Direct-Writing and Quantitative Characterization of Soft Material Tubes

by

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A thesis submitted in conformity with the requirements for the degree of Master of Applied Science
Institute of Biomaterials and Biomedical Engineering
University of Toronto

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Abstract

Soft materials tubes and their formation have important implications in a wide range of applications across the field of tissue engineering. Presented here are improvements and further characterization made using a microfluidic-based platform for the continuous formation of collagen tubes with gelation timescales up to several minutes and write speeds ranging from 3-7mm/s. A bioprinting cartridge consisting of three microfluidic channel layers are aligned by a unifying radial outlet that, when infused, forms cylindrical sheaths of concentric flow to extrude tubular constructs. Tubes can vary in sizes by manipulating focusing fluid flow rates to yield tubes of various dimensions, which can be approximated using analytical models. Assessment of gelation timescales can be performed via \textit{in situ} measurements by translating optical measuring units to measure pH gradient, turbidity and diameters. This platform could have the potential of extruding cell-laden tubes to bioprint tubular microtissues.
Acknowledgments

I would like to acknowledge Dr. Axel Guenther for his support and supervision. Thank you to my colleagues, past and present, for their assistance, advice and suggestions that was invaluable to my research. To my family and friends, thank you for your boundless patience, kindness and moral support that kept me motivated throughout the completion of my degree.
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1 Introduction

1.1 Background

The human body is abounding with soft material tubes that vary in size (diameters, wall thickness, length), spatial composition and consequently differ in mechanical properties, some examples include blood vessels, airway conduits present in the respiratory tract as well as ductal tissue found in the biliary tract (Figure 1a).

In developing tissue engineering approaches it is imperative that biomaterials can achieve similar native extracellular matrix (ECM) spatial organizations as seen in vivo. Recapitulating hierarchical architectures on soft material constructs has important implications in promoting cell adhesion, proliferation, migration, differentiation and alignment [1-2].

Recapitulating microscale architecture is important in achieving tubular constructs which can closely mimic mechanical and cellular behavior on the macroscale. Formation of tubular structures have many applications such as tissue-engineered blood vessels (TEBV)[3-4], perfusible microtissues [5], and vascularized biomaterials [6]. Many approaches have been demonstrated for the continuous and discontinuous formation of soft material tubes. Diameters, materials, heterotypic composition in radial (R), circumferential (C), axial (A) directions, in addition to dimension control that have been demonstrated for in-plane and off-plane preparation and molding of tubular structures are summarized in Figure 1b. Tubular constructs have been made with micro to millimetre scale diameters. Various materials have been used to demonstrate tube formation. Proper selection and subsequent characterization of materials is a crucial aspect in the pursuit of forming biomimetic constructs.
Figure 1. Brief Summary of Tubular Structures and Formation Approaches. (a) Comparison of select tubular structures in the human body. Blood vessels [7], Airways [8-9], Bile duct [10-12]. (b) Comparison of tubular formation methods and techniques. In-plane extrusion [13-23], Off-plane extrusion [24-29], Molding [30-35]. Green boxes represent the formation method discussed herein.
Arianna McAllister, Mark D. Jeronimo and Haotian Chen of the Guenther lab developed a microfluidic approach for the extrusion of homogeneous and heterogeneous hydrogel tubes [24-27]. Their approach involved a multilayer microfluidic device which produced alginate-based tubes by co-axially extruding the rapid gelling aqueous alginate with an ionic crosslinking solution of calcium chloride. Their approach could predictably and controllably form tubes ranging from 600µm-3mm. Using this method, formation of heterotypic tubes was demonstrated with spatial composition varying in radial, circumferential and axial directions. While alginate is a widely used and well-characterized biopolymer used in many biomedical applications, an important drawback of its use is its limited long-term stability in physiological conditions often causing hydrogels to dissolve due to the release of divalent ions into the surrounding media [36]. Additionally, alginate lacks specific adhesion sites for mammalian cells, which is essential for cell proliferation, migration and adhesion [37].

Here, I present further development of this microfluidic approach for the formation of soft material tubes. Improvements reported in this thesis report include: tubular formation using the slow-gelling protein-based biopolymer, collagen I, improvement of flow distribution and formation yield through improvements made to the microfluidic device design and its fabrication including quantitative characterization in situ and post-printing.

Collagen I, referred to here-in this thesis as collagen, is the most abundant protein found in the human body and is a major ECM component in many extracellular tissues particularly blood vessels. It is characteristic for its high tensile strength and obvious biocompatibility; collagen is widely favored in tissue engineering approach for its natural ability to promote cellular attachment and growth [38]. Directing cell growth on oriented engineered substrates was first identified as contact guidance [39]. Many techniques have been subsequently developed to form anisotropic collagen constructs with highly aligned collagen fibers [2] [38-44]. A highly favored biomaterial mainly attributed for its hierarchical composition. Collagen fibrils (50-500nm) composed from collagen molecule (1.3nm) [45] are the building block of many composite materials whose function are greatly dictate by fibrillar arrangement.
1.2 Objectives

1. Consistent, controllable formation of collagen tubes.

This microfluidic platform provides a method for the continuous formation of soft materials tubes ranging size. Improvements to the bioprinting platform can be made to improve device footprint and design to improve tube formation yield. This bioprinting platform should be capable of forming tubes using the slow-gelling biopolymer, collagen I.

2. Quantitative in situ and post-printing characterization of collagen tubes.

To develop methods and consequently the ability to study the gelation timescales of collagen tubes in situ as they are being extruded. Post-printing tubes should be readily handleable for post-printing characterization to assess for the influence of gelation processes on dimensions and mechanical properties.

2 Contributions

I acknowledge the contributions and collaborations made and results presented herein this thesis report. In collaboration with Navid Hakimi device design iterations were made (Section 3.2). Device simulation results were obtained in collaboration with Richard Cheng (Section 3.2.1). Dimensionless models were completed in collaboration with Shashi Malladi presented in Section 3.3. Numerical simulation results presented in Section 4.1.3, Appendix A7 were completed by Chen Fan. Spectral data smoothing was completed using MATLAB code developed by Moien Alizadehgiashi [46]. All aspects of the tube forming technology described in this thesis report are protected under US Patent 20,160, 068, 385 [26].

2.1 New contributions of this work

Unique contributions made in the development of this work include: the robust and consistent formation of collagen tubes, improvements to the bioprinting cartridge (both in its fabrication and design) and consequently the extrusion process and lastly the methods described for the in situ characterization of tube formation.
3 Direct-Writing of Soft Material Tubes

3.1 Methods and Materials

Formation of collagen tubes is a continuous process that requires a microfluidic device, or bioprinting cartridge, through which a vertical confinement is reversibly attached. The bioprinting cartridge is infused via PEEK tubing which is epoxied to the inlets. This device reversibly attaches to a borosilicate glass confinement which is an extension of the central outlet through which the nascent tube passes as it is being extruded. The device inlets are infused using OEM syringe pumps (Harvard Apparatus, Massachusetts, USA) with the focusing and biopolymer solutions. The microchannels within the microfluidic device are arranged to radially distribute each solution around the common outlet. Upon exiting the outlet the solutions meet, establishing concentric sheaths of focusing fluid which surround the biopolymer layer flowing vertically and downward through the confinement. The interaction of the focusing fluid and biopolymer layer forms a pH gradient which leads to self-assembly of collagen monomers when the biopolymer solution reaches neutral pH. Subsequently, gelation of collagen tubes commences. The collagen tube continues to gel within the confining tube. The length of the confinement ($L_C$) in combination with flow parameters ($Q_i, Q_o, Q_M$) dictates the residence time ($t_{residence}$). This impacts the time necessary for the collagen tubes to be sufficiently gelled before exiting and collecting into a dish or beaker containing the focusing solution. The

Figure 2. Direct-Writing of Soft Material Tubes. (a) Bioprinting cartridge (microfluidic device) for continuous, template-free formation of soft material tubes. (b) Continuous strand of tube contained in a Petri dish. (c) Confocal image of a cross-section of a soft material tube. Scale bar: 500µm.
density difference between the biopolymer and the focusing solutions is small; however, the
densities of the focusing and biopolymer solutions can be matched such that the sole factors
influencing the extrusion velocity are the input flow rates and gravity. The newly formed collagen
tubes are left to continue the gelling for 1h, before being collected for post-processing.

