High-Fidelity Printing Strategies for Printing 3D Vascular Hydrogel Structures

K. Pataky[1], M. Ackermann[1], T. Braschler[1,] M. Lutolf[2], P. Renaud[1], J. Brugger[1]; 1. Institute of Microengineering – EPFL 2. Institute of Bioengineering - EPFL; Lausanne, Switzerland.

Abstract

Vascularization arguably poses the most significant hurdle for the success of most biomaterials-based tissue engineering therapies. In this work, we report two printing strategies that permit the 3D Inkjet printing of fluorescent alginate hydrogels into overhanging structures and closed lumens that could serve as vessel mimetics. The first is 4-Matrix printing, where the order of droplet printing in each printing layer is optimized so that new droplets do not coalesce with un-gelled droplets on the surface. The second is the use of incremental droplet spacing to print overhanging and closed structures in order to reduce the degree of down-wall flow.

Printed 3D structures were examined by 3D confocal microscopy in order to determine the effectiveness of these printing strategies. The results are promising and might be applied to other rapidly gelling hydrogel systems.

Introduction

Recently, 3D inkjet printing has received attention as a possible Tissue Engineering tool for producing complex tissue mimetics, precursors, and ultimately artificial organs [1]. However, to print bulk tissue mimetics or full-size organ precursors, it is necessary to create a vascular network to provide a convective nutrient and waste transport network for cells within the bulk [2]. Vascular structures also provide shear-flow cues for differentiation [3] and vascular remodeling [4].

To this end, two challenges exist in 3D hydrogel printing which are not typically encountered in 3D printing or rapid prototyping. Firstly, printed hydrogels typically exhibit some degree of surface flow, or even complete spreading, before gelation. Secondly, the diameters of vascular structures found in the body are on the order of several inkjet droplet diameters or even smaller [5, 6]. This means that minor deviations of droplet trajectory or surface flow during printing can drastically change the diameter of or even collapse vascular structures.

In this work, we report on two printing strategies that improve the resolution of 3D inkjet printing of alginate hydrogels. The first is 4-Matrix printing – so called because the image to be printed is sub-divided into 4 inter-spaced images that are printed sequentially. As a result, printed droplets have time to gel on the substrate before droplets are printed adjacent to them. The second printing strategy is depositing droplets in incremental spacing (smaller than the main droplet spacing in the bitmap matrix) when printing or bridging overhanging structures. Pattern fidelity is improved drastically with these printing strategies and they permit the fabrication of overhanging structures and closed lumens that could serve as vessel mimetics for angiogenesis and tissue engineering.

By controlling the timing, spacing, and distribution of printed droplets very precisely, we have succeeded in printing closed vessel mimetics transversally as opposed to axially; the current method reported in literature [7]. The method presented here also differs from those in which cross-linking agents are printed into liquid precursors to form shells [8]. As opposed to printing the vessels themselves, it is portions of the vessel walls and surrounding bulk which are printed – like bricks in a building.

Materials and Methods

Alginate Printing

Alginate was selected as a model system for this work because it is well-characterized from its widespread use in the food industry, it is biocompatible, and it gels rapidly in the presence of bivalent cations. The alginate (A0682, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) was mixed to 0.8% wt. in de-ionized water for all printing experiments. The alginate was conjugated with fluorescein so that printed structures could be characterized by laser scanning confocal microscope. All confocal microscope images were made in standard phosphate buffered saline (PBS). Printed droplet spacing in the 2D and 3D images presented in this work was 40 µm unless otherwise indicated.

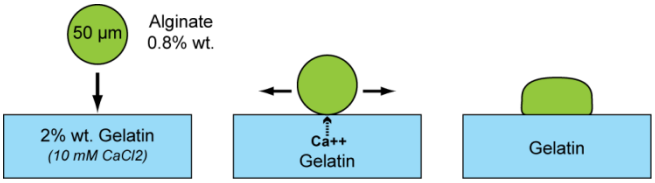

Figure 1: *LEFT: An alginate droplet is printed on a substrate. CENTER: The droplet spreads on the substrate as Ca^{2+} ions rush in. RIGHT: The droplet maintains a substantial portion of its height, allowing 3D structures to be printed.*

Hydrogel Substrates

In order to gel the alginate at the desired sites on the surface, alginate was printed onto Ca^{2+} laden gelatin substrates (Figure 1). The substrates were prepared by mixing a 2% wt. gelatin solution (48724, Fluka, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) containing 10 mM $CaCl_2$ 0.9% wt. NaCl and heating to boiling, after which the gels were cooled for several hours in a refrigerator.

