Image based printing of structured biomaterials for realizing complex 3D cardiovascular constructs

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Abstract

Considerable effort has been focused on printing synthetic biodegradable scaffolds for hard tissue applications. The requirements for soft tissues, in particular cardiac tissue are somewhat unique since the cellular arrangement is more essential for achieving function. The goal of this study was to develop a technique that allows aligning of cardiomyocytes in printed channels.

A fabrication chamber was filled with a 2% alginic acid solution, a liquid that is known to crosslink under mild conditions to form a biodegradable hydrogel scaffold. An ink cartridge was filled with calcium chloride cross-linker. By printing a variety of concentrations of cross-linker, the fabrication process could be optimized by comparing the fidelity of the printed pattern to the designs.

Adult feline cardiomyocytes were added to the biomaterial and the resulting cardiac constructs were electrically stimulated. In response, the cardiac constructs contracted rhythmically and synchronously. The shortening extent of the edge was up to 7.0% of the total length of the constructs compared to 3.5 % in constructs without channels. In this study printed alginate gels demonstrated that by engineering the microstructures of the printed alginate scaffolds improved function can be achieved.

Introduction

Recently there has been an increased interest in building biomaterial scaffolds with controlled architecture for tissue engineering applications. Most commonly hierarchical scaffold designs are built using layer-by-layer fabrication processes known collectively as "solid free-form fabrication" [SFF] reviewed recently in several articles [1,2,3,4,5]. All SFF systems build a 3D structure by layering a 2D material onto a moving platform. Commercially available systems either photopolymerize liquid monomer; sinter powdered materials; process material either thermally or chemically as it passes through a nozzle; or print material, such as chemical binder, onto powders.

Many of these SFF technologies can offer effective ways to precisely control matrix architecture [size, shape, interconnectivity, geometry and orientation] of a scaffold, yielding biomimetic structures of varying design and material composition. Hierarchical design of the scaffolds with micron to millimeter features have demonstrated that enhanced control over mechanical properties, biological effects and degradation kinetics of the scaffolds is possible [4]. Moreover, SFF techniques can be easily automated and integrated with imaging techniques to produce constructs that are customized in size and shape allowing tissue-engineering grafts to be tailored for specific applications or individuals [1].

Since fabrication feasibility has been amply demonstrated, the critical issue has shifted to showing that designer scaffolds outperform traditional scaffolds. In spite of advances, the current SFF techniques have yet to lead to harmonically organized complex tissue constructs. The primary hurdles to be overcome are the same as in the classical tissue engineering approach: the building of capillaries and the exact placement of cell populations throughout the scaffold. Additionally, the toxic solvents or high temperatures are still widely used in most SFF techniques are not suitable to many engineered tissue devices and limit their further applications in tissue engineering[6]. Thus, adapting existing SFF technologies to tissue engineering continues to be a genuine challenge.

One recent advance in this area has been the combined printing of cells and biomaterials using inkjet printing technology to generate 3D scaffolds and cellular structures [7,8]. This advance raises the possibility of spatially controlling not only the scaffold structure, but also the type of tissue that can be grown within the scaffold and the thickness of the tissue as capillaries and vessels may be constructed within the scaffolds. A fundamental advantage of this process is the simultaneous delivery of scaffolding materials, living cells, nutrients, therapeutic drugs, growth factors, and other important chemical components at the correct time, and position, in the correct amount, and within the correct environment to form living cells/ECM [or scaffold] for in vitro or in vivo growth. However, there are some technical barriers to the development of this emerging inkjet printing technology in developing the appropriate biomaterial ink and image processing algorithms to realize functional, viable, and functionally vascularized 3D constructs. This paper details some promising biomaterials inks and a rudimentary image processing to achieve aligned, functional cardiovascular tissue engineering constructs.

Materials and Methods

Preparation of cell sources: Adult feline cardiomyocytes were prepared according to the protocols described before in detailed [9]. In brief, 10 adult cats of either sex [1.8-4.0 kg] were anesthetized with meperidine 92.2 mg/kg im], acepromazine maleate [5 mg/kg im], and ketamine hydrochloride [50 mg/mg im] and heparinized [1,000 U iv]. A left thoracotomy was performed, the heart was rapidly removed, the aorta was cannulated, and the coronary arteries were perfused retrogradely for 10 min, first with a recirculating HEPES-Krebs buffer [in mM: 140 NaCl, 4.8 KCl, 2.4 $MgSO_4$, 1.2 NaH_2PO_4, 4.0 $NaHCO_3$, 0.5 $CaCl_2$, 12 HEPES, and 12.5 glucose], second with a non-recirculating buffer of the same composition without supplemental calcium, and third with a recirculating calcium-free buffer supplemented with 1.6 mg/ml

collagenase B [Boehringer-Mannheim]. Perfusion was terminated when the heart was flaccid. The heart was removed from the cannula, and the cardiac tissue was minced and then gently sieved through a 210-µm nylon mesh to isolate the cardiomyocytes. After centrifugation the cells were washed with the HEPES-Krebs buffer and 1% BSA and resuspended in HEPES-Krebs buffer plus 1% BSA.

