Perfusion 3D Bioprinting with Gelatin and Hydrogel for Vascular Tissue Engineering

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Introduction

Vascular disease is one of the leading causes of morbidity and mortality in developed countries, and thereby necessitates the replacement of diseased artery or vein through surgical intervention. Tissue engineering offers an alternative approach of designing biomaterials for small diameter artery regeneration.

Vascular graft infections occur infrequently, with an incidence that varies by surgical site but in general ranges from 0.5% to 6%. Infections of vascular grafts are associated with significant mortality and morbidity risk. Vascularization is also a major problem in tissue engineering, especially for engineering tissues that are thicker than approximately 200 μm. Insufficient vascularization limits the oxygen and nutrient transfer to cells, which can lead to hypoxia and formation of non-uniform tissue structures. Vasculature-like structures are usually multilayer and hollow. They have a complex shape with varying diameters throughout the body. Thus, it is quite difficult to imitate this complex 3D structure using materials similar to native vessels such as hydrogels. Though a few techniques have been explored to fabricate this complex 3D structure, direct bioprinting of such a complex 3D structure on a solid platform in a single step has not been realized.

Natural vascular tissues serve as a unique experimental tool for investigating fundamental mechanisms of vascular function and maturation process under 3D flow conditions. The methods have great potential in vascularized tissue fabrication as the vascular channel is simultaneously created while cells and matrix are printed around the channel in desired 3D patterns

1. Specific Aims

1.1 Applying 3D Printing Tissues Process to Produce Specific Vascular Tissue Which Can Reflect The In Vivo Responses of Individual Characteristics.

Fabrication of vascular system is one of the main challenges in 3D printing, because isolated cells cannot live in spaces of less than 3 mm of volume. Vascular channels transport oxygen, growth factors and nutrients and remove the waste solutions for living cells. Therefore, well-designed blood vessel tree of capillaries and microvessels are required for operating large tissues or organs. Moreover, sufficient mechanical properties are also needed for vascular tissue engineering to tolerate physiological pressures and surgical connections (Jang et al., 2018)

1.2 Conduct Specific Characterization to Identify Biocompability for Manufactured Vascular Tissue.

Creating functional vasculature represents one of the most fundamental challenges in tissue engineering. For large engineered tissue constructs, vascularization is pivotal for maintaining viability, especially in the case of highly metabolic cardiac tissue. Strategies to stimulate the vascularization of implanted tissue substitutes include chemical modification of biomaterials, optimization of pore sizes to facilitate blood vessel ingrowth, and incorporation of pro angiogenic growth factors. However, the recruitment of native endothelial cells and subsequent physiological growth of new blood vessels occurs at a prohibitively long time-scale, preventing sufficient mass transfer throughout the large constructs (Wüst, Müller & Hofmann, 2011).

1.3 Identify Cell to Cell Interaction and The Host Response to Scaffolds.

Vascular implants elicit the host immune response, often resulting in engraftment impediment or rejection. Besides this negative effect, however, the immune system components also yield a positive influence on stem cell recruitment and differentiation, allowing tissue regeneration and healing. Thus, a balanced cooperation between proinflammatory and proresolution players of the immune response is an essential element of implant success. The scaffold, immune, and stem cells are linked by a three-way interaction, and many efforts are being made for scaffold-appropriate design and functionalization in order to drive the inflammation process toward regeneration, vascularization, and implant success (Crupi, Costa, Tarnok, Melzer & Teodori, 2015).

2. Clinical Impacts

Vascular graft infections occur infrequently, with an incidence that varies by surgical site but in general ranges from 0.5% to 6%. Infections of vascular grafts are associated with significant mortality and morbidity risk and cost an estimated $640 million annually in the United States. Clinical presentation varies by time elapsed from implantation and by surgical site. (Kilic et al., 2015). With those statistics, a new approach could save many people from suffer from vascular graft infection. Also it could avoid economic loss due to the expensive vascular graft surgery.

Although 3D bio-printing technology has great potential in creating complex tissues with multiple cell types and matrices, maintaining the viability of thick tissue construct for tissue growth and maturation after the printing is challenging due to lack of vascular perfusion. Perfused capillary network can be a solution for this issue ("3D bio-printing of multi-layered cell-laden fluidic hydrogel scaffold", 2010).

