THREE-DIMENSIONAL CELL-HYDROGEL PRINTER USING ELECTROMECHANICAL MICROVALVE FOR TISSUE ENGINEERING

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ABSTRACT

In this study, we report a newly developed three-dimensional (3D) biological printer using non-contact, electromechanical microvalves with a nozzle diameter of 150 μm. To control and utilize this printer for life science applications, we developed an easy-to-use control software with a graphic user interface (GUI). First, using the printer, we tested the viability of dispensed mammalian cells after printing, and there was no significant difference in viability between dispensed cells and conventionally plated cells. Next, we constructed a 3D hydrogel scaffold by printing collagen hydrogel precursor layer-by-layer with linear patterns of gelatin inside. Using the same scheme, neurons were printed and patterned in multi-layered collagen scaffold. The on-demand capability to print cells and hydrogels in multi-layered hydrogel scaffold offers flexibility in generating artificial 3D tissue composites.

KEYWORDS

Tissue engineering; 3D freeform fabrication; Cell printing; Hydrogel scaffold; Electromechanical microvalve

INTRODUCTION

Conventional *in vitro* cellular studies are often conducted in two-dimensional (2D) environments where the properties of cells-of-interest can be significantly different than three-dimensional (3D) *in vivo* conditions. To address this issue, soft-lithographic and microfluidic technologies have been applied to construct miniaturized biomimetic cellular composites [1]. These technologies, operating in nano- or micro-scale, are employed to control cellular environments precisely, but require individual photomasks and fixed-form molds to generate each pattern. Therefore, the needed flexibility lacks in constructing a biomimetic environment and in positioning cells within the 3D scaffold.

Recently, a new breed of technique called "3D freeform fabrication" has emerged with an aim for direct cell printing along with hydrogel-based scaffold materials to construct tissues and organs in 3D [2]. However, there have still been a limited number of demonstrations enabling on-demand generation of volumetric cell-scaffold composites.

In this study, we report a 3D biological printer capable of printing cells and hydrogel materials using an independently-controlled, 4-channel microvalve array dispensers (Fig. 1). Using the printer, cells and hydrogels could be dispensed as droplets, and 3D hydrogel-cell composites were constructed in on-demand fashion without using the traditional lithographic approaches.

EXPERIMENTAL

3D Cell-Hydrogel Printer

The hardware setup of 3D cell-hydrogel printer was developed based on the electromechanical microvalves (SMLD Fritz Gyger AG, Thun-Gwatt, Switzerland) that are capable of dispensing liquids on the order of 10 nl droplet volume (Fig. 1). The diameter of the dispenser nozzle is 150 μm and the valve on/off duration was controlled by a standard transistor-transistor logic (TTL) signal. It also has an attachable thermo-electric device which can be heated or cooled for dispensing temperature-sensitive hydrogel materials such as gelatin (gel-sol transition occurring in the body temperature). A liquid reservoir (a set of disposable syringes as 'printer cartridges') was connected to the microvalve through a Teflon perfluoroalkoxy polymer resin (PFA) tube and pressurized. An electronic pressure regulator was applied for each dispensing channel to adjust the pressure (0–15 psi). By adjusting the valve opening duration and applied pressure the droplet volume was controlled.

The dispenser unit was installed on robot arms (Newmark Systems, CA) with an accuracy of 5 μm, oriented on the horizontal (X-Y) axes. The position and timing of droplet dispensing were controlled by a computer.

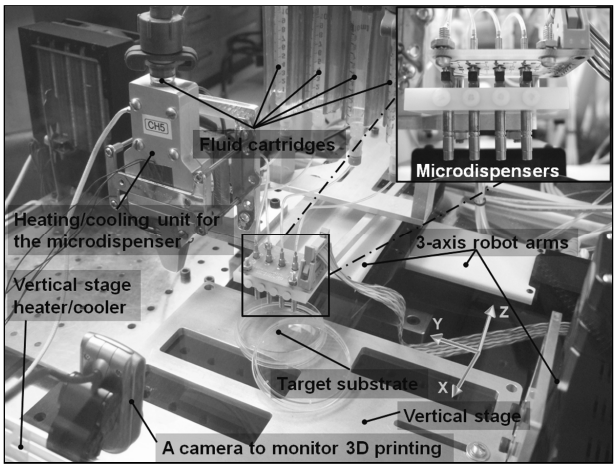


Figure 1: The 3D direct cell printer platform. Inset: 4 channel microdispenser array.