The substrates served as a Ca^{2+} reservoir during printing to gel the alginate – first to gel the alginate arriving on the gelatin surface, and second to maintain the free Ca^{2+} concentration in the printed alginate layers to gel subsequent alginate layers.

Inkjet Setup

The 50 µm inkjet head (MD-K-140) and inkjet driver electronics (MD-E-201-H) were supplied by Microdrop Technologies GmbH (Nordstedt, Germany). Three motorized MTS50 stages (Thorlabs GmbH, Munich, Germany) actuated the printing substrate in X, Y, and Z directions during printing. LabView (National Instruments, Austin, Texas) was used to provide printing trigger signals and motion commands based on input bitmap file stacks.

Results

Droplet Gelation & Coalescence

It is known that droplet spacing and printing period have an effect on the morphology of lines of inkjet printed materials [9]. However, the alginate system used here is slightly different than those in which inks dry at a surface or a molten droplet solidify because alginate gelation is driven by a gradient of Ca^{2+} ions rather than a temperature gradient.

To investigate the extent of the difference, 50 µm diameter alginate droplets were printed at 10, 25, and 40 µm spacings and printing periods of 100, 200, 300, 400, 500, and 600 ms (Figure 2). The lines were imaged using a fluorescent stereo-microscope and were analyzed for bulging, smooth line, and stacked coin features which are seen in the inkjet printing of polymer materials [10, 11]. Soltman and Subramanian offer a detailed description of these effects [10]. Bulging results from axial flow (coalescence) due to surface tension [9]. Smooth lines occur when droplets coalesce but insufficiently to experience any major axial flow [10]. Stacked-coin structures occur when there is nearly no coalescence between droplets, such that individual droplet shapes are still visible [10].

At 10 µm spacing and with a 600 ms period, bulging was still visible, although the bases of the incident droplets were sufficiently gelled that an underlying line structure was apparent. At 25 µm droplet spacing, no smooth lines were observed, and the transition from bulging to non-bulging lines seemed to appear just after the 500 ms printing period (2 Hz). For the droplets spaced at 40 µm, bulging was no longer visible with a 300 ms printing period.

Unlike the case of inkjet printed lines of polymers in organic solvents, smooth lines are not observed in the alginate system and stacked coin structures are actually observed within beaded lines. This suggests that the droplet-substrate interface is gelled upon contact, but that the rest of the droplet may still reflow if its viscosity is sufficiently low (limited gelation).

In a standard row-by-row inkjet printing situation, this reflow would force the user to wait a set period of time between droplets to ensure gelation and avoid coalescence. 4-Matrix printing is discussed in the following section as a means to avoid droplet coalescence while eliminating this wait time.

4-Matrix Printing

In a basic 'linear' printing program, a droplet is dispensed at a point and then the system moves to an adjacent position to print the next droplet. In order to prevent coalescence (bulging) in this situation, it would be necessary to wait a fixed time between droplets.

To address this issue, we developed a program to divide each bitmap matrix into four sub-matrices that are printed sequentially. The sub-matrices each contain the matrix points of alternating rows and columns such that no sub-matrix contains adjacent droplets (Figure 3).

In order to assess the improvement in pattern fidelity using 4-Matrix printing, the letters "EPFL" were printed using a standard linear printing process and 4-Matrix printing (Figure 3). Both images were printed using the same 40 µm droplet spacing, the same inkjet droplet generation parameters, and the same bitmap files. Droplet coalescence in the linear printed "EPFL" is very evident and results in a reduction of edge straightness, corner definition, a reduction of the size of bordered structures (the "P")