3. Research Strategy

3.1. Background

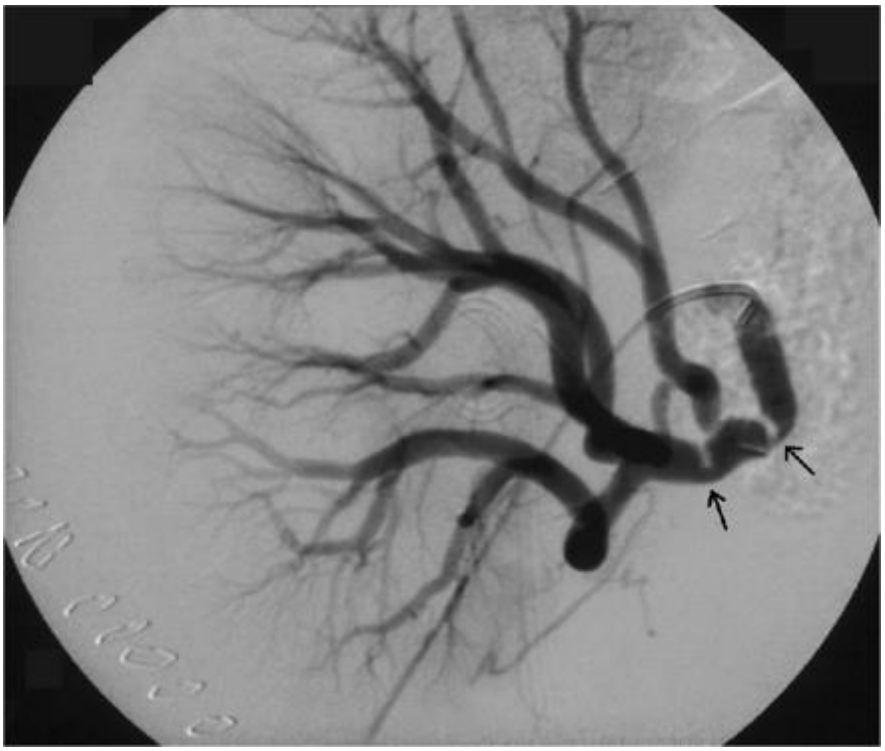
Synthetic grafts for replacing occluded arterial vessels were first introduced in the 1950s following surgical complications associated with harvesting vessels, the frequent shortage of allogeneic grafts, and immunologic rejection of large animal-derived vessels. Synthetic vascular grafts continue to exhibit a number of shortcomings that have limited their impact.

According to Enomoto (2010) smaller vascular grafts made from conventional synthetic biomaterials, such as expanded polytetrafluoroethylene (ePTFE) and polyethylene terephthalate (Dacron), particularly those <5 mm in internal diameter, are associated with a high incidence of thrombosis, especially when the distal anastomosis is below the knee.

Autologous vein has been the preferred graft material for infrainguinal arterial reconstruction since the use of reversed saphenous vein. Unfortunately, 20–30% of vein grafts develop stenoses in the first postoperative year, due low-risk for the development of vein-graft stenoses need to be defined to myointimal hyperplasia. The majority of graft stenoses are asymptomatic until they progress to graft occlusion (Nghiem, 2008).

A wide variety of factors causing vascular thrombosis in the microvascular free flap reconstruction have been encountered. The most frequent situation in our experiences has been vascular kinking, especially in drainage veins with thin walls. Vascular redundancy after recanalization and flap inset position also increases the risk (Horng, Chen, Lee & Horng, 2010).

Vasculature-like structures are also usually multilayer and hollow. They have a complex shape with varying diameters throughout the body. Thus, it is quite difficult to imitate this complex 3D structure. Synthetic materials such as silicone tubes were initially used to mimic blood vessels in vitro but their poor permeability and compatibility restrict their further applications (Mironov et al., 2009).



Angiogram demonstrating the kinks (marked by arrows) in the transplant renal artery.

3.2. Significance

By using gelatin as biomaterials and hydrogel (collagen) as scaffold for vascular tissue we can replace the synthetic vascular graft. The superiority of gelatin and hydrogel has been proven to overcome the dawbacks of synthetic vascular graft. Three dimension perfusion main features are using phase-changing hydrogels without harsh chemicals as well as a dispensing technology that is gentle enough for the cells. An important advantage of this technology is its ability to simultaneously deposit live cells, growth factors along with biomaterial scaffolds at precisely controlled locations to mimic the native tissue architecture. With that advantages manufacturing small vessels like arterioles, venules, and capillaries with enough precision to match individual patients' needs are possible (Lee et al., 2014).