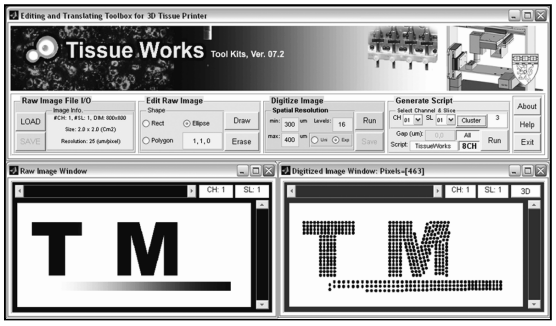
The target printing substrate was located on the stage, the position of which was controllable with a robotic arm oriented along the vertical axis.

Software Development

We also developed comprehensive in-house software with a user-friendly GUI (Fig. 2) to operate our cell-hydrogel printer. First, input data generator was implemented using MATLAB (Mathworks, Natick, MA). Various image sources such as radiological data, 3D layer-by-layer image sets, or 2D images can be loaded into the MATLAB software as grayscale and bitmap format, and then droplet dispensing coordinates were sampled from the loaded images based on the user-defined printing resolution. The coordinates of the dots were clustered and sorted depends on the geometry to minimize the printing time, and the optimized coordinates and printing sequence were used as input data for printer controller.

Visual Basic (Microsoft) was used to generate printer control code and to control the actual printing actions of 3-axis robot movements and droplet dispensing. The robot movements can also be controlled manually and printing progress was visualized as dots on the printed coordinate in real-time for users.

(a) Input data generator



(b)The printer controller

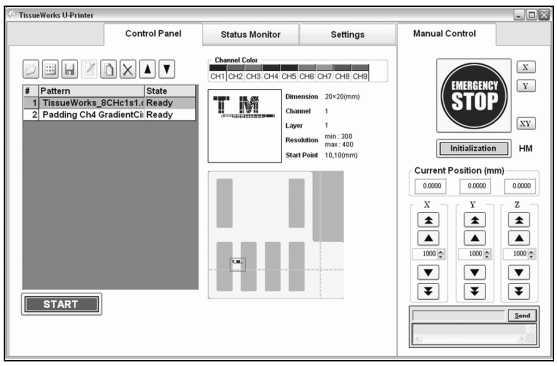


Figure 2: Software interfaces. (a) Input data generator prepares the coordinates for droplet dispensing from bitmap image. b) The printer controller orchestrated the dispensing of materials based on the information relayed from the input data generator.

RESULTS

Viability Test of Printed Cells

To investigate the viability of printed cells, three cell lines of human dermal fibroblasts, human epidermal keratinocytes, and rat embryonic day 18 neurons were used. Table 1 shows the viability of the cells dispensed with electromechanical microvalves using a pressure of 1.2 psi and valve opening time of about 500 µs. There was no significant difference in cell viability between printed cells and control cells (manually plated) for the three tested cell lines (student t-test, $p>0.05$). For printing cell viability test, cell suspension of 1×10^6 cells/mL was used for fibroblasts and keratinocytes, and 3×10^6 cells/mL was used for preparing neurons in culture media. In order to discourage the cell-aggregation, mechanical agitation was gently applied to cell-containing syringes using a motorized agitator. We did not detect any presence of nozzle clogging in these cell concentrations during the several hours of cell dispensing.

Table 1: Dispensed cell viability of human dermal fibroblasts, human epidermal keratinocytes, and rat embryonic day 18 neurons. Cell suspensions of 1×10^6 cells/mL were used for fibroblasts and keratinocytes, and 3×10^6 cells/mL was used for preparing neurons.

Cell Lines (All primary)	Viability of control cells	Viability of printed cells	Student t-test (n=30)
Human fibroblasts	96.6 ± 3.9%	95.0 ± 2.3%	n.s.; p > 0.05
Human keratinocytes	85.5 ± 7.1%	83.9 ± 5.7%	n.s.; p > 0.05
E18 rat neurons	75.2 ± 2.3%	78.6 ± 0.6%	n.s.; p > 0.05

n.s.: not significant

Printing of Hydrogel Scaffold

The collagen hydrogel precursor (2.23 mg/mL; diluted with phosphate buffered saline (PBS)) was used to construct a hydrogel scaffold. The collagen hydrogel precursor was maintained at a pH of 4.5, and kept in an ice before dispensing. 0.8 M sodium bicarbonate ($NaHCO_3$) solution in distilled water was prepared to be used as a crosslinking agent for collagen hydrogel precursor. Each printed collagen layer was coated with a nebulized $NaHCO_3$ solution for collagen gelation. 7 % (w/v) gelatin in distilled water was prepared at 40 °C to maintain liquid-phase before printing and loaded into a heated dispenser unit.

