Inkjet printing of cells and biopolymers

Skander Limem[a], Paul Calvert[a], Hyeon Joo Kim[b], David L. Kaplan[b]; a: Department of Materials and Textiles, College of Engineering, University of Massachusetts Dartmouth, North Dartmouth, MA 02747, USA; b: Departments of Biomedical Engineering and Chemical and Biological Engineering, and Bioengineering & Biotechnology Center, Tufts University, Medford, MA 02155, USA

Abstract

We are using inkjet printing to deposit patterns of silk, collagen and ionic complexes of polypeptides. These guide the growth of cells deposited on the patterns. Yeast has been printed onto agar and the effect of overprinted biopolymer layers on the growth of yeast is being studied. This method allows organized structures of differing cells and biopolymers to be printed in order to study cell-cell interactions.

Introduction

There is much current interest in "bioprinting" methods to deposit biopolymers and cells in 3D structures that may then be grown to form tissue or organs [1]. There is also increasing interest in "quorum sensing" and other forms of communication between cells of the same or different species. Certainly in the development of human tissues, but also in bacteria and yeast, the form and rate of growth depends not just on the chemical environment but also on the number and type of other organisms present. Structured patterns of cells could be used to study these effects. Inkjet printing is proving to be a versatile and simple approach to building these 3D structures.

Our approach uses modified thermal inkjet cartridges because these can be readily replaced if the nozzles become clogged. Recently we have been particularly focusing on "reactive" printing where multiple inks react on the substrate to form new materials.

Inkjet printing setup

A custom made inkjet printing system and a modified HP 51649A ink color cartridge were used to print layer-by layer poly-L-lysine and poly-L-glutamic acid structures. The printing setup, shown in Figure 1, consists of a PC controlled dual stepper motor system (MD-2b, Arrick Robotics, TX, USA) coupled with a precision XY table (MSt) (MAXY4009W1-S4, Velmex Inc., NY, USA). The interactive motion control MD2 software was used to program the desired patterns from a notebook computer. Before use, HP 51649A cartridges were cut open then foams were removed from the consisting three reservoirs. The cartridges were emptied then rinsed repeatedly with deionizer water to ensure no ink was left. Three Tygon tube pieces of 1inch long by ¼ inch internal diameter were cut then glued with two part epoxy adhesive onto the filters present in each reservoir. Custom foams were added in each tube to stabilize the pressure while printing. The modified HP 51649A ink color cartridges were mounted on HP20 cartridge holder and firmly clamped. A custom made inkjet driver with a built-in square pulse generator coupled with a custom adapter from a flexible printed circuit to a parallel port bus (FPC/Bus) cable was used to drive manually selected nozzles.

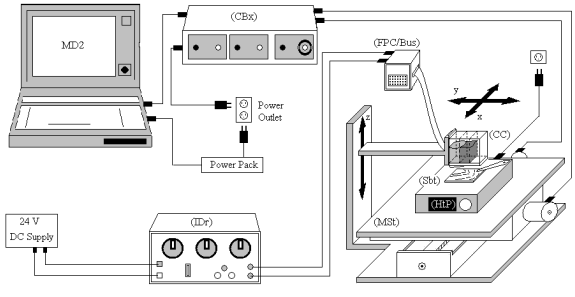

Figure 1: *Inkjet printing system*

Inkjet printing of silk and collagen

Silk fibers are formed in nature by spinning from a metastable liquid crystalline solution that is stored in the silk gland. This solution solidifies as shearing in the spinneret of the insect extends the folded silk chains into the insoluble beta-sheet structure. Silk can be resolubilized in dilute acid and then neutralized to make an unstable printable solution.

Cocoons of B. mori silkworm (Institute of Sericulture, Tsukada, Japan) were boiled for 20 minutes in an aqueous solution of 0.02M sodium carbonate, and then rinsed thoroughly with water to extract the glue-like sericin proteins. The extracted silk was then dissolved in 9.3M lithium bromide solution at 60°C yielding a 20% w/v. This solution was dialyzed in water using Slide-a-Lyzer dialysis cassettes MWCO 3500 (Pierce Biotech. Inc., IL, USA) for 48 hours. The final concentration of water based silk solution is about 8 wt% [2]. The silk solution was further diluted to 0.6% w/w for use as "bio-ink" [3].

As shown in figure 2a, these silk solutions can be printed as lines onto glass or plastic. After printing, the patterns were dried and then immersed three times in 70% / 30% ethanol / water solution for 15 minutes at room temperature. This step locks in the structural state of the protein to avoid resolubilization in culture medium, while also helping to sterilize the material.