3D thermal inkjet printers: Canon Pixima ip1500 printers were modified with the use of the methods previously described [10]. Moreover, in order to build 3D structures, a z-axis module was added into the modified printers. The z-axis module has been described previously [11]. A sterile 50 ml conical tube [Fischer Scientific] was customized and used as the elevator chamber. It was pre-filled with an alginate hydrogel solution An inkjet cartridge was emptied and rinsed thoroughly with double distilled water, sonicated using an ultrasonic sonicator to remove any ink or blockages, sprayed with 100% ethanol and the whole assembly of the printer along with the cartridge was placed overnight in a Class II hood under ultraviolet light prior to use [12].

Printing of hydrogels: A test patterns of parallel lines with 42 microns diameter [0.12 pt] were printed layer-by-layer by spraying a crosslinker onto liquid hydrogels. The hydrogels consisted of 0.1% laminin and alginate of varying amounts from 0.5% to 12% dissolved in phosphate buffered saline (PBS). Laminin is an adhesive protein of the basement membrane. Alginate is a linear carbohydrate copolymer derived from seaweed that is known to crosslink under mild conditions to form gels of several 100 kPa in modulus and strengths. The crosslinker was calcium chloride which was varied in concentration from 0.1 to 0.5 mmol/ml. Resulting patterns were analyzed under a phase contrast microscope. Two control groups were used, one group did not include laminin in the hydrogel; additionally the second group was not patterned.

Alignment and functional characterization of cells on printed hydrogels: Isolated cardiomyocytes were either flown under gravity over patterned hydrogels, or entrapped between two consecutive layers of hydrogels. All samples were placed in a custom-designed study chamber, superfused with cardiomyocyte culture medium, maintained at pH 7.4, 37°C, and electrically stimulated to contract at 1 Hz or other designed frequencies. The transients of the printed construct contraction were determined by using computer assisted videomicroscopy and IonOptix® video edge-detection protocols [IonOPtix, Milton, MA].

Results and Discussion

One objective of this study was to determine if varying hydrogel and crosslinker concentrations has an effect on the resulting structures, in terms of microstructure as well as overall resolution. A systematic variation of both the hydrogel and crosslinker should sufficiently change the kinetics of the gelation to effect fusion of neighboring drops. Table 1 is showing representative images of resulting samples as function of crosslinker and hydrogel concentration. Concentrations below 0.3 mmol/l of $CaCl_2$ and below 1% alginate did not result in gels with appropriate strength. Line patterns printed with alginate concentrations above 6% and $CaCl_2$ concentrations above 4 mmol/ml resulted in structures that appeared as lines in the phase contrast microscope; concentrations below 2% alginate and 2 mmol/ml $CaCl_2$ appeared as droplets. Further analysis by SEM revealed that the droplets and lines observed in the phase contrast images are hollow and contain no hydrogel material. In fact, the material gelled around the periphery of the line or dot. This can be explained by a surface gelling mechanism, in which the surface of the drop gels fist, forming a capsule or shell. This shell will prevent the larger alginate molecules from penetrating into the drop, but will allow the smaller calcium ions to diffuse to the outside.

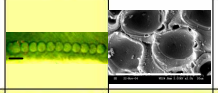
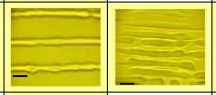
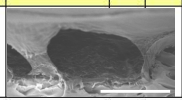
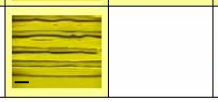
Alginate (%) / $CaCl_2$ (mmol/ml)	0.5	2.0	3.0	5.0	6.0	8.0	10	12
0.1	X	X	X	X	X	X	X	X
0.2	X	X	X	X	X	X	X	X
0.3	X							
0.4	X							
0.5	X							

Table 1. *Microscopic views of printed alginate biomaterials. Images are phase light micrographs, top view, 40x; the high resolution images are SEM cross-sectional micrographs of printed structures. To fabricate alginate channels, best results are obtained with 8% alginate solution and 0.4 mmol/ml $CaCl_2$. [Scale bar = 50 microns]*

The alginate in the periphery of the drop is subsequently crossslinked, resulting in solid material surrounding the shell. The diffusion is concentration driven; thus, by changing the concentrations of hydrogel and crosslinkers, a variety of structures can be obtained, ranging from amorphous to dots to channels. Understanding this concentration dependence is important for I age based scaffold design as virtual pixels are translated into hollow dots, and vectors into channels.

Interactions of cardiomyocytes with channels are shown in Figure 1. As the channel diameters can be of similar size than the basal dimensions of the cells, they tend to align under flow conditions into the channels. Control samples without laminin or channels did not show preferential alignment.