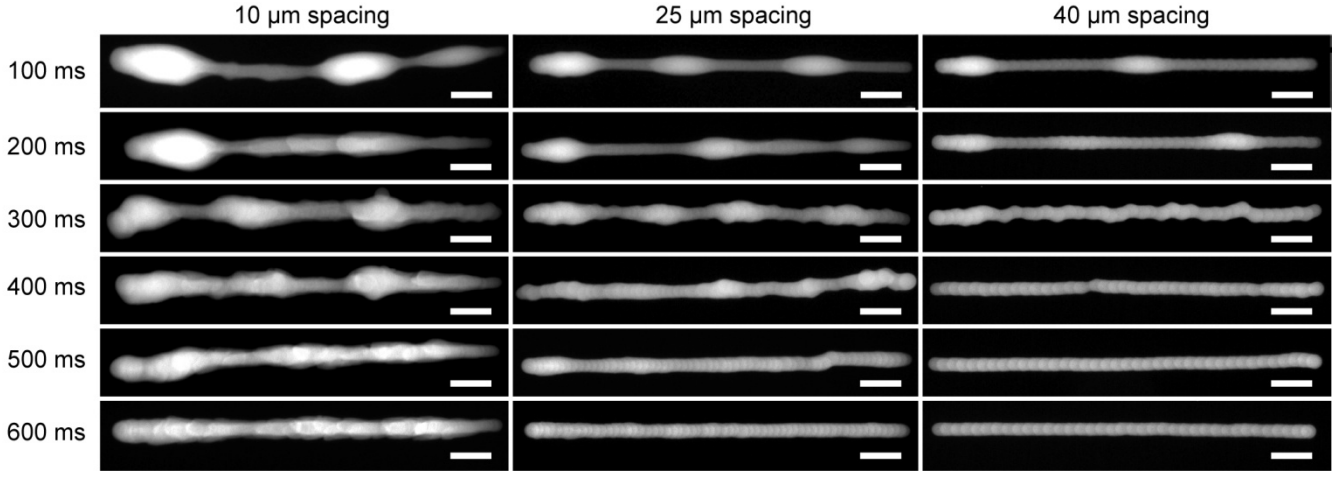

Figure 2: *1200 µm long alginate lines printed with 10, 25, and 40 µm droplet spacing. Printing periods of 100, 200, 300, 400, 500, and 600 ms were tested. Beading is visible at low printing periods and small droplet spacing, but as expected reduces with an increased delay between droplets and spacing between droplets. Scale bar = 150 µm.*

and general horizontal 'banding' due to the coalescence of droplets printed by row. In the 4-Matrix printed "EPFL" pattern, the sidewalls, corners, and bordered structures are maintained. There is no banding, and individual droplets remain visible within the letters.

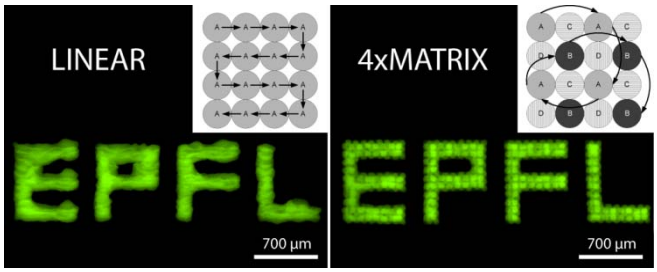

Figure 3: *TOP: Schematics of linear and 4-Matrix printing. In 4-Matrix printing, the original image is divided into 4 interspersed matrices: A, B, C, and D. First the A matrix is printed, followed by B, then C, and D such that droplets on the surface gel completely before new un-gelled droplets are printed adjacent to them. BOTTOM: The letters "EPFL" produced by linear and 4-Matrix printing. 4-Matrix printing improves edge and corner quality. It also reduces 'banding' to the point that individual droplets are visible.*

Overhanging Structures

In spite of the rapid gelation of alginate and the advantages of 4-Matrix printing, a certain degree of surface flow does occur during printing. When printing basic 3D alginate shapes such as cubes, we found that surface flow results in only a minor loss of pattern fidelity as the sidewalls remain fairly vertical. However, when printing overhanging structures or closed structures using standard droplet grid spacing, we found that the overlap for a droplet spacing of 40 µm was not sufficient to ensure gelation because printed droplets tended to run down the sidewall. Generally speaking, this decreased the height of the over-hanging structure and often resulted in its collapse.