To begin, flow of the focusing fluid is initialized to ensure that no bubbles, if any, are present within
the microchannels and confinement. Once the confinement is filled with the focusing solution, the
device is then infused with the biopolymer solution of collagen. As the confinement is directly
attached to the device newly forming tubes can then directly pass continuously through the
confinement unaffected by leakage or potential air films.

To enable in-situ characterization, a camera is set up perpendicular to the confinement to visualize
the tube formation process. Side imaging is achieved with Q-Imaging QICAM Fast
1394 and QCapture software (QCapture Pro 7, QImaging, CA).

**Figure 3. Experimental setup.** (a) Angled view of experimental setup. Bioprinting cartridge (1)
is held by an optical apparatus to accommodate the length of the confinement (2) $L_C$. is
reversibly inserted directly into the bioprinting cartridge. (3) Fluidic connections via syringe
pump infuse the bioprinting cartridge, red, blue and green lines represent $Q_o$, $Q_m$, $Q_i$, respectively. Scale bar: 25mm. (b) Photograph of bioprinting cartridge. Scale bar: 25 mm. (c) In-
situ image taken perpendicular to the direction of extrusion, visualizing tube passing through the
confinement. Scale bar: 1mm.
Figure A2 shows a photograph of the experimental setup with cameras mounted for side imaging.

3.1.1 Flow Confinement

The formation process of tubes begins when the matrix solution interacts with the focusing fluids upon exiting the outlet of the bioprinting cartridge. All fluids are then guided through a tubular confinement (DC), made of borosilicate glass, which confines the concentric sheath flow as it extrudes downward. This thereby stabilizes the tube geometry as it gels. The confinement has a diameter of 3.3mm that closely matches the outlet of the bioprinting cartridge which has a diameter of 3.175mm. The confinement is necessary for tube formation in order to stabilize the nascent forming tube as it continues to gel. The confinement can vary in length (Lc=2.8cm-1.2m). For a given set of defined input flow rates, Lc can be increased to provide adequate residence time to enable sufficient gelation.

3.1.2 Biopolymer Matrix

To form soft material tubes with predictable dimensions, rapid and slow gelling biopolymers can be used. In this report, template-free formation of collagen I tubes was characterized. As many mammalian tubular structures contain collagen, which naturally promotes cell attachment and growth [26], it is an ideal protein-based polymer to study due primarily to its physiological relevance. Beginning as a transparent liquid, the acidic monomeric collagen solution is infused through the bioprinting cartridge with a buffered alkaline focusing solution. A pH gradient is established between the focusing and matrix solutions which causes the pH of the collagen solution to approach neutrality. Following this pH change, the self-assembly of collagen monomers occurs within the cylindrical matrix sheath leading to the development of fibrils. Fibril formation during the printing process is aided by the presence of PEG in the focusing solution which functions as a molecular crowding agent. This crowding agent causes an osmotic pressure gradient by physically confining the collagen as it extrudes downwards as well as causing the release of H$_2$O out of the biopolymer matrix solution. As collagen remains liquid in acidic conditions, the formation of collagen tubes in this aforementioned manner does not require temperature control and as such can be conducted at room temperature. In future, if the incorporation of cells within the biopolymer matrix is desired, it would require the collagen solution to be near neutral pH and thus would need to be kept cool at 4°C to prevent premature gelation within the fluidic connections or bioprinting cartridge.
3.1.3 List of Materials and Equipment

A three layer microfluidic device, fabricated from polydimethylsiloxane (PDMS), comprises the bioprinting cartridge. The biopolymer which infuses the matrix layer is a 5 mg/mL aqueous collagen solution made from High Concentration, Rat Tail, Collagen I (Corning, MA, USA). The stock collagen solution ranging from 8-11 mg/mL is diluted to its final concentration using pH 2. At room temperature (21°C) the dynamic viscosity is $\mu_{\text{collagen}} = 74.5$ mPa·s with a density $\rho = 950$ kg/m$^3$. The inner and outer focusing layers are infused with a solution of 10% aqueous of poly(ethylene glycol) (PEG) solution (Sigma-Aldrich, ON, Canada) with buffering salts. PEG is used in solution as a molecular crowding, and gelation triggering agent [47]. Firstly, 4.14mg/mL diphosphate monobasic sodium, 12.07 mg/mL dibasic sodium phosphate, 6.86 mg/mL TES sodium salt (Sigma-Aldrich, ON, Canada) and 7.89 mg/mL sodium chloride (Bioshop Canada Inc.) were added to DI water and stirred till thoroughly dissolved. Before adding 111g of PEG, the salt solution is corrected to a pH of 8. The end product is a focusing solution of 10% w/v PEG, pH 8 with a dynamic viscosity, $\mu_{\text{focusing}} = 19.0$ mPa·s and a density $\rho = 1019.98$ kg/m$^3$. Raw viscosity data can be referred to in Figure A12. All solutions that are infused into the bioprinting cartridge pass through the confinement made from borosilicate glass (Kavalierglass of North America, Inc., Prague, Czech Republic) with inner diameter of 3.3 mm and an outer diameter of 5 mm. Upon exiting the confinement the tubes are collected in a dish containing the same PEG solution used for focusing. The input flow rates are controlled using OEM syringe pumps (Harvard Apparatus, Holliston, MA, USA) and is controlled with a custom GUI using LabVIEW (National Instruments, Austin, TX, USA). In-situ imaging is recorded with a Q-Imaging QICAM Fast 1394 camera (Q-Imaging, Surrey, BC, Canada).

3.2 Tubular Bioprinter Cartridge Design

The bioprinting cartridge described here is a three-layer microfluidic device fabricated from PDMS. This cartridge, Figure 3b, is infused with focusing and matrix solution at their respective inlets. Fluids pass through resistance channels and meet at a common outlet where each fluid is distributed radially and extrude downwards to begin the tube formation process. The three fluidic layers are arranged such that the matrix layer is encased by a focusing layer on the top and bottom. All layers are identical to each other. Microchannels are fabricated to a depth of 150 µm. Features layers are 100 µm apart.
Figure 4. Bioprinting cartridge for the direct-writing of soft material tubes. (a) 3D rendered image of the bioprinting cartridge. Scale bar: 25mm. (b) Assembly of (1) inner (green), (2) matrix (blue) and (3) outer (red). Scale bar: 13.8mm.
3.2.1 Device Modifications

A major alteration to the tube formation process was improving the flow distribution of the bioprinting cartridge as well as reducing its device footprint and consequently dead volume. In old generation devices, poor resistance near the outlet lead to many issues with achieving consistent tube formation, particularly with uneven distribution of fluid around the outlet. These issues were observed experimentally; incomplete tube formation (circumferentially), uneven wall thicknesses, and inability to consistently achieve tube formation without manual manipulation. New generation cartridges were designed to approximate a biomimetic design principle that mimics the structure of vasculature [48]. For a constant-depth, d, rectangular microchannels, where d=150 µm, the bioprinting cartridge has n=4 bifurcations. At the inlet, n=0, the parent microchannel separates to two daughter microchannels, n=1, where width, w, is conserved at w=550 µm. Subsequently, at n=2, there are 4 daughter microchannels of w=400 µm. At n=3, there are 8 microchannels with w=300 µm. Finally at n=4 there are 16 daughter microchannels, w=124.5 µm, meeting at 4mm diameter ring which enable all solutions in their respective layers to distribute evenly before exiting at the 3.175mm diameter outlet. Comparison of the pressure distribution for the new generation device design against the previous generation device design were performed in COMSOL Multiphysics® (Comsol, Inc., MA, USA). Results of the simulation are shown in Figure 6. Microchannel designs