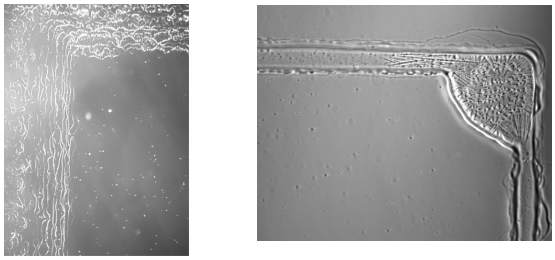

Figure 2 *(a, left) Printed silk (b, right) Printed collagen Linewidths 0.5 mm*

In the same way, collagen can be printed from a 0.1% w/w solution of collagen type I from rat tail in 0.05% HCl solution, figure 2b.

For both silk and collagen, the metastability of the protein solution tends to lead to clogging of the nozzles, such that long term printing of these solutions is not possible without stopping to clean the nozzles ultrasonically. It would be much preferable to print stable solutions and induce irreversible precipitation separately after printing.

Reactive printing of polypeptide complexes

Aqueous saline solutions of poly-L-lysine hydrobromide (PLL), poly-L-glutamic acid sodium (PGA) were deposited simultaneously using slightly modified inkjet color cartridges. After annealing overnight at 90°C, the resulting structure was insoluble in water. The X-ray elemental analysis conducted on these printed structures before and after annealing and washing showed that all bromide, chloride, and sodium ions were washed away hence suggesting that the residue is an insoluble complex of poly-L-lysine and poly-L-glutamic acid.

Poly-L-lysine hydrobromide, mol. wt. ≤ 15,000), and poly-L-glutamic acid sodium salt mol. wt. 3,000-15,000) (Sigma-Aldrich Inc., St Louis, MO) and sodium chloride were used to prepared two water based ink solutions: a polycationic ink, PLL, and a polyanionic ink, PGA. The PLL ink consisted of 0.25% w/v poly-L-lysine hydrobromide with 0.25% w/v sodium chloride in deionized water, while PGA- ink had 0.25% w/v poly-L-glutamic acid sodium with 0.25% w/v sodium chloride in deionized water. PLL and PGA inks were then loaded respectively in two reservoirs of the color cartridge. Before inserting pressure stabilizing foams, a vacuum pump was used to drain about ½ ml of each loaded ink to ensure the removal of residual water from previous cleaning steps.

Two nozzles were manually selected to fire simultaneously: one nozzle ejecting ink from reservoir (R) and the other from reservoir (B). The slope between the firing nozzles is calculated and input in the MD2 motion software to drive the XY stage such that the printed lines from the associated nozzles are superposed to allow the mixing of both PLL and PGA inks. The printed pattern was a parallelogram. The tangent of its small internal angle θ equaled the slope between firing nozzles with respect to the motion stage (X, Y) coordinate system. The stage surface temperature was maintained at 45 ± 4°C to speed up the water evaporation from printed lines.

Printed structures were annealed at 90 ± 2°C for 24 hours in VWR® oven (Sheldon Manufacturing Inc., Cornelius, OR). Deionized water was sprayed repeatedly onto annealed samples to wash out non-assembled components. Samples were then immersed in water bath for 15 minutes then dried in room temperature prior to examination.

The XY motion stage was programmed to move along the slope defined by the two printing nozzles. Figure 4 shows light microscope images of three different sections of the parallelogram top side after 5 layer-by-layer (LBL) printing cycles. It can be seen in the middle section, shown in Figure 4.b that the PLL and PGA printed lines were superposed and a color change was observed from light gray for individual inks, as shown in Figure 4.a and 4.c, to dark gray in the middle section.

To analyze the printed lines, the EDS attachment of our scanning electron microscope was used to determine the elemental composition of the layers. If the cationic and anionic polymer were fully reacted, only C,N and O would be detected without any of the original counter-ions (Na and Br). The theoretical elemental composition of LBL printed self-assembly, was calculated based on the chemical formula and weight composition of PLL and PGA inks. Table 1 shows theoretical and experimental values for lines as printed and after annealing and washing.

A layer-by-layer self-assembled ionic structure was produced from 0.5 % w/v poly-L-lysine hydrobromide and 0.5 % w/v poly-L-glutamic acid sodium inks with no added salt. Figure 3 shows the confocal micrograph of the self-assembled structure formed after 10 printing cycles that was annealed and washed. The printed line presented a characteristic valley and basin topography. The average maximum ridge height was 5 μm and the maximum height in between reached 1 μm.