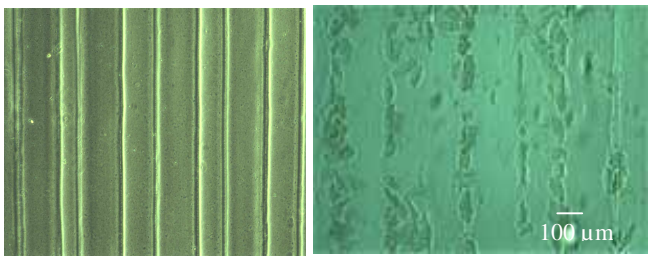


Figure 1. *Phase contrast microscopy of a alginate scaffold immediately after printing and after flowing of cardiomyocytes across the surface. [top view, 40x]*

The scaffolds were stimulated at 1Hz and contractility recorded as described above. Pure alginate constructs did not show

much contractility, as expected from the weak attachment known to exist in pure alginate. However, the individual cardiomyocytes contracted about 9.8%, thus confirming viable, functional cells. Alginate/laminin constructs with channels and aligned cardiomyocytes contracted 7% and individual cardiomyocytes inside the channels contracted on average 7%. It is hypothesized that the somewhat lower contractility of the individual cells is due to the attachment to the hydrogel construct. Constructs without the channel architecture and unaligned cardiomyocytes contracted about 3.5%.

The printhead of this printer has a single row face plate with 300 orifices each ~30 microns in diameter. The orifices in each row are separated by 30 microns and are able to eject 15 pl drops with a resolution of 30 microns. Print speeds of up to three million drops per second have been reported resulting in an hourly fabrication throughput of 150 ml volume at 30 microns resolution per printhead. More recently HP and Canon introduced printers that are capable of delivering close to $100x10^6$ drops per second, with a 2-5 pl drop volume which could potentially result in a ten-fold increase in fabrication throughput.

Conclusions

We described an image based fabrication method to construct hydrogel structures with 30 micron resolution. The method is of particular interest to tissue engineering since the pixel data prescribes holes, pores or empty channels in the final material, all known to be of utmost importance in the field of tissue engineering by allowing maintenance of cell viability and function. Using this image based approach to create porous materials may be useful in designing and building larger and more complex 3D structures that may otherwise not be possible to fabricate. Furthermore, this method may be incorporated with image based diffusion optimization algorithms [13] to lead to optimized, hierarchically designed tissue engineering scaffolds.

Cardiomyocytes align within the 30-50 microns channels of alginate hydrogels and show improved function under stimulation, when compared to unaligned cells. The adhesive basement membrane protein laminin was needed to facilitate cardiomyocyte attachment to the material. The improved function of the pseudo-tissues is encouraging and may ultimately serve to better understand the engineering of a cardiac tissue engineered implant.

References

[1] D. W. Hutmacher, M. Sittinger, and M. V. Risbud, “Scaffold-based tissue engineering: rationale for computer-aided design and solid free-form fabrication systems" Trends Biotechnol., 22, 354 [2004]

[2] V. L. Tsang and S. N. Bhatia, “Three-dimensional tissue fabrication" Adv. Drug Deliv. Rev., 56, 1635 [2004]

[3] W. Y. Yeong, C. K. Chua, K. F. Leong, and M. Chandrasekaran, “Rapid prototyping in tissue engineering: challenges and potential" Trends Biotechnol., 22, 643 [2004]

[4] K. F. Leong, C. M. Cheah, and C. K. Chua, “Solid freeform fabrication of three-dimensional scaffolds for engineering replacement tissues and organs" Biomaterials, 24, 2363 [2003]

[5] E. Sachlos and J. T. Czernuszka, “Making tissue engineering scaffolds work. Review: the application of solid freeform fabrication technology to the production of tissue engineering scaffolds" Eur. Cell Mater., 5, 29 [2003]

[6] V. Mironov, “Printing technology to produce living tissue" Expert. Opin. Biol. Ther., 3, 701 [2003]

[7] W. C. Wilson, Jr. and T. Boland, “Cell and organ printing 1: protein and cell printers" Anat. Rec. A Discov. Mol. Cell Evol. Biol., 272, 491 [2003]

[8] V. Mironov, T. Boland, T. Trusk, G. Forgacs, and R. R. Markwald, “Organ printing: computer-aided jet-based 3D tissue engineering" Trends Biotechnol., 21, 157 [2003]

[9] M. R. Zile, M. K. Cowles, J. M. Buckley, K. Richardson, B. A. Cowles, C. F. Baicu, G. Cooper, IV, and V. Gharpuray, “Gel stretch method: a new method to measure constitutive properties of cardiac muscle cells" Am. J. Physiol, 274, H2188 [1998]

[10] E. A. Roth, T. Xu, M. Das, C. Gregory, J. J. Hickman, and T. Boland, “Inkjet printing for high-throughput cell patterning" Biomaterials, 25, 3707 [2004]

[11] T. Boland, X. Cui, B. Damon, and T. Xu, “Application of Inkjet Printing to Tissue Engineering" Biotechnology J., [2006] in press

[12] T. Xu, J. Jin, C. Gregory, J. J. Hickman, and T. Boland, “Inkjet printing of viable mammalian cells" Biomaterials, 26, 93 [2005]

[13] S. J. Hollister, “Porous scaffold design for tissue engineering" Nat. Mater., 4, 518 [2005]

Author Biography

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