Assistant Professor, where he received

tenure in 2005. He holds an adjunct appointment as Associate Professor at the Medical University of South Carolina's College of Graduate Studies. Thomas is the Director of a NSF/NIH funded Bioengineering and Bioinformatics Summer Institute, whose primary mission is to introduce senior undergraduate and junior graduate students with science and engineering backgrounds to the interdisciplinary research projects in the bioengineering and bioinformatics areas. Thomas' research interests are applying engineering principles to automate, predict and build three dimensional structures with that show biological function. He has received numerous awards and was featured on CNN and the Discovery Channel for his ground braking innovations using inkjet printers to assemble cells and biomaterials into viable and functioning structures. He is the author of more than 45 publications, including 3 invited reviews and chapters, and he has delivered more than 25 invited presentations. He is a member of the AVS, MRS, the Society for Imaging Science and Technology (IS&T) and the Tissue Engineering and Regenerative Medicine International Society (TERMIS).