**Figure 5. Fabrication of bioprinting cartridge.** A layered view of a bioprinting cartridge rendered in SolidWorks. Three features layers: inner, matrix, outer are aligned and bonded atop a device base which is bonded to a confinement base. Scale bar: 25mm.
were modelled for pressure distribution simulation flow of the focusing solution at its $Q_{I,O}=500\mu$L/min. In the previous generation devices there is a 98.7% decrease in pressure from the inlet ($P=15.5$ kPa) to the last bifurcation ($P=0.2$ kPa). Examining the new generation device design there is an 83.8% decrease in pressure from the inlet ($P=13.5$ kPa) to the last bifurcation ($P=2.2$ kPa). Comparing the results from these simulations shows a ten-fold improvement in the pressure distribution in the new generation devices. In each bifurcation there is a direct relation between pressure distribution and volumetric flow rate, as pressure drives flow, greater and more even distribution is seen on-chip due to the hydraulic resistance that is maintained through succeeding daughter channels. Experimentally, these design changes enabled tube formation which could be self-starting and required very little manual manipulation to initiate the tube formation process. Furthermore, limited backflow and clogging within the channels was observed. The bioprinting cartridges were also improved in their footprint. Previous PDMS generation devices were 3x4” in footprint, while new generation devices are 1x1” with an on-chip dead volume of 7μl. The reduction in footprint enables conservation of reagents on-chip and also has important implications for the potential manufacturing of devices to thermoplastics, as well as its integration into commercial bioprinting systems as a tubular forming print head.

Another important modification to the tube formation platform was the ability to reversibly attach the confinement directly to the outlet of the device, without the need for a weight or applying pressure to the device. This modification enables more robust tube formation as the possibility for air film formation between the device and the confinement is eliminated. Fluids can pass undisturbed from the outlet of the device and through the confinement. This enables the elimination of the reservoir, which required a large and unnecessary amount of reagent and eliminates a potential source of contamination for future iterations where cells may be incorporated to the matrix solution for the formation of cell-laden tubes. In previous, the bioprinting cartridge sat atop a printing stage (containing the confinement) which was immersed in a fluid filled reservoir. Due to the dimensions of the reservoir, there were inherent limitations in increasing the confinement length. For a slow-gelling polymer such as collagen, this limitation decreases the available stability and residence time attributed to the length of the confinement. Inconsistent tube formation, crimping and non-uniform wall thickness were some issues that arose in this described limitation. Therefore, it was imperative that to enable sufficient time for the newly forming tubes to gel the possibility to alter the length of the confinement for any given length was needed.
3.2.2 Bioprinting Cartridge Fabrication

The multilayered microfluidic bioprinting cartridge is fabricated using the method of multilayer soft lithography [21]. The device design is made using AutoCAD 2016 (Autodesk) and is then printed on a photomask transparency at 23400 DPI (CAD/ART Services, OR, USA). The photomask is used to UV expose the channel features onto an SU-8 2050 (MicroChem, MA, USA) negative photoresist coated silicon wafer which has been spin-coated to a desired thickness of 150 µm. After UV exposure the wafers are baked and developed. The UV exposed resist that remains functions as the master, which forms the features, when casted with PDMS, of each layer of the bioprinting cartridge. Microfabrication processes are verified by profilometer scans of the developed silicon master.

The bioprinting cartridge is fabricated using spin-coated and cast PDMS layers. PDMS kits (Sylgard 184 Silicone Elastomer kit, Dow Corning, Midland, MI, USA), purchased from Krayden, Inc, contain a base and curing agent that are mixed at a ratio of 10:1. The top (inner focusing) layer is formed by casting a 3 mm layer of PDMS, the matrix and outer focusing layers are spin-coated, the bottom layer consists of a thin 1-3mm layer of PDMS to seal the outer focusing layer. To begin, the top layer is fully cured at 80°C, the matrix and outer focusing layer are partially cured at 80°C. The top layer is carefully aligned with the outlet of the partially cured subsequent layer, the layer is sealed with PDMS is sealed then baked to form a permanent bond. This process is repeated till all feature layers are bonded. The inlet holes are formed. The last feature layer is then sealed by corona treating the bottom layer with the device base layer. The entire device is sealed and baked to bond the device base with the bottom layer. The outlet hole is then formed. The confinement base is formed by fully curing a 1cm thick base and forming a center hole. The device is corona treated to the confinement base. PEEK tubing is epoxied to the inlets and a thin patch of PDMS is corona treated to the top of the device to seal the punched outlet hole. Once the epoxied has cured, the bottom of the newly formed device is sealed with tape and the device is covered entirely in PDMS. The device is then left to bake overnight. Figure 5 shows a SolidWorks® (DS SolidWorks Corp., MA, USA) rendered image of the fabrication process.
Figure 6. COMSOL simulation of new and old generation devices. Simulation of pressure distribution for focusing fluid ($Q_{I,O}=500\mu\text{l/min}$, $\mu_{\text{focusing}}=19\text{ mPa}\cdot\text{s}$) from inlet to last bifurcation. (Left) Image result for new generation device design with pressure difference, $\Delta P = 11.325\text{ kPa}$. (Right) Image result for old generation device design with pressure difference $\Delta P = 15.230\text{ kPa}$. Scale bar: 13.8mm.

3.3 Modeling Tube Formation

In producing a robust platform for the formation of soft material tubes it is important that they can be formed in a predictive and scalable manner. An analytical model was previously developed and explained to predict tube dimensions [25]. In order to compare predictability of forming tubes of varying $Q_T$ and material the aforementioned models have been derived in dimensionless form. Their derivations are found in Appendix A4. The dimensionless parameters are dimensionless outer diameter ($D^*_o$), dimensionless inner diameter ($D^*_i$), dimensionless outer flow rate ($Q^*_o$), dimensionless inner flow rate ($Q^*_i$), and dimensionless matrix flow rate ($Q^*_M$). Dimensionless tube diameters are defined from experimental data as $D^*_o = \frac{D_o}{D_c}$ and $D^*_i = \frac{D_i}{D_c}$ where $D_c$ is the inner diameter of the confinement. Dimensionless flow rates are defined as $Q^*_o = \frac{Q_o}{Q_T}$, $Q^*_i = \frac{Q_i}{Q_T}$, where $Q_T = Q_o + Q_i + Q_M$. Figure 7 shows the results of collagen tube diameters formed at varying values of $Q^*_o$ against model values. Diameters formed at $Q^*_o = 0.2$, 0.4, 0.6 follow the general trend seen in both solid and liquid
model, where $D_O^*$ values increase with decreasing values of $Q_O^*$. However, dimensionless diameter values do not exhibit complete agreement with model values.

Figure 7. Dimensionless plot of collagen tubes formed at $Q_O^*=0.2, 0.4, 0.6$ for constant $Q_M^*$. Diameter results plotted for tubes formed at $Q_O^*= 0.2, 0.4$ and $0.6$ for constant $Q_M^*=0.16$, where $Q_T=1200\mu l/min$, $Q_M=200\mu l/min$, $D_C=3.3mm$. Experimental $D_O^*$ values (left y-axis) are represented by the red squares. Experimental $D_I^*$ values (right y-axis) are represented by the green circles. Thick red and green lines represent solid model $D_O^*$, $D_I^*$ values, respectively. Thin red and green lines represent liquid model $D_O^*$ and $D_I^*$ values, respectively.

Experimental $D_O^*$ and $D_I^*$ values for $Q_O^*=0.2, 0.4, 0.6$ are directly compared against their model predictions in Table 1. Complete agreement is not seen between both models, with the experimental values more closely approximating the liquid model than the solid model. There are some possible explanations that can be given for the discrepancy seen between model and experimental values. Both models described in this thesis report model tube formation either as a cylindrical liquid sheath (liquid model) or as a rigid cylinder (solid model). In both models, viscosity is assumed to be constant which is not the case experimentally. During tube formation, the collagen solution begins as a liquid but transitions, with an increase in viscosity, to a gelled tube as it extrudes within the
This change in viscosity is not represented in either model. The slight difference in density between the focusing (\(\rho=1020 \text{ kg/m}^3\)) and matrix (\(\rho=950 \text{ kg/m}^3\)) solution could also affect the predictability of tube formation and its diameters as buoyant forces may play a role, but was not quantified. In both models tube extrusion is considered axisymmetric, which does not occur experimentally as tubes form a winding motion similarly described by Mahadevan et al [49]. This winding motion or “coiling” could be influenced by these density differences and viscous changes experienced by the tubular matrix, which compete with the downward axial forces governed by the volumetric infusion flow rates, \(Q\), and gravity, \(g\). Additionally, the \(D_C\) chosen is slightly larger than the diameter of the device outlet, this has an impact on the experimental \(D_O^*\), \(D_I^*\) values described herein.

