Differentiation of Bone Marrow Stem Cells on Inkjet Printed Silk Lines

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

Water based silk solutions were successfully inkjet printed for the first time into patterns of parallel lines onto vinyl plastic substrates. Human bone marrow stromal cells (hMSCs) were seeded on the silk printed patterns and cultured in the presence of 100 ng/ml of bone morphogenic protein (BMP-2). After one week of culture cell growth and attachment showed site specificity on the silk printed lines. Both alkaline phosphatase activity and cell morphology indicated hBMSCs differentiation into osteogenic cells along the silk printed lines. After 4 week of culture, the cellular bridging of adjacent silk printed lines took place for all interline distances lower than 1.25 mm. Therefore, commercial inkjet printing technology can produce complex viable cellular patterns with 111 ± 24 μm lateral resolution, through the deposition of bioactive materials. The results provide a first step toward cell specific control using 3D inkjet printing techniques using biocompatible gel systems to regulate cell functions.

Introduction

Tissue engineering, the science and engineering of forming functional living tissues and organs, utilizes various processing techniques to generate temporary scaffolds for transplanted cells to attach, proliferate, and differentiate into tissue-like functions. In tissue engineering, scaffolds are required to accommodate mammalian cells and guide their growth and tissue regeneration in three dimensions [1].

Most attempts to form scaffolds use biocompatible and/or biodegradable synthetic polymers (e.g. silicones, saturated aliphatic polyesters, polyurethanes, and polyhydroxyalkanoates) [2, 3] and bioceramics including bioinert (e.g., alumina and zirconia), resorbable (e.g., tricalcium phosphate), bioactive (e.g., hydroxyapatite, bioactive glasses, and glass-ceramics), or porous materials for tissue ingrowth (e.g., hydroxyapatite-coated metals). Various natural polymers, including proteins, have been investigated for scaffold fabrication. Two groups of proteins have been heavily studied: collagen [4, 5], and silk [6]. Silk is a natural protein spun into fibers by several Lepidoptera larvae. The most common type of silk is that from the silkworm, Bombyx mori, which has been exploited for centuries in the textile industry and used as biomedical suture material [6]. Silk consists of two structural proteins: fibroin and sericin. Fibroin is the structural component giving silk fibers their strength and toughness and consists of a heavy chain (~390 kDa) and light chain (~25 kDa) linked by a single disulfide bond. The sericins are hydrophilic proteins functioning as glues to hold the fibroins in bundles [7]. In addition to the remarkable mechanical properties of silk fibers [6], fibroin was shown to proteolytically degrade with predictable rates [8].

Many polymer processing techniques are available for forming materials for cell and tissue growth, including fiber bonding, solvent casting and particulate leaching, membrane lamination, melt molding, polymer/ceramic fiber composite-foam processing, phase separation, gas foaming using CO_2 gas at high pressure, emulsion freeze drying, freeze drying [9], and electrospinning [10]. Inkjet printing might be used to print the scaffold polymers, to print the cells or both.

A modified thermal inkjet desktop printer (HP 550) was effective in printing Chinese Hamster Ovary (CHO) and embryonic motor neuron cells into predefined patterns on soy agar and collagen gels. Over 90% of the printed cells were not damaged during the nozzle firing [11]. Cell-printing technology using thermo-reversible gels offers possible solutions to subtly combining cells, growth factors and scaffolds into an architecture permitting their unrestricted interaction, especially when distinct cell types are required in anatomically specific sites to achieve defined biological functions. Though in its very early stages, organ printing, based on thermal inkjet printing technology, also offers potential [12, 13].

As a first step towards organ printing, in the present work we studied the feasibility of printing water based silk solutions into defined patterns, and monitored the growth and differentiation of human bone marrow stem cells cultured on these thermal inkjet printed silk surfaces. We also studied cellular bridging and fusion of cell sheets between neighboring parallel silk lines. These results provide a first glimpse into modes of controlling cell geography in 3D printed gel systems, to regulate cell-cell interactions through such technology, and ultimately to consider multilayer gel systems toward organ related reconstruction and functions. These options derive from the use of biocompatible silk-based polymeric systems, the sol-gel phase transition of these proteins and their structural stability in the solid state to serve as slowing degrading biomaterial matrices.

Materials & Methods

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% [8]. The silk solution was further diluted to 0.6% w/w for use as "bio-ink" [14].

A custom made inkjet printing system and a modified HP 51626A ink cartridge were used to print the line patterns from the water based silk solutions. The cartridge was rinsed thoroughly with water prior to the silk solution introduction.