4. Innovation

The main features of perfusion 3D printing are using phase-changing hydrogels without harsh chemicals as well as a dispensing technology that is gentle enough for the cells. An important advantage of this technology is its ability to simultaneously deposit live cells, growth factors

along with biomaterial scaffolds at precisely controlled locations to mimic the native tissue architecture (Yanagawa, Sugiura & Kanamori, 2016).

3D perfusion bio-printing method to construct a perfused vascular channel within thick collagen scaffold. The vascular channel is constantly perfused and resides on a biologically relevant and porous matrix in which other cell types can be easily introduced to form desired tissue structures during the process of bio-fabrication (Lee et al., 2014).

The cell migration into the collagen matrix was exclusively occurred by angiogenic invasion and sprouting. Single cell migration into the matrix was rarely observed. Luminal structure was developed in these sprouts, and the existence of lumen was confirmed by injecting fluorescent microbeads. It resulted the vascular channel maintained its structural integrity for long-term tissue culture under the flow condition (Urbaczek et al., 2017).

5. Approach

5.1. Rationale

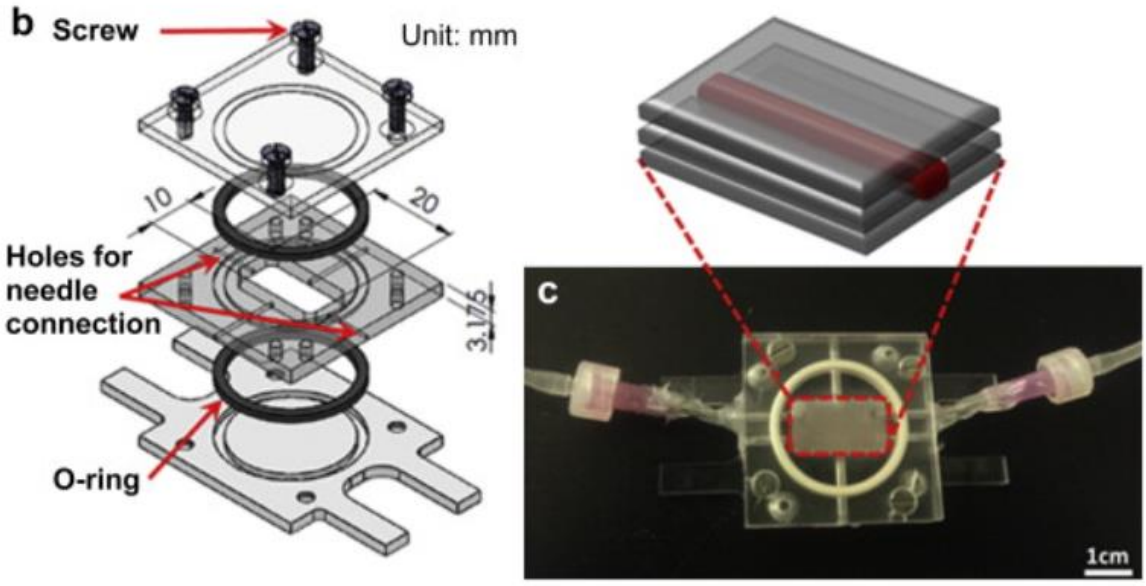
Natural polymers generally show excellent biological performances; specifically, they do not activate chronic inflammation or toxicity. Collagen is the major ECM protein in the body that supplies mechanical support to many tissues. Collagen demonstrates low antigenicity, low inflammatory response, biocompatibility, biodegradability, and excellent biological properties. Collagen type I is one of the main components of the vascular wall, whereas it is widely used as scaffold for vascular tissue engineering applications (Catto, Farè, Freddi & Tanzi, 2018).

3D Bio printing has been used for decades for many application but they still have some of the drawbacks especially for vascular tissue engineering.

Bioprinting method	**Inkjet 3D bioprinting**	**Laser-assisted 3D bioprinting (LAD)**	**Stereolithography (SLA)**
Description	Thermal, piezoelectric, or electromagnetic forces expel successive drops of bio ink onto a substrate	Bioink and cells are suspended on the bottom of a ribbon and when vaporized by a laser pulse, are propelled to a receiving substrate	Use digital light to cure bioink in a layer by layer fashion
Disadvantages	Lack of precision in droplet placement and size, need for low viscosity bioink	Time consuming, high cost	Use of high intensity UV light, lengthy post-processing, lack of compatible materials

5.2. Experimental for Specific Aims

5.2.1 Experimental Equipment



(b) Custom-designed flow chamber consists of three transparent polycarbonate pieces, two o-rings for sealing, and screws.