<table>
<thead>
<tr>
<th>(Q_O^*)</th>
<th>(D_I^*) (experimental)</th>
<th>(D_I^*) (liquid model)</th>
<th>(D_I^*) (solid model)</th>
<th>(D_O^*) (experimental)</th>
<th>(D_O^*) (liquid model)</th>
<th>(D_O^*) (solid model)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>0.574±0.028</td>
<td>0.632</td>
<td>0.704</td>
<td>0.678±0.027</td>
<td>0.766</td>
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</tr>
</tbody>
</table>

Table 1. Comparison of \(D_O^*\) and \(D_I^*\) experimental values for tubes formed at \(Q_O^*\)= 0.2, 0.4, 0.6 with liquid and solid model values.

Scalability of this tube formation platform was examined by performing order of magnitude extrusion experiments using alginate, where \(Q_T=15\text{mL/min}\). The results for \(D_O^*\) and \(D_I^*\) values were plotted and can be found in Figures A5, A6.
4 Quantitative Characterization of Soft Material Tubes

Understanding the gelation timescales of biopolymeric tubes is vital to improve the printing process. For slow gelling protein-based biopolymers such as collagen many parameters influence their gelation kinetics, namely, ionic strength, temperature, pH and monomer concentration [50].

To better understand the processes occurring during and after the tubular formation process a series of characterization experiments were performed. Firstly, to assess the pH change experienced by the forming collagen matrix, in situ fluorescence measurements were completed. Secondly, to monitor the gelation development of the forming tubular matrix, in situ turbidity measurements (IST) were conducted. Post-printing collagen tubes were processed and assessment for their dimensions during the post-processing phase, in addition to their mechanical strength was conducted.

4.1 In situ Characterization of Tube Formation

Acidic collagen remains a clear, liquid solution at room temperature and was thus utilized to demonstrate collagen tube formation in the absence of a temperature controlled printing system. During the formation process, the acidic collagen is increased to near neutral pH by the buffered focusing solution, which remains constant at a pH of 8. This increase in pH to neutrality induces collagen self-assembly, a spontaneous process where available collagen monomers in solution are developed into fibrils [51]. During this process of self-assembly the collagen matrix rapidly increases in viscosity, which is exhibited by its increasing turbidity [50]. It is proposed that collagen-based tubular constructs are formed by three governing mechanisms: (1) hydrodynamic focusing imposed by the concentric flow from the bioprinting cartridge (2) induction of pH change to neutral, initiating gelation (3) the use of a macromolecular crowding agent to form an osmotic pressure gradient thereby expelling water out of the collagen matrix. To elucidate the gelation timescales during the tube formation process, a series of in situ measurements were taken along Lc. pH change as related to fluorescence was measured as was the progression of turbidity of the forming tube.
4.1.1 *In situ* fluorescence measurements

**Figure 8. Experimental setup for in situ fluorescence measurements.** (a) Isometric view of experimental setup. (1) Bioprinting cartridge (microfluidic device) (2) Confinement, LC (3) FITC Filter cube (4) Optical Cage (5) Entry of incident light source (6) Optical post (7) Detection to Fluorescence Spectrometer (b) Top view of experimental setup. Scale bar: 50mm.

To observe the pH gradient, pH sensitive particles were used. FITC (fluorescein isothiocyanate) is a common fluorophore known for its pH sensitivity in the range of 3-9, with greatest sensitivity and consequently intensity between pH 6-9 [52]. 1μm size melamine resin marked FITC particles (Sigma-Aldrich, ON, CA) were incorporated in the collagen matrix solution at a concentration of 1% v/v, and were consequently embedded in the collagen tubular matrix during the formation process. To examine the pH gradient, a series of optical cages were set up to enable in-situ fluorescent measurements to be performed. A 30 mm optical cage (Thorlabs, USA) is enclosed with machined nylon plates (MIE Machine Shop) with center holes top (D<sub>top</sub>=5mm) and bottom (D<sub>bottom</sub>=6mm). The machined nylon plates allow z-direction translation of the measurement unit around and along the length of the confinement. Connected to this cage is a filter cube cage (DFM1-Kinematic Filter Cube, Thorlabs, USA). This filter cube contains a FITC filter set (Thorlabs, USA): FITC excitation filter (λ<sub>ex</sub>= 475±17.5nm) FITC emission filter (λ<sub>em</sub>=530±21.5nm) and dichroic filter (λ<sub>reflection</sub>=470-490nm, λ<sub>transmission</sub>=508-675nm). A blue (λ<sub>nomin</sub>=470nm) LED (Thorlabs, USA) is threaded to a 1m long multimode fiber optic cable with a core diameter of 1000 μm, numerical aperture (NA)
NA=0.22 (Ocean Optics, USA) by a terminated fiber adapter (Thorlabs, USA). The opposite end of the fiber is threaded to a fixed focus collimation package (Thorlabs, USA) of focal length (f) f=7.86mm, NA=0.51, with an anti-reflective (AR) coating ranging from 350-700nm. The collimated beam is directed past the FITC excitation filter to the dichroic filter which then reaches the confinement contained in the adjacent cage. The emission from the FITC embedded particles within the collagen matrix of the forming tube passes through the dichroic filter and FITC emission filter. The intensity from this emission is recorded in-line by a multimode fiber optic cable with a core diameter of 1000 µm (Ocean Optics, USA) connected by a terminated fiber adapter (Thorlabs, USA). The emission is recorded as spectral data using software (SpectraSuite, Ocean Optics, USA). FITC intensity values at 530nm (524.9-530.9) were averaged for each position recorded and plotted. Standard deviation was taken for each measurement to be the full width half maximum (FWHM) of smoothed spectral data in the emission region. Calibration experiments were attempted in order to directly relate the pH experienced by the collagen matrix as a function of position, z, and time. FITC particles were incorporated with focusing fluid at varying pH values and loaded into the confinement and measured using the fluorescence measurement unit. However, there were order of magnitude differences in intensity from the calibration intensity values and the experimental data. Collagen stream alone, did not seem to contribute to the intensity discrepancy. As pH could not be directly plotted with position, FITC intensity was plotted with position. To demonstrate the pH sensitivity of FITC particles, 0.1% v/v FITC was incorporating into focusing solutions of pH 2-8. They were loaded into a cuvette and their emission data was collected on a spectrometer. Photoluminescence (PL) intensity curves were plotted, the results are shown in Figure A7. The PL data indicate there are minute differences in intensity between FITC intensity at various pH values.

4.1.2 In situ turbidity measurements

Monitoring the progress of tube formation is important in understanding the behavior of the biopolymer (whether slow or fast gelling) and their gelation timescales. This also has important implications on the design of the printing platform. Flow rate parameters do not successfully yield consistent tubes [25] below the flow rate conditions reported in this thesis report; therefore, $L_C$ primarily dictates the residence time afforded to developing tubes.

A common method for observing the progression of gelation, for slow-developing biopolymers such as collagen, is measuring the turbidity of the developing gel or construct. Early studies observed collagen self-assembly processes by monitoring turbidity [50] [53]. Monomeric collagen solution
progresses from a clear, colourless solution to a noticeably turbid gel where its development has been extensively supported to follow a sigmoid growth curve with distinct lag, growth and plateau phases [50] [53-56]. During the lag phase, nucleating structures are said to form. Following the lag phase, a growth phase begins with a rapid increase in turbidity occurring. This increase in turbidity is attributed to the self-assembly of collagen monomers whereby they become fibrilar structures [53-54]. Subsequently a plateau phase begins when available collagen monomers in solution become depleted.