The water based silk solution was printed onto 22 x 22 mm2 clear vinyl plastic cover slips (Structure Probe Inc., PA, USA). 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. The patterns were then dried under aseptic conditions and then rinsed with 1x phosphate buffer solution (PBS) (Gibco, USA) prior to cell seeding.

Human bone marrow stem cells, were seeded onto the printed patterns and cultured for up to 28 days. Six samples (3 for hematoxylin and eosin staining and 3 for alkaline phosphatase activity) were removed from each set respectively after 1 day, 1 week, 2, and 4 weeks for analysis. The alkaline phosphatase is commonly used as an early bone differentiation marker [15].

Results

Water soluble silk solution (0.6% w/w) was printed onto the vinyl plastic substrates to form a series of parallel lines with a gradient in separation distance. Figure 1 shows the SEM images of the silk printed lines in the tight (1.a), middle (1.b and 1.d), and wide (1.c) zones. The printed lines demonstrated irregular edges and small proximal spots resulting from satellite droplets. The measured line average width is 111±24 μm. Due to the surface tension, the silk lines in the tight zone (T) (Fig 1.a) merged to form a thin mono-layered silk film of 6.25 mm length and up to 1.5 mm width. After printing for some hours the nozzles would become clogged with solid silk.

Cell proliferation on the control surface is shown in Fig. 2. The initial cell seeding concentration was 100,000 cells/well (~10,000 cells/cm^2). However the average cell concentration measured on samples after 1 day of culture was 5,600 ± 1,000 cells/cm^2 because of the preferential cell attachment to the well base. Cell growth reached 40,000 ± 2,645 cells/cm^2 after 4 weeks of culture.

After one day of culture, the cells did not show preferential attachment or alignment based on visual assessments via optical microscopy. The cells were randomly distributed across the vinyl plastic cover slip for both printed and non-printed substrates. However, after one week of culture, the cell attachment, proliferation and morphology became specific on the silk printed substrates in comparison to the controls. The images of the hematoxylin and eosin stained samples cultured for 1, 2, and 4 weeks are shown in Figure 3 (light microscope) and 4 (digital picture).

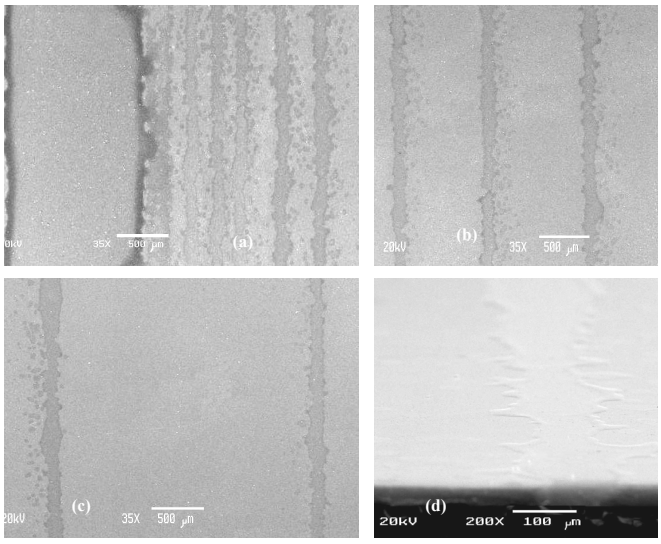

Figure 1. *SEM images of the silk printed pattern. (a) Tight; (b) Middle; (c) Wide zone; and (d) 10º tilted view of a single silk printed line.*

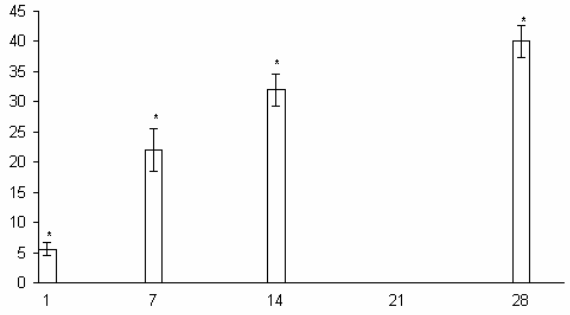

Figure 2. *Growth of cultured hBMSCs on vinyl plastic control substrate. (*) Data are shown as mean ± standard deviation.*

The cells migrated and attached along the silk lines in the printed samples (Figure 3.a-i). The cells orbed to form nodule like structures [16] only on the silk printed lines (Figures 3.d, e, h) indicating possible calcification sites. Calcium deposits were also observed and were profuse along the silk lines rather than on control surfaces (Figures 3.i). The cells reached confluence in the control samples after 2 weeks of culture. Cultured cells also exhibited ALP activity specifically on the printed silk line. The ALP activity was more pronounced after 4 weeks of culture. In the printed samples, a few cells attached away from the silk pattern.