Table 1: Composition of PGA-PLL complexes

Atomic composition	As printed		Annealed and washed	
	Theoretical	Experimental	Theoretical	Experimental
C	*19.0*	41.8 ±1.5	*54.4*	45.8 ± 2.6
O	*10.1*	16.5 ± 1.4	*28.9*	33.2 ± 1.9
N	*5.8*	9.3 ± 1.0	*16.6*	20.5 ± 1.1
Na	*24.1*	12.8 ± 1.3	*0.00*	0.1 ± 0.1
Cl	*31.1*	15.8 ± 1.5	*0.00*	0.0 ± 0.1
Br	*9.8*	3.8 ± 0.9	*0.00*	0.3 ± 0.1

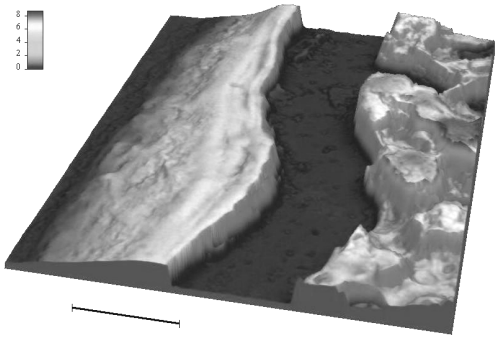

Figure 3: *Confocal image of annealed and washed line. Bar = 25 μm*

Printing of Yeast Cells

S. cerevisiae (533W strain) colony were transferred from a plate culture into 10 ml complete YPD (dextrose) media placed in a conical tube. After 14 hours of culture in 30°C at 150 rpm shaking, the S. cerevisiae reaches mid-Log phase of its growth curve, is centrifuged the supernatant aspirated. The S. cerevisiae is re-suspended and mixed in phosphate buffer saline (PBS), re-centrifuged and re-suspended in 10 ml of PBS. The cell concentration achieved after 14 hours of culture and PBS washing is about $1.5x10^9$ cells/ml. This cell suspension is diluted to prepare 4.5 ml solution with $5x10^6$ cells/ml final concentration of S. cerevisiae. At this concentration and under uniform condition, one S. cerevisiae cell would be deposited for every four printed drops. Figure 4 shows yeast printed onto agar in a Petri dish at 100,200 and 400 drops per secondYeast-biopolymer sandwich

Figure 4: *Inkjet printed yeast*

Yeast-biopolymer sandwich

As shown in figure 5 yeast cells were printed onto a printed silk layer on agar and then overprinted with another 50 layers of silk. It was expected that the trapped yeast would grow much more slowly that that exposed directly to the nutrients in the growth medium. Figure 6 shows the resulting yeast growth at 24 and 52 hours. The short sides of the parallelogram have only yeast and the long side have yeast entrapped by silk. In this experiment no difference was seen; apparently the silk blanket does not limit uptake of the nutrients to the growing cells. The cells surrounded by the blanket can be seen in figure 7.

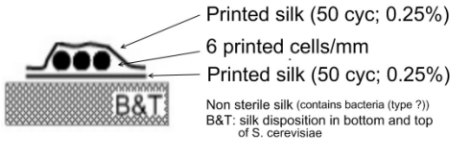

Figure 5: *Cell sandwich*

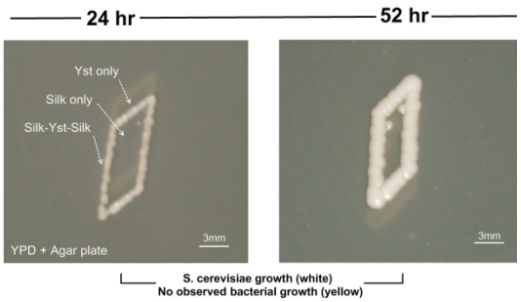

Figure 6: *Yeast grows equally well inside or outside the sadnwich*

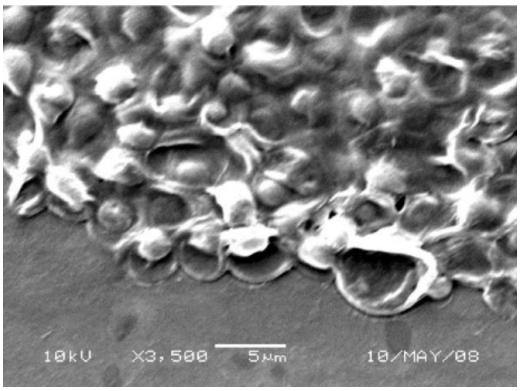

Figure 7: *Yeast between silk layers*

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Author Biography

Paul Calvert is Professor and SkanderLlimem is a graduate student in the Department of Materials and Textiles at University of Massachusetts Dartmouth. Hyeon Joo Kim is a postdoctoral fellow and David Kaplan is a Professor in the Bioengineering & Biotechnology Center, Tufts University