(c) Actual picture of flow chamber. The chamber is connected to the perfusion system through needles installed on the side of chamber.

The printing platform consists of a robotic stage with 3-axis motors, an array of dispenser with microvalves, and an attachable temperature-controlling unit. The liquid-based materials including cell suspensions, soluble chemicals, micro-beads suspension, and hydrogel precursors can be printed while maintaining cell viability. A user-friendly software interface enables on-demand printing of spatial patterns of the materials in 3D at sub-cellular accuracy. In this printing platform, air pressure is applied on the loaded materials and a droplet of the material is dispensed when the microvalve is opened for short time (100–800 μs). The volume of dispensing droplet (i.e. drop size) is adjusted by controlling of valve opening duration and the air pressure. The printing pressure and dispenser valve opening times are crucial parameters for the proper printing of various biomaterial hydrogel and cells (Lee et al., 2014).

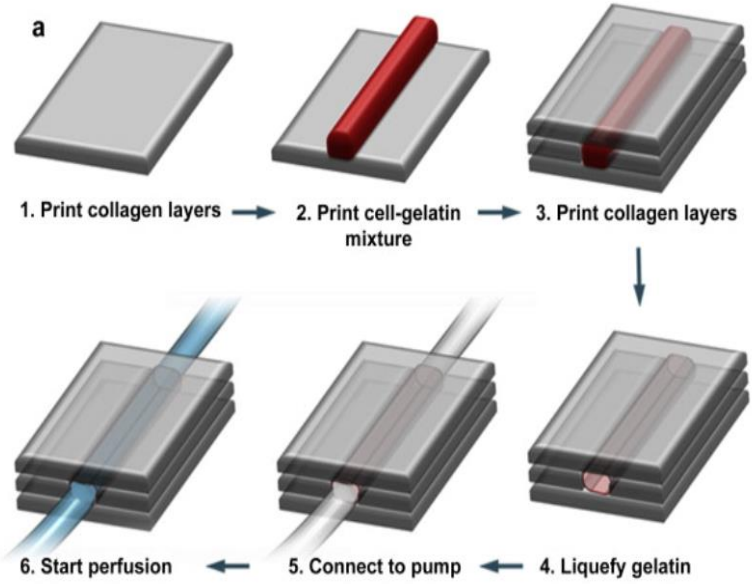
5.2.2. Experimental Material

Collagen hydrogel precursor (Rat tail) is used as a scaffold material for printing. Gelatin from porcine skin was used as a sacrificial material to create fluidic channels. Human umbilical vein endothelial cells (HUVECs) are cultured to form vessel (Lee et al., 2014).

5.2.3. Cell Culture and Hydrogel Preparation

For this experiment, Human umbilical vein endothelial cells (HUVECs) were cultured and the culture media was changed every two days. HUVECs were routinely passaged onto tissue culture flasks and discarded after 8 passages to ensure representation of key endothelial characteristics (Yao et al., 2012). Right before cell printing, cells were harvested using and then maintained as cell suspensions on ice until ready to be used. Collagen hydrogel precursor (Rat tail) was used as a scaffold material for printing. The collagen precursor from stock was diluted using acetic acid and maintained on ice until it was loaded into a syringe printing. Gelatin from porcine skin was used as a sacrificial material to create fluidic channels (Lee et al., 2014).