**Figure 9. Experimental setup for in situ turbidity (IST) measurements.** (a) Isometric view of setup (1) Bioprinting cartridge (2) Confinement, Lc (3) Photodetector (4) Optical cage (5) Entry of fiber optic coupled light source (6) Optical Post (b) Side view of IST setup. (5) Illustrates incoming collimated beam in purple and absorbance signal in white gathered by the photodetector. Scale bars represent 50mm.

*In-situ* turbidity measurements (IST) were performed with a simple set up similar involving an optical cage through which a collimated light source and photodetector are attached and arranged in-plane. A deep UV ($\lambda_{nominal}$=340nm) LED (Thorlabs, USA) is threaded to a 1m long multimode fiber optic cable with a core diameter of 1000 µm (Ocean Optics, USA) by a terminated fiber adapter (Thorlabs, USA). The other end of the fiber is connected to a terminated fiber adapter and a series of 1” diameter lens tube that is connected to the optical cage. The deep UV light is collimated using a UV fused silica plano-convex lens, $f$=35.0mm (Thorlabs, USA) with an AR coating ranging from 245-400nm. The collimated beam is directed at the confinement and transmitted light is received by an amplified photodetector (Thorlabs, USA) that is threaded to the optical cage and is 180° to the
incident collimated beam. The voltage ($V$) generated from the transmitted light is read on an oscilloscope (Tektronix, USA).

To quantify IST, the voltage recorded on the oscilloscope is used to calculate absorbance ($A$), where

$$A = -\log \left( \frac{V}{V_0} \right) = \alpha l$$

with $V$, the measured voltage at indicated positions along $L_C$, $V_0$, the voltage recorded in the reference solution (confinement solely containing the focusing solution), where $\alpha$ is the absorption coefficient (cm$^{-1}$), and $l$ is pathlength in cm. Turbidity, $\tau$, was then calculated as

$$\tau = \left( \frac{A}{l} \right) \ln(10).$$

The optical measuring unit is translated in the $z$-direction around the confinement ($L_C=0.6m, 0.8m, 1.2m$). The progression of turbidity within the confinement can be seen in Figure 10.
4.1.3 Results from *in situ* measurements

Figure 10 shows plots of the results from the *in situ* fluorescence and IST measurements. FITC intensity, IST and pH were plotted with position and time (based on \( Q_M = 200 \mu \text{L/min} \)). Due to experimental limitations the first fluorescence measurement was taken at

Figure 10. *pH*, FITC intensity and IST as a function of confinement position and time. Flow conditions for the *in situ* characterization were \( Q_T = 1200 \mu \text{L/min} \), where \( Q_{LO} = 500 \mu \text{L/min} \), \( Q_M = 200 \mu \text{L/min} \). Green line represents COMSOL® simulation results for the pH gradient which occurs *in situ*. Green squares represent FITC intensity measured at indicated positions. Blue circles represent IST data measured at the indicated positions. Hollow blue circles represent IST data with \( L_C = 0.6 \text{m} \). Solid blue circles represent IST data measured with \( L_C = 0.8 \text{m} \). Double circles represent IST data measured using \( L_C = 1.2 \text{m} \). Blue curve represents sigmoid fit of IST data. Coefficient values (± one standard deviation) of sigmoid curve: base= 0.031296 ± 0.00902, max= 0.18444 ± 0.0129, \( x_{\text{half}} = 45.67 ± 1.81 \), rate=12.33 ±1.84. Vertical lines represent key time points identified after IST analysis. The yellow line represents lag time, \( t_{\text{LAG}} \). Green line represents inflection time, \( t_{\text{INF}} \). Red line represents plateau time, \( t_{\text{PL}} \).

Figure 10 shows plots of the results from the *in situ* fluorescence and IST measurements. FITC intensity, IST and pH were plotted with position and time (based on \( Q_M = 200 \mu \text{L/min} \), \( D_{LO(\text{avg})} \) for \( Q_O^* = 0.4 \) and \( L_C \)). Due to experimental limitations the first fluorescence measurement was taken at
0.03m, with fluorescence measurements taken every 1cm till 0.1m. At 0.1m fluorescence measurements were taken every 0.025m till 0.2m to directly compare with the IST measurement positions. IST measurements began at 0.05m with measurements taken every 0.025cm, equivalent to 5 seconds. Simulation results, completed by Chen Fan, indicate that the pH change experienced by the collagen matrix occurs within 0-0.1m equivalent to the first 20 seconds within all fluids first contacting. After 0.1m (20 s) it is expected that the pH is maintained near a pH 8, due to the pH of the buffered focusing solution. These results gave the basis for the experimental positions recorded for the fluorescence measurements. It was expected that in the first 0.1m of the confinement there would be an increase FITC intensity with steady plateau >0.1m. Results from the FITC intensity measurements show an increase in fluorescence with position between x=0.03m and x=0.125cm. It appears that the fluorescence steadies past x=0.125cm, as expected; however, at x=0.2m, it is observed that the FITC intensity decreases. A possible explanation for this decrease could be that the fluorescence is blocked by the increasing turbidity of the forming tubular matrix.

The results from the IST measurements display a sigmoidal shape. Key time points from the IST data were identified based on the sigmoid fit curve shown Figure 10. Lag time, tLAG, was defined as the time or position where the IST value increases at least 5% from the initial IST measurement taken. Inflection time, tINF, was determined as the maximum value from the first derivative curve of the sigmoid fit. Plateau time or tPL was determined as the position or time where 95% of the IST developed from the last measurement taken. The IST data indicates that the tLAG begins around 0.05m (10s). With tINF, beginning at x=0.45m (93s) and tPL at x=0.775m (160s). Key events that occur during collagen self-assembly are attributed to an increase and plateau in turbidity [50-51] [53-56]. During the lag phase (tLAG), nucleating structures are said to form. Collagen monomers self-assemble into fibrils during the growth phase anchor to the nucleating structures. This self-assembly process is marked by the rapid increase in turbidity (tINF). Once tPL is reached it is said that collagen monomers are depleted in solution which is characterized by a plateau in turbidity [51]. While turbidity measurements continue to be the gold standard for monitoring collagen gelation [57-60], the events can only be deduced by analysis of the IST data. In order to confirm the events in situ, direct visualization using methods such as confocal reflectance microscopy (CRM) [51] [61-63] would be needed to observe and image the aforementioned events specific to the formation method. This method of IST measurements could be useful for the characterization of other biopolymers that undergo a turbid change during their sol-gel transition during the formation process.
4.2 Quantitative Assessment of Collagen Tubes Post-Printing

Following printing, tubes are collected for post-processing. Tubes are washed twice with deionized water and then incubated at 37°C in Fibrillogenesis Inducing Buffer (FIB) for 48 hours [47]. Tubes were measured for their tube dimensions during formation and after 48 hours post-printing to assess the influence of the post-processing protocol on tube diameters. Post-processing, tensile measurements were performed on tubes.

Figure 11. Cross-section confocal images of collagen tubes post-processing. (4X magnification) (a) Collagen tube embedded with 1% v/v FITC particles formed at $Q_0^*=0.2$. Scale bar: 500 um. (b) Collagen tube embedded with 1% v/v FITC particles formed at $Q_0^*=0.4$. Scale bar: 500um. (c) Collagen tube embedded with 1% v/v FITC particles formed at $Q_0^*=0.6$. Scale bar: 500 um.

Tube dimensions can be tuned by manipulating the flow conditions of the focusing solutions. Figure 11 shows confocal images of tubes formed at the three studied flow conditions, $Q_0^*=0.2$, 0.4, and 0.6 respectively. Post-printing tubes are collected for processing, where they are washed and placed in a solution of FIB. This process ensures that collagen tubes are sufficiently gelled. Tube dimensions were measured in situ with side camera imaging as seen in Figure A2. This was deemed as 0h where the initial diameter of the tube is set during the formation process. As the gelation timescale of collagen can be on the order of minutes to hours, they were collected and assessed for their dimensions 48h post-processing. As tubes continue to gel, it is expected that a diameter reduction would be seen as it is expected that water is being removed/expelled from the collagen matrix solution possibly compacting tube wall. Tubes can be imaged on a cannulation stage at 0m water level or alternatively through conducting a 3D scan of the construct using confocal. These are
the preferred methods to image, as they require very little manipulation as to not influence the tube diameters due to their soft, pliable nature. The results of this assessment can be found in Figure 12. Tube were measured for their $D_0^*$ and $D_t^*$ at 0h and 48h as well as their cross sectional area at the aforementioned time points. $D_0^*$ values seem to show a decrease across all conditions of $Q_0^*$. While there are some irregularities in $D_t^*$ values, it seems that the cross-sectional area does not exceed values set initially at formation, while a small decrease in cross sectional area in all flow conditions was noted.