Individual cells and small cell clusters were present in the space between the printed lines and away from the patterns throughout the culture period. However, the results of 1 week culture compared to 2 weeks indicate that the cell density dropped notably in the unpatterned spaces and increased from 16,800 ± 2,000 cells/cm^2 to 36,000 ± 2,516 cells/cm^2 on the silk lines.

When the cells growing on adjacent silk printed lines fused together to form a continuous sheet covering all or part of the interline space, the lines were referred to as "bridged". The lines

were "fully bridged" when the interline space was thoroughly covered with cells, and "partially bridged" when cells fused in one section or more of the interline space. Figure 8 shows the culture time and type of cellular bridging of neighboring printed silk lines. All lines in the tight zone separated with less than 250 µm were bridged within the first week of culture. The lines separated with a distance varying from 375 µm to 625 µm were partially bridged within one week and fully after 2 weeks of culture. The lines separated by 750 µm to 1000 µm were partially bridged within 2 weeks and fully after 4 weeks of culture. The rest of the middle zone (1125 and 1250 µm) was only partially bridged after 4 weeks. For the wide zone (> 1250 µm), no bridging occurred after 4 weeks of culture.

Discussion

Successful cell patterning using inkjet deposition of 0.6% water based silk solution is reported. Unlike the vinyl plastic control surface, cell attachment of hMSCs followed by differentiation became protein pattern specific after 1 week of culture. The cells migrated from the bulk plastic surface and attached preferentially to the printed silk fibroin protein surfaces where they proliferated for the rest of the culture period. The attachment and proliferation of cells was also notable along the edges of the vinyl plastic cover slip used as printing substrate. This could be due to the surface roughness and higher surface area at the edges. These results suggest that non-treated vinyl plastic surfaces are not cell repellent which allowed the fusion of cells to form continuous sheets over four weeks and full bridging between the printed lines of the silk pattern. Full bridging occurred only for interline distances lower than 1 mm.

Although inkjet printers can be designed to handle liquids with viscosities up 100 cP [17], continuous printing of water based silk solution requires concentrations lower than 0.6 %. For concentrations higher than 1% w/w, the firing nozzle clogged within the first few minutes of printing. When the concentration was between 0.6 % and 1%, silk printing continued up to 15 minutes and then stopped and was accompanied by satellite formation.

In the HP 51626A thermal inkjet print head, a heating element placed in the nozzle wall heats the solution in the nozzle up to 300ºC in less than 5 µs, leading to the nucleation and growth of a vapor bubble of "ink". Based on the data obtained, it was concluded that the elevated temperature did not damage the silk fibroin proteins used in the study or the ability of the cells to attach to and differentiate on this protein.

Conclusion

A custom printing setup using a modified commercial thermal inkjet cartridge was successfully used to produce defined patterns of silk fibroin protein with 111 µm lateral resolution. Human bone marrow stem cells were seeded and cultured in the presence of 100 ng/ml of BMP-2. Cell growth and differentiation were specific to printed silk surfaces. Flexibility, low cost, speed, ease of use and sterilization, and little effect on solute structure make thermal