5.2.4. Experimental Procedure



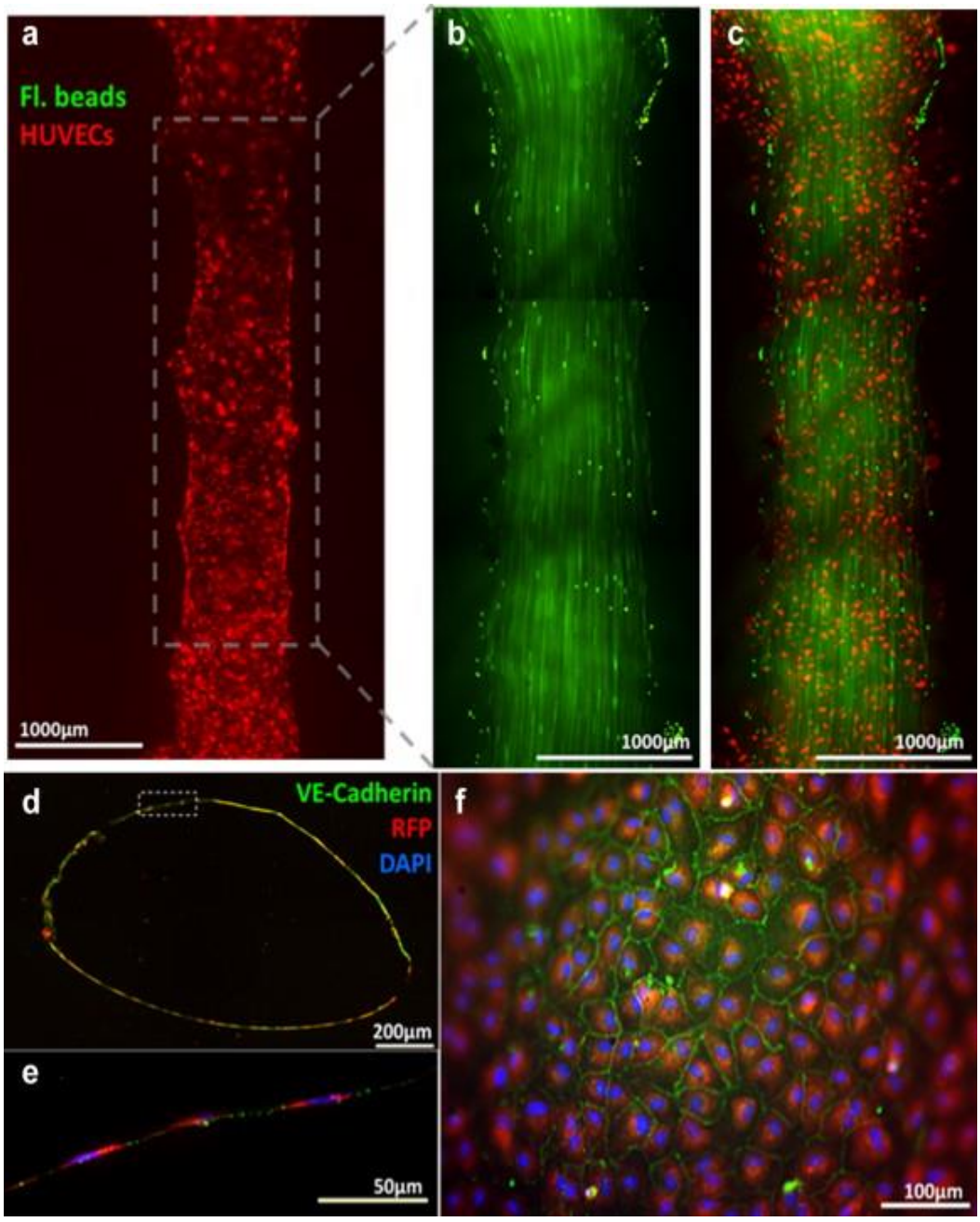
Schematics of the vascular channel construction procedure using cell–gelatin mixture

By a layer-by-layer approach, we created a fluidic vascular channel within a 3D collagen matrix using collagen precursor, gelatin, and HUVECs. The collagen precursor was printed on a flow chamber and polymerized by $NaHCO_3$ nebulization. The collagen precursor was printed on a flow chamber and polymerized. This collagen printing step was repeated 5–6 times to fill the bottom half. Gelatin and HUVECs is printed in a straight pattern (fig a.2). The gelatin solidified within 1–2 min when it was exposed to room temperature The flow chamber was kept in 4 ℃ for 10 min to complete gelatin solidification and to provide a higher degree of rigidity to the gelatin pattern. More collagen layers were printed to cover the gelatin pattern in full (fig a3). The flow chamber s sealed and incubated at 37 ℃ for 30–40 min to complete the collagen gelation and conversely, to liquefy the gelatin (Fig a.4). During this incubation process, HUVECs captured within the gelatin sank down slowly and attached to the inner surface of the channel. The flow chamber is flipped over every 10–15 min to allow cells to attach to both bottom and top surfaces of the channel. The flow chamber was connected to a dispensing pump and gentle media flow (∼0.3 mL/min) was applied to wash out the gelatin and to obtain a perfused vascular channel (Fig. a 5–6). After this step, the flow chamber was connected to a dispensing pump and gentle media flow (∼0.3 mL/min) was applied to wash out the gelatin and to obtain a perfused vascular channel (Fig. 1a, steps 5–6). For the channel samples for dynamic culture, flow rate was gradually increased until it reached 10–15 dyn/cm2 of shear stress. Flow rate calculation was based on channel dimensions. 1.5–4.0 mL/min of flow was applied, depending on channel dimensions (Lee et al., 2014).