**Figure 12. Characterization of collagen tube diameters post-printing.** White category bar represent tubes formed at $Q_0^*=0.2$. Diagonally striped category bar represent tubes formed at $Q_0^*=0.4$. Grey category bar represent tubes formed at $Q_0^*=0.6$. (a) $D_0^*$ measured at 0h (*in situ*) and 48h (confocal, cannulation) $Q_0^*=0.2$ ($D_{0,0h}=2237\mu m\pm90\mu m$, $D_{0,48h}=2149\mu m\pm75\mu m$) $Q_0^*=0.4$ ($D_{0,0h}=1871\mu m\pm161\mu m$, $D_{0,48h}=1861\mu m\pm77\mu m$) $Q_0^*=0.6$ ($D_{0,0h}=1426\mu m\pm56\mu m$, $D_{0,48h}=1414\mu m\pm151\mu m$) (b) $D_t^*$ measured at 0h and 48h. $Q_0^*=0.2$ ($D_{t,0h}=1897\mu m\pm94\mu m$, $D_{t,48h}=1914\mu m\pm103\mu m$), $Q_0^*=0.4$ ($D_{t,0h}=1618\mu m\pm147\mu m$, $D_{t,48h}=1491\mu m\pm68\mu m$), $Q_0^*=0.6$ ($D_{t,0h}=1036\mu m\pm54\mu m$, $D_{t,48h}=1249\mu m\pm136\mu m$) (c) Cross-sectional area of tubes measured at 0h and 48h. $Q_0^*=0.2$ ($A_{0h}=11.0\pm0.4 \times 10^{-7} \text{ m}^2$, $A_{48h}=7.5 \pm 0.5 \times 10^{-7} \text{ m}^2$), $Q_0^*=0.4$ ($A_{0h}=6.9\pm1.0\times10^{-7} \text{ m}^2$, $A_{48h}=5.1\pm3.5\times10^{-7} \text{ m}^2$), $Q_0^*=0.6$ ($A_{0h}=7.5\pm0.4 \times 10^{-7} \text{ m}^2$, $A_{48h}=3.5\pm0.7 \times 10^{-7} \text{ m}^2$)

Collagen tubes were measured for tensile strength in the axial and circumferential direction. Uniaxial tensile measurements were performed on 1cm segments of collagen tubes formed at $Q_0^*=0.2$, 0.4 and 0.6. Tube segments were clamped on horizontally on both ends of a custom tensile testing machine. Force balance and position was zeroed initially before tubes were controllably pulled at a rate of 0.1mm/s until failure. Samples were liquid immersed during tensile measurements.
Displacement and force were measured and subsequently used to calculate the stress and strain by comparing the initial sample length.

To measure circumferential tensile properties of collagen tubes, segments 1-2cm in length, were mounted to a cannulation stage. See Figure A9 for setup. Tubes were cannulated on both ends of tubing and the edges are fixed to prevent leakage. One end was connected to a syringe, which remained at water level (0m). A hydrostatic reservoir that was connected to the opposing end was lifted in increments of 0.025m and tubes were measured at each change in hydrostatic pressure. Their diameters were measured and used to calculate hoop stress and ultimately circumferential modulus. Tubes were considered to act as a thick-walled cylinder as the approximation for stresses in thin-walled cylinders does not hold because the radius of the extruded tubes does not exceed ten times the wall thickness.

Hoop stress was calculated using Lamé’s equations for hoop stress, \( \sigma_\theta \),

\[
\sigma_\theta = \frac{r_i^2 P_{in} - r_o^2 P_o}{r_o^2 - r_i^2} + \frac{(P_{in} - P_o)r_i^2 r_o^2}{(r_o^2 - r_i^2)r^2}
\]

(1)

\[
E = \frac{\sigma}{\varepsilon} = \frac{\sigma_\theta}{\Delta r/r}
\]

(2)

With, \( \Delta r = r_p - r_o \)

(3)

Where \( r_p \) is the outer radius of the pressurized tube.
Figure 13. Axial and circumferential tensile properties of collagen tubes post-processing. White category bars represent axial elastic moduli, grey category bars represent circumferential elastic moduli for tubes formed at $Q_0^*=0.2$ ($E_A=25.3\pm9.1$ kPa, $E_C=16.7\pm0.9$ kPa), $0.4$ $E_A=22.5\pm7.2$ kPa, $E_C=14.0\pm2.0$ kPa and $0.6$ ($E_A=25.3\pm2.5$ kPa, $E_C=10.2\pm3.0$ kPa) respectively at $9.8$ kPa.

Circumferential and axial tensile measurements are shown in Figure 13 indicating greater tensile strength in the axial direction than circumferentially. This result is expected given that there is no imposed fibril alignment in the circumferential direction. The results from the cannulation/pressurization experiments, in future, could be performed using a tube hosting device. Tube hosting devices were designed in SolidWorks (DS SolidWorks Corp., MA, USA) and 3D printed (3D Systems, SC, USA). An enclosed design, Figure A9 was made with superfusion and fixation channels and perfusion inlet/oulets configured for direct loading of a tube. Another design, Figure A10 was made in open well configuration with fixation and perfusion channels. Using a tube hosting device, one could controllably perfuse, superfuse and fix tubular constructs at defined conditions by supplying inlets with positive hydrostatic pressure (perfuse and superfuse) and negative hydrostatic pressure (fixation). Once loaded, tubes could be actively perfused at defined flow rates using syringe pumps and supplied with perfuseate and/or superfuseate. Possible applications include: cell seeding of acellular tubes, degradation studies, and mechanical characterization. If tubes are cell-laden, they can be pressurized, perfused and superfused with relevant culture media, which could maintain the construct integrity and cell viability in incubation.
5 Summary

5.1 Conclusion

Soft materials tubes and their formation have important implications in wide range of applications across the field of tissue engineering. Understanding and characterizing their formation is important to give proper design rationale and to precisely achieve spatial compositions which closely mimic native constructs. Using a microfluidic-based platform, continuous formation of soft material tubes can be achieved using biopolymers with gelation timescales ranging from seconds to several minutes with write speeds from 3-7mm/s. A compact PDMS bioprinting cartridge is composed of three layers of microfluidic channel with a unifying radial outlet that, when infused, forms cylindrical sheaths of concentric flow to extrude slow-gelling biopolymers, such as acidic collagen, co-axially, with a pH buffered focusing solution that contains a macromolecular crowding agent. By inducing a rapid pH change, the tubular collagen matrix undergoes self-assembly upon reaching neutrality. During this sol-gel transition the tubular matrix undergoes a rapid increase in turbidity. Soft material tubes can vary in sizes by manipulating focusing fluid flow rates to yield tubes of various dimensions. Dimensionless model was used to predict dimensions of extruded tubes based on dimensionless flow parameters, which follow a general agreement with experimental results of extruded collagen tubes.

To assess gelation timescales, pH gradient, turbidity and diameters were measured in situ by translating optical measuring units. These in situ measurement methods can enable the characterization of other biopolymers, to test the compatibility of this bioprinting platform. Furthermore, this platform can also be capable of extruding cell-laden tubes by incorporating cell and relevant materials into the matrix solution.