To construct a multi-layered hydrogel scaffold, first, the surface of a tissue culture dish was coated with a nebulized $NaHCO_3$ solution (each droplet less than 2 µm in diameter) using an ultrasonic transducer (SU-1051W, Suspentown, CA) operating at a resonance frequency of 2.5 MHz. Next, an initial layer of collagen was printed to fill

the 10 × 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. Cells or different scaffold materials other than primary hydrogel (collagen) were printed on to and embedded in this semi-gelled collagen layer. Nebulized $NaHCO_3$ solution was applied on the top surface to crosslink the printed collagen layer. The coating of $NaHCO_3$ also served as the binder and crosslinker for the subsequent collagen layers. The process was repeated to construct multi-layered cell-hydrogel composites.

Fig. 3a shows constructed 3D collagen (rat tail origin, type I) hydrogel structures created by using the printer, along with its printing schematics. Five layers of collagen were printed layer-by-layer with embedding a gelatin line (3rd layer from bottom) and colored microspheres squares in place of cells (2nd and 4th layer). Before the gelatin line printed, the 3rd layer of collagen was patterned as a wall while leaving space for the gelatin patterns.

After crosslinking the collagen pattern, gelatin was printed into the groove with a printing resolution of 400 μm under an operating pressure of 7 psi and a valve opening time of 400 μm, and cooled to room temperature (20 ˚C). The additional layer of collagen was printed on the top of the hydrogel layer containing the gelatin patterns. Colored microspheres were printed over collagen precursor before crosslinking and embedded in the collagen layer.

Fig. 3b is a 3D collagen hydrogel scaffold with multi-layered gelatin lines. Two gelatin lines, which were embedded in 2nd and 4th layer of collagen, distinctively appeared as two multi-layered orthogonal lines.

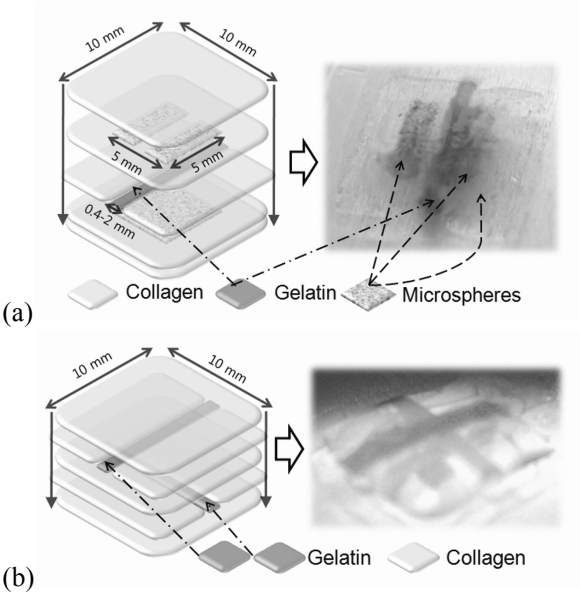


Figure 3: Printed, multi-layered collagen hydrogel scaffold (a) with a gelatin line (middle) and colored microspheres (squares) in place of cells for visualization (b) with two gelatin lines.

Construction of 3D Cell-Hydrogel Scaffold

We also tested the developed direct cell printing platform to pattern and culture neural cells in a 3D multi-layered collagen gel. Rat tail origin type I collagen was used as a hydrogel precursor. First, the collagen precursor was diluted to 1.12 mg/mL with 0.02 N acetic acid solution (CH_3COOH) and 1× PBS (volume ratio of 1:1:2) and kept on an ice bath. The dilution factor was determined from the collagen density that showed the neurite outgrowth among three different densities of collagen [3]. The pH of the prepared collagen precursor was approximately 4.5, and it remained uncrosslinked for liquid-phase dispensing.

Rat embryonic day 18 neurons (BrainBits LLC, Springfield, IL) were suspended in Neurobasal Media (Gibco, Invitrogen, San Diego, CA) with 2% B27 supplement (Gibco), 0.5 mM glutamine (Gibco), and 25 μM glutamate (Gibco), and were loaded into a fluid cartridge of the printer (see Fig. 1) after dilution to a concentration of 3.0 × 10^6 cells/mL.

For 3D cell-hydrogel scaffold construction, as described above (see printing of hydrogel scaffold section), the surface of the tissue culture dish was coated with nebulized $NaHCO_3$ solution, and the uncrosslinked collagen precursor was printed on the coated surface and crosslinked to form a gel due to the pH change. The process was repeated to construct multiple layers of collagen and cells. Each collagen precursor layer was printed to occupy a 10 × 10 mm^2 square area using the inter-dispensing distance (spatial resolution) of 600 μm. The droplets of collagen precursor were printed with pressure of 1.8 psi and a valve opening time of 500 μs, and the droplets of neuron suspension (3.0 × 10^6 cells/mL) were printed with a pressure of 1.2 psi and a valve opening time of 500 μs.