5.2.5. Viability Test

5.2.5.1. Beads injection

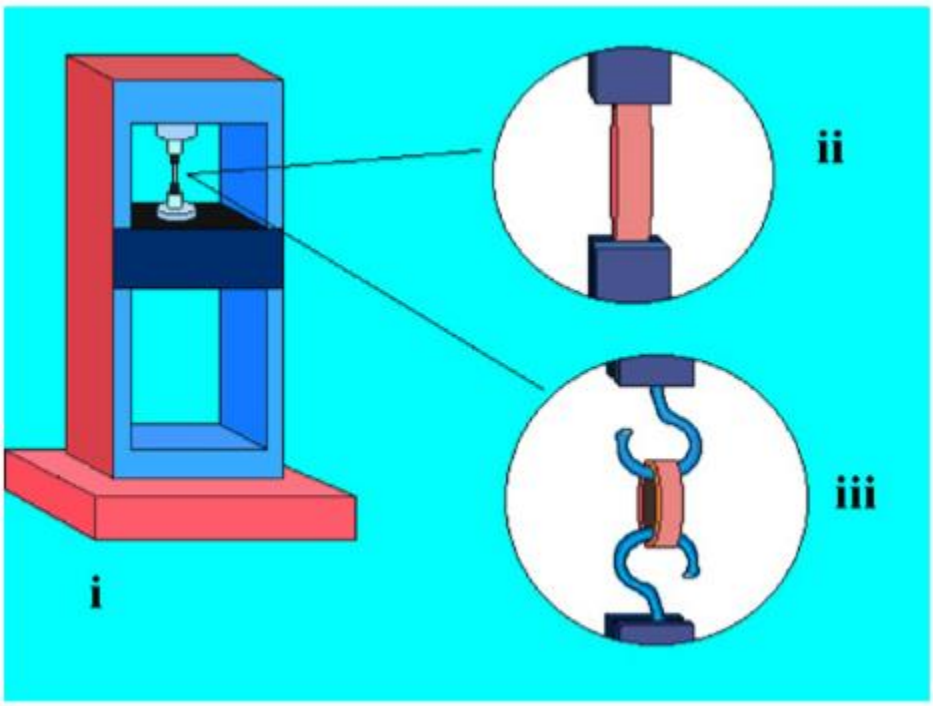
To visualize the flow pattern, green fluorescent beads were added to the culture media. The media containing green beads was perfused with 10–15 dyn/cm^2 of flow rate, and the beads flow pattern was imaged using a wide-field fluorescent microscope. To verify the existence of luminal structure in angiogenic sprouts, 10 μm size of Polystyrene Microspheres (Invitrogen) were injected into the vascular channel through the luer-connection.



(a) Fluorescent images of vascular channel system on Day 1 of dynamic flow culture. Large image of endothelial cells (red) seeded on the printed channel. (b) Laminar flow in the vascular channel was visualized by motion of green fluorescent beads. Discontinuity of flow pattern is due to stitching (mosaic) of multiple images. (c) Overlay image of the flow of green beads and printed endothelial cells. (d) Cross-section of vascular channel after 5 days of dynamic culture. (e) Magnification of dotted inset area in (d). Endothelial cells formed a monolayer along the inner surface of the channel. (f) Fluorescent image of inner surface of vascular channel after 5 days of dynamic culture. The edge of the image is out of focus due to a curved surface of the channel. (d–f) blue: DAPI nuclei staining; red: RFP-transfected HUVEC; green: VE-cadherin

5.2.5.2. Mechanical Test

After fabrication, conduits were crosslinked and soaked in CaCl2 solution for 24 hours. Soaking them in CaCl2 solution minimized the effect of residence time A Biotense Perfusion Bioreactor was used to evaluate tensile strength characteristics. Each sample was a maximum of 30 mm long and mounted on rectangular mini sandpaper in order to prevent slippage during the test. Upon applying the mechanical load, conduits were ruptured in the middle or near edges. Displacement and load information data were recorded by a data acquisition system.