In order to reduce the degree of down-wall flow, we developed a process improvement for printing by incremental steps (between the points of a standard printing array). Instead of merely printing droplets every 40 µm, we implemented the option of printing at 10 µm incremental steps. Consequently, there was more overlap between adjacent droplets near the edge of the overhanging wall, and less of the deposited droplets were deposited over the lip of the overhang.

To demonstrate the effectiveness of this process improvement, a closed lumen was printed. The first seven layers of the structure each consisted of a 15 x 15 droplet square with a 5 x 5 droplet void in the middle. Simply by adding incrementally spaced droplets in the subsequent layers, the lumen was successfully closed and retained its shape in a liquid environment (PBS) during confocal microscopy. 4-Matrix printing was used to maximize the pattern fidelity of each printed layer. The results are presented in Figure 4.

Down-wall flow is clearly visible in the center void of the second confocal image slice in Figure 4. The top slice was taken roughly at the height of the 8th image of the bitmap stack. The lumen was completely closed; a result of the down-wall flow from bitmap images higher in the stack.

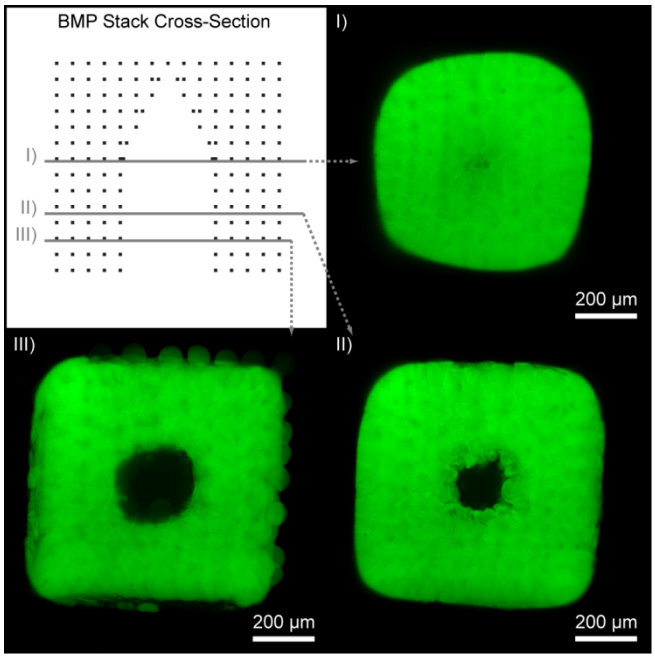

Figure 4: *Cross-section of stack of bitmap images used to print overhanging closed-box structure with fluorescent images of confocal slices taken from the corresponding printed structure. Black squares represent individual droplets. The typical height of a printed alginate layer in the stack is roughly 30 µm. The spacing between droplets in the grid is 40 µm with incrementally spaced droplet centers separated by 10 or 20 µm. At slice I) there is enough down-wall surface flow from upper layers to close the box completely. At slice II), down-wall flow of distinct droplets is evident. At slice III) near the base, there is little observable down-wall flow and opening dimensions are as expected.*

Conclusion

In this work, we have reported on two strategies that improve printing fidelity dramatically when inkjet printing complex 2D and 3D alginate structures.

First, 4-Matrix printing was shown to dramatically improve pattern fidelity by all but eliminating coalescence of partially gelled droplets on a substrate without an added wait time. Next a closed lumen was printed transversally by overlapping droplets at finer spacing than the standard printing grid. This reduced down-wall flow so that the lumen would not collapse during printing of the overhanging sidewalls. Despite a relatively limited number of applications of alginate in tissue engineering, the printing strategies presented here could potentially be applied to other rapidly gelling hydrogel systems such as pH cross-linked gels or alginate functionalized PEG [12].

The ability to print closed lumens transversally is a key first step towards producing vascularized bulk tissue mimetics and full-size organ precursors by inkjet printing. Ongoing work will focus on the methods required to create complex interconnected vascular structures.

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Author Biography

Kris Pataky is a Ph.D. student in the Microsystems Laboratory (LMIS1) under the guidance of Prof. Juergen Brugger at the Swiss Federal Institute of Technology in Lausanne (EPFL). His research focuses on the Inkjet printing of cells and hydrogels and the development of novel micro and nanotools for the study of stem cell niches. His undergraduate thesis (Engineering Science, University of Toronto) was on the development of an Inkjet alignment system and cell printing protocol.