Following the extrusion process, tubes are collected for processing, where they are washed and incubated in a buffer at physiological temperature for 48 hours. Tubes were assessed for their diameter change before and after this processing. Tube segments can be cannulated and pressurized using a positive pressure hydrostatic reservoir. Tube diameter changes were measured with changes in hydrostatic pressure. Pressurizing soft material tubes in this manner is useful for calculating circumferential tensile properties of tubes and the potential to perfuse tubular constructs at defined flow rates for applications such as cell seeding, material degradation studies, tube wall transport measurements.
5.2 Future Work

In this work tubes were formed using PDMS bioprinting cartridges by infusing acidic pH collagen solution with PEG-based focusing solution. Often PDMS device fabrication is a low-yield and time-consuming process that can incur misalignment at the outlet that can ultimately affect the quality of tube formation. Furthermore, PDMS devices cannot withstand large amounts of pressure before device failure and delamination. This limits the infusion rates/pressure that can be achieved. In future, device fabrication could be made from biocompatible thermoplastics that can be made via hot embossing or injection molding to improve device yield. Device fabrication could ultimately be scaled in these aforementioned processes. Acidic pH collagen solution was used as the tube formation method is currently performed at room temperature. In the absence of temperature control, neutral pH collagen is prone to premature gelation and clogging of bioprinting cartridges, which greatly limits the ability to form tubes. In future, temperature control using thermoelectric cooling/heating can be used to control components of the tube formation process (bioprinting cartridge, syringes, confinement) at defined temperatures. This would allow the use and formation of collagen tubes formed from neutral pH collagen. As temperature plays an important role in the gelation of fibrous protein-based polymers such as collagen, the effect of temperature induced gelation could also be performed using the IST measurement methods described in this thesis report. An important step forward, the use of neutral pH collagen could provide suitable conditions for the inclusion of cells and cellular materials to form cell-laden tubular microtissues.
References


http://www.nature.com/nmat/journal/v12/n6/abs/nmat3606.html#supplementary-information


http://www.nature.com/nmeth/journal/v10/n8/abs/nmeth.2524.html#supplementary-information


Appendix A1- Experimental Setup for *in situ* fluorescence measurements

(a) Side-view of main components: (1) Bioprinting cartridge (2) Confinement, L_C (3) Optical cage (4) FITC filter cage (5) Emission fiber coupled to spectrometer (6) Collecting dish (7) Optical post with translating measurement unit (8) Syringe pumps (b) Top view of measurement unit. (1) Bioprinting cartridge (3) Optical cage (4) FITC filter cage (5) Emission fiber coupled to spectrometer (9) Fiber coupled to light source for excitation

**Figure A1. Experimental setup for *in situ* fluorescence measurements.** (a) Side-view of main components: (1) Bioprinting cartridge (2) Confinement, L_C (3) Optical cage (4) FITC filter cage (5) Emission fiber coupled to spectrometer (6) Collecting dish (7) Optical post with translating measurement unit (8) Syringe pumps (b) Top view of measurement unit. (1) Bioprinting cartridge (3) Optical cage (4) FITC filter cage (5) Emission fiber coupled to spectrometer (9) Fiber coupled to light source for excitation
Appendix A2 Experimental setup for *in situ* turbidity measurements

**Figure A2. Experimental setup for *in situ* turbidity measurements.** (a) Side-view of main components: (1) Bioprinting cartridge (2) Confinement, L_C (3) Photodetector that receives (4) Fiber coupled light source (5) Dish for collecting newly formed tube (6) Syringe pumps (7) Optical post with translating measurement unit (8) Oscilloscope receiving signal from photodetector (9) Camera arranged for *in situ* imaging. (b) Angled view of measurement unit. (1) Bioprinting cartridge (2) Confinement, L_C (3) Photodetector (4) Fiber coupled light source
Appendix A3– Fabrication Protocols

Fabrication Protocols

Mask writing:
1. Mask features are designed in AutoCAD (AutoDesk, California, USA)
2. Transparency photomasks printed at 23400 DPI and delivered by CAD/Art Services (OR, USA)

Master fabrication – 150 µm film thickness:
1. A 76.2 mm (3”) silicon water (Wafer World, Florida, USA) is rinsed with isopropyl alcohol (IPA) and acetone, and then dehydrated for 15 minutes on a hot plate (model HP30A, Torrey Pines Scientific, CA, USA) at 200°C. Subsequently silicon wafers are placed on a hot plate and allowed to cool at 65°C for 1 minute.
2. Spin 75 µm layer of SU-8 2050 (MicroChem, Massachusetts, USA)
   a. Recipe: Step 1: 500/5/5, Step 2: 1900/30/5.
3. Bake at 65°C/95°C for 5/15 minutes.
4. Repeat step 2.
5. Bake at 65°C/95°C for 15/45 minutes.
6. Align mask photomask on cured SU-8 wafer and exposed at 250J/cm² with UV source.
7. Post exposure bake (PEB) on a hot plate for 15 minutes at 95°C. Cool wafer on a hot plate at 65°C for 1 minute.
8. Develop the resist in a bath (1cm deep) of SU-8 Developer (MicroChem, Massachusetts, USA) for ten minutes.
9. Rinse with SU-8 Developer and IPA. Blow dry.
10. Place developed wafer on a hotplate at 95°C/175°C for 5/15 minutes. Cool developed wafer on a hotplate at 65°C for 1 minute.

Multilayer device fabrication:
A tube extrusion device contains 4 layers of PDMS; from the top down they are the inner flow, matrix flow, and outer flow and bottom layers. A combination of casting and thin film casting is used.

Figure A2. Experimental setup for in situ turbidity measurements. (a) Side-view of main components: (1) Bioprinting cartridge (2) Confinement, L_C (3) Photodetector that receives (4) Fiber coupled light source (5) Dish for collecting newly formed tube (6) Syringe pumps (7) Optical post with translating measurement unit (8) Oscilloscope receiving signal from photodetector (9) Camera arranged for in situ imaging. (b) Angled view of measurement unit. (1) Bioprinting cartridge (2) Confinement, L_C (3) Photodetector (4) Fiber coupled light source
to control layer thickness and separation.

1. Prepare a 10:1 (bulk:curing agent) mixture of PDMS (Ellsworth, Wisconsin, USA)
2. In an empty 150mm x 15mm petri dish (ThermoFisher Scientific, Mississauga, CA), cast a thin (0.5-1mm) layer of PDMS. This is the bottom layer
3. In another petri dish, cast This is the confining layer which will hold the confinement
4. Form a shallow dish surrounding the inner flow master using aluminum foil. Cast it with approximately 3 mm of PDMS
5. Form a thin film on matrix and outer by spin-coating:
   a. Recipe: Step 1: 200/5/5, Step2: 400/30/5.
6. Place all four layers and any remaining PDMS in a dessicator and degas for 45 minutes.
7. Move all layers to an 80°C oven:
   a. Inner flow layer is fully cured; 15 minutes.
   b. Matrix layer is partially cured (meaning the surface is still slightly adhesive to enable bonding); 5-7 minutes.
   c. Outer flow layer is partially cured; 5-7 minutes
8. Carefully peel the inner flow layer away from the master, align the central features with those of the matrix layer (which is partially cured and still attached to its master). Gently place the inner flow layer in the aligned position. Push away any trapped air, lightly, with a scalpel.
9. Coat the edges with PDMS.
10. Place the newly attached layers in the oven to bake for 15-20 minutes.
11. Peel the two layer device away from the matrix layer master and repeat step 8-10, but bonding with the outer layer.
12. Peel the three layer device away from the outer layer master.
13. Using a 1/16” (1.588mm) manual punch, make inlet holes for the inner, matrix and outer layers.
14. Corona plasma treat the surface of the bottom base layer in a hatch pattern to evenly treat surface.
15. Repeat step 14 for the bottom of the three-layer device.
16. Place the three layer device on top of the bottom base layer.
17. Seal the edges with PDMS and place in the oven for 15-20 minutes.
18. Cut the fully sealed device out of the dish and carefully punch the central hole through the bottom of the device. Use a 1/8” (3.175mm) punch and punch through all layers.
19. Take the thick 1cm confining layer and punch a 5mm hole.
20. Repeat step 14 again but this time bonding to the confining layer and aligning the outlet holes together.
21. Seal with PDMS and bake for another 15-20 minutes.
22. Cut out the fully made device and apply adhesive tape to the bottom to keep the outlet holes free of debris until use.
23. Cut three 3cm long pieces of 1/16” OD PEEK tubing (UpChurch Scientific, Washington, USA) and insert them into the inlet holes.
24. Fix the inlet tubing in place with quick setting epoxy. Allow to dry.
25. Cut a small square of thin cured PDMS (~1mm thick) and use it to cover the outlet hole on the top of the device. Seal the edges with PDMS.
26. Place the device in a dish and cover with PDMS.
27. Bake overnight at 80°C.
Appendix A4- Derivation of Analytical Models for Tube Formation

Describes here are the analytical models related to predictability of tube dimensions during formation. Two models are presented: (1) Model 1 (Solid Model) and its dimensionless derivation (2) Model 2 (Liquid Model) and its dimensionless derivation.