Tissue mounting in materials testing machine for evaluation of mechanical properties. Diagram illustrates: (i)
Instron Model 1122 materials testing machine tower, (ii) a longitudinal vessel strip mounted between grips, (iii) a transverse vessel section mounted onto hooks that are gripped by machine. The longitudinal strips were used to perform mechanical tests along the vessel axis (parallel to blood flow), while transverse vascular rings were used to test mechanical properties normal to the direction of blood flow.

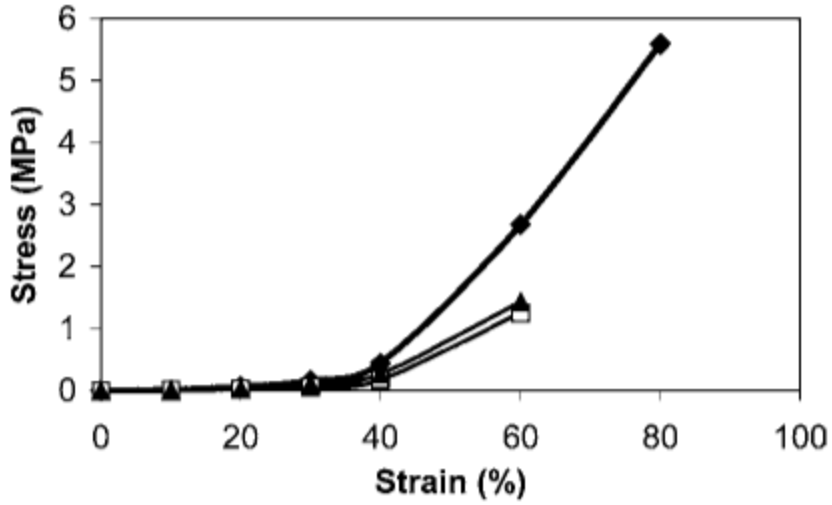
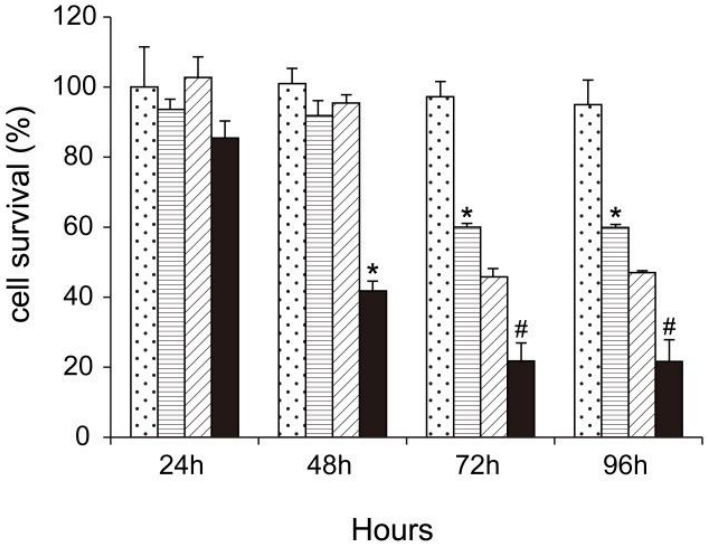


Diagram illustrates separation of total, elastic, and viscous curves from incremental stress–strain testing

The strain-rate dependence of the stress–strain behavior of tissue samples, were determined using a strain-rate of 1000%/min. The calculated moduli of these tests were then compared with the results from the 10%/min strain-rate tests. The moduli were fit using a least squares method for the 1000%/min (Snowhill & Silver, 2005).

5.2.5.3. MTT Assay



We can use the MTT method to detect the effects of combined treatment on cell growth. A significant degree of proliferative inhibition was observed after 72 hours (Yao et al., 2012).

5.3. Potential Problems and Strategies

This mechanism, as also other 3D Bioprinting procedures have some drawbacks that need to be overcome. Biomaterials from gelatin tend to have low viscosity and mechanical integrity. To overcome this limitation in the future it is possible to combine with some composite to increase mechanical strength (Graham et al., 2017). To avoid clogging the lowest pressure that showed stable dispensing without clogging can be used for the printing of collagen and gelatin. Also specific biodegradable inert compound to reduce friction will help to avoid clogging (Catto et al., 2015).

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