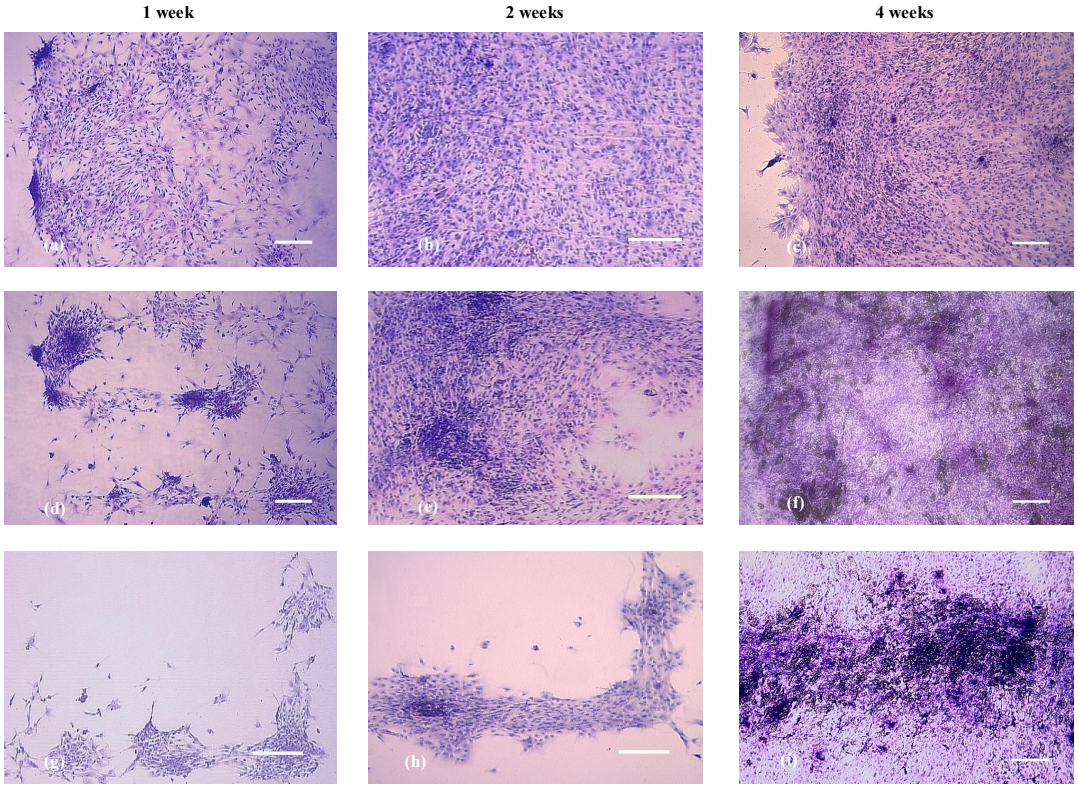

Figure 3 *Light microscope images of printed silk patterns seeded with hBMSCs and stained with H&E of (a), (b), (c)* tight; *(d), (e), (f) middle; and (g), (h), (i) wide zones; and (j), (k), (l) control substrate (silk free), respectively after 1, 2, and 4 weeks of culture. (Scale bar: 500 µm).*

inkjet printing a promising technology for understanding tissue development and building complex 3D cellular constructs.

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Biography

Paul Calvert is department chair and Skander Limem is a PhD candidate in the Department of Materials and Textiles at University of Massachusetts Dartmouth. David Kaplan is Chair of the Department of Biomedical Engineering at Tufts University and Hyeon Joo Kim is a postdoctoral fellow.

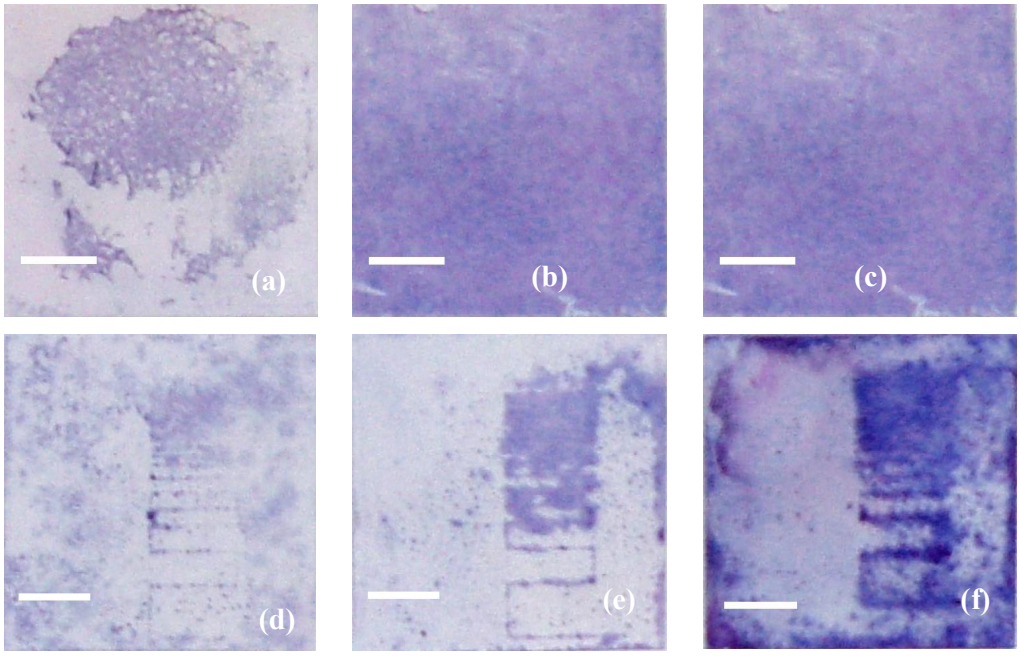

Figure 4. *Digital images of (a), (b), and (c): control (silk free), versus (d), (e), and (f) silk printed patterns on vinyl substrates seeded with hBMSCs and stained with H&E respectively after 1, 2, and 4 weeks. (Scale bar: 500* μm).

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