Fig. 4 shows that constructed scaffold with 8 multiple layers of collagen which embeded neurons in 2nd, 4th, and 6th layers. Between the cell layers, one collagen layer was inserted to secure the cross patterning of cells. After printing, the neural cell-collagen composites were cultured at 37 ˚C and 5% CO_2 in Neurobasal media with 2% B27 supplement, 0.5 mM glutamine, and 25 μM glutamate. A half of the media was replenished with fresh media without glutamate every 3 or 4 days. The cells patterned in the collagen hydrogel scaffold were cultured for 7 days. After culture, neurons were visualized with DiI staining. In constructed 3D neuron-hydrogel scaffold shown in Fig. 4, the multiple cell layers formed a single neuronal cross pattern, which was thicker than a single cell layer.

To confirm the capability of generating distinctive cell layers using the printer, neurons were embedded in 1st and 7th layers of an 8 multi-layered collagen scaffold (Fig. 5). Fig. 5 shows the controlled distance between cell layers by inserting 5 collagen layers, and the two distinct cell layers were clearly visible. These cells were also cultured for 7 days and were subsequently visualized using a live cell

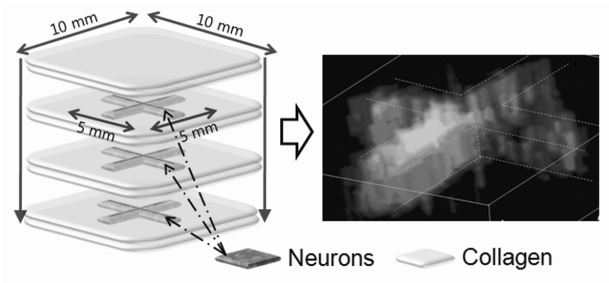


Figure 4: 3D rendering of a printed, multi-layered 3D collagen hydrogel scaffold with cross pattern of DiI stained rat embryonic neurons.

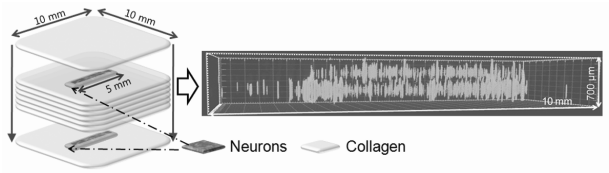


Figure 5: 3D rendering of the multi-layered 3D collagen hydrogel scaffold with distinguishable multi-layered parallel lines of live stained rat embryonic neurons.

staining method with Calcein AM (Fig. 5).

DISCUSSION

Tissue engineering ultimately aims to replace damaged or non-functional tissues with engineered tissue products made of cells for normal functional potency. By constructing an artificial tissue by strategic positioning of cells in proper biocompatible scaffolds, tissue engineering techniques can offer 3D cellular environments that mimic the *in vivo* conditions of healthy tissue physiologically or geometrically. The construction of such cell-hydrogel composites will also serve as a useful biomedical research tool to study cell-to-cell interactions, cell-to-extra cellular matrix (ECM) interaction, or to be used in potential pharmaceutical screening.

We developed a 3D cell-hydrogel printer and applied it to multi-layered collagen hydrogel scaffold and gelatin patterning. Two different types of phase-transition hydrogel materials, one being temperature-sensitive (gelatin) and the other being chemically crosslinkable (collagen), were used in our 3D hydrogel scaffold construction. The ability to print two different types of hydrogel to construct a 3D scaffold structure suggests that various scaffold options are available. And neural cells were patterned in 3D collagen scaffold by using the printer and cultured up to 7 days. To the best of our knowledge, it is one of the first demonstrations of a multi-layered construction of a neural cell structure by printing both neural cells and hydrogel scaffold material. We believe that the presented on-demand 3D printing technique will serve as a flexible tool needed for the creation of such as neural composites.

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 generating high cell viability upon printing. Since the gelatin line patterns were created based on the sequential dispensing of the spherical droplets, there was a degree of variability of line width when the droplet landed on a substrate. In addition, the gelatin lines were created on the collagen grooves that are also created by printing collagen matrix. Since the printing resolution, viscosity (it would merge the dispensed droplets, thus changing the intended morphology), and printed droplet volume may influence the printed morphology, a further investigation is indicated to optimize the dimension and shape of printed hydrogel lines. Such optimization/calibration is commonly employed for the dispensers of commercial ink-jet printers. The use of different dispensers with the capability picoliter-range droplets will potentially generate a smaller line width.

CONCLUSION

Our on-demand printing method was effective for creating a unique cell patterned 3D structure in a high throughput manner (each layer of structure can be printed under a minute). The method will provide much needed flexibility in tissue engineering and render new opportunities for studying cell-to-cell and cell-to-environment interactions in 3D and, with even potential opportunity for creating artificial tissues for clinical applications.

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