**Model 1 (Solid Model)**

The first model assumes gelation of the soft material tube at the interfaces $r = r_i$ and $r = r_0$. With $G = \Delta p/\Delta x$, the boundary conditions are:

$$r = 0 \quad \frac{dV_i}{dr} = 0, \quad (A1)$$

$$r = r_i \quad V_i = V_M \quad (A2)$$

$$r = r_O \quad V_o = V_M \quad (A3)$$

$$r = r_C \quad V_o = 0 \quad (A4)$$

$$G_i = G_O \quad (A5)$$

$$G_M = 0.5(G_i + G_o) \quad (A6)$$

**Inner Focusing Fluid**

From the general solution of the Navier-Stokes equation and with $G_f = \Delta P_f/\Delta x$, the radial variation of the inner focusing fluid can be expressed as:

$$V_i(r) = -\frac{G_i}{4\mu_i} r^2 + B_i \ln(r) + C_i \text{ and} \quad (A7)$$
\[
\frac{dV_I}{dr} = -\frac{G_I}{2\mu_I} r^2 + \frac{B_I}{r}
\]

With Eq. (A1):

\[B_I = 0\]  \hspace{1cm} (A8)

With Eq. (A2):

\[C_I = V_M + \frac{G_I}{4\mu_I} r_I^2\]  \hspace{1cm} (A9)

With (A7) follows for the radial variation of the velocity within the inner focusing fluid:

\[V_I(r) = V_M + \frac{G_I}{4\mu_I} \left(r_I^2 - r^2\right)\]  \hspace{1cm} (A10)

and for the volumetric flow rate:

\[Q_I = 2\pi \int_0^r V_M r + \frac{G_I}{4\mu_I} \left(r_I^2 - r^2\right) r \, dr\]  \hspace{1cm} (A11)

\[Q_I = \pi r_I^2 V_M + \frac{\pi G_I r_I^4}{8\mu_I}\]  \hspace{1cm} (A12)

**Biopolymer Matrix Solution**

We consider the biopolymer matrix solution as a rigid structure that flows with a constant velocity \(V_M\):

\[Q_M = 2\pi \int_{r_I}^r V_M r \, dr = \pi \left(r_O^2 - r_I^2\right) V_M\]  \hspace{1cm} (A13)
Outer Focusing Fluid

Applying the general solution for the outer focusing fluid yields:

\[ V_o(r) = \frac{G_o}{4\mu_o} r^2 + B_o \ln(r) + C_o. \]  (A14)

With Eq. (A3) follows

\[ -\frac{G_o}{4\mu_o} r_o^2 + B_o \ln(r_o) + C_o = V_m. \]  (A15)

With Eq. (A4) we obtain

\[ -\frac{G_o}{4\mu_o} r_c^2 + B_o \ln(r_c) + C_o = 0. \]  (A16)

Subtract Eq. (A16) from Eq. (A15):

\[ \frac{G_o}{4\mu_o} (r_c^2 - r_o^2) + B_o \ln\left(\frac{r_o}{r_c}\right) = V_m. \]  (A17)

\[ B_o = \frac{V_m - \frac{G_o}{4\mu_o} (r_c^2 - r_o^2)}{\ln\left(\frac{r_o}{r_c}\right)}. \]  (A18)

Eq. (A18) in Eq. (A16) lead to:

\[ -\frac{G_o}{4\mu_o} r_c^2 + \frac{V_m - \frac{G_o}{4\mu_o} (r_c^2 - r_o^2)}{\ln\left(\frac{r_o}{r_c}\right)} \ln(r_c) + C_o = 0. \]  (A19)
Substituting $B_o$ and $C_o$ in Eq. (A14):

$$V_o(r) = \frac{G_o}{4\mu_o} \left( r_o^2 - r_c^2 \right) + \frac{4\mu_o V_m - G_o \left( r_c^2 - r_o^2 \right)}{4\mu_o} \ln \left( \frac{r}{r_c} \right).$$  (A21)

For the volumetric flow rate of the outer focusing liquid follows:

$$Q_o = 2\pi \int_{r_o}^{r_c} \left[ -\frac{G_o}{4\mu_o} \left( r_o^2 - r_c^2 \right) + \frac{4\mu_o V_m - G_o \left( r_c^2 - r_o^2 \right)}{4\mu_o} \ln \left( \frac{r}{r_c} \right) \right] r \ dr$$  (A22)

$$Q_o = \frac{G_o \pi}{8\mu_o} \left( r_c^2 - r_o^2 \right)^2 + \frac{\pi \left( 4\mu_o V_m - G_o \left( r_c^2 - r_o^2 \right) \right)}{2\mu_o \ln \left( \frac{r_o}{r_c} \right)} \left[ \frac{r_o^2 - r_c^2}{4} - \frac{r_o^2 - r_c^2}{2} \ln \left( \frac{r_o}{r_c} \right) \right]$$  (A23)

**Force Balance on Tube Wall**

Shear forces applied at tube outer and inner wall balance pressure forces acting on cross-sectional area:

$$2\pi r \mu_t \frac{dV_o}{dr} \bigg|_{r_o} - 2\pi r_o \mu_o \frac{dV_o}{dr} \bigg|_{r_o} = \pi \left( r_o^2 - r_i^2 \right) \frac{G_i + G_o}{2}$$  (A24)
Equation (A24) can be further simplified by evaluating the derivatives of Eq. (A10) and Eq. (A21) at radii \( r_I \) and \( r_O \), respectively:

\[
-G_I r_I^2 + G_O r_O^2 - \frac{4\mu_o V_M}{r_c^2 - r_O^2} G_O \left( r_c^2 - r_O^2 \right) = \left( r_c^2 - r_O^2 \right) \frac{G_I + G_O}{2}
\]  \hspace{1cm} (A25)

and with Eq. (A5) we obtain:

\[
G = \frac{4\mu_o V_M}{r_c^2 - r_O^2}
\]  \hspace{1cm} (A26)

Substituting Eq. (A26) into Eq. (A12) and Eq. (A23) gives:

\[
Q_I = \pi r_I^2 V_M \left( 1 + \frac{\mu_O r_I^2}{2\mu_I (r_c^2 - r_O^2)} \right)
\]  \hspace{1cm} (A27)

\[
Q_O = \frac{\pi (r_c^2 - r_O^2) V_M}{2}
\]  \hspace{1cm} (A28)

Equations (A13), (A27) and (A28) can be converted to a dimensionless form using the above conventions and solved analytically to predict \( D_I^* \), \( D_O^* \) and \( V_M^* \) for given flow rates \( Q_I^* \), \( Q_O^* \), and \( Q_M^* \):

\[
D_I^* = \frac{2Q_O^* Q_I^* - 2Q_I^* \sqrt{Q_0^*(Q_0^* + Q_I^*) + 2Q_I^* \sqrt{Q_0^*(Q_0^* + Q_I^*)} - 2 \sqrt{Q_0^*(Q_0^* + Q_I^*) + 2Q_I^*}}}{3Q_0^2 + 2Q_0^* Q_I^* + 2Q_0^* - Q_I^2 + 2Q_I^* - 1}
\]  \hspace{1cm} (A29)
\[ D_o = \sqrt{\frac{4Q_0^* + 2Q_i^* - 4Q_0^* \sqrt{Q_0^*(Q_0^* + Q_i^*)}}{3Q_0^* + 2Q_0^* Q_i^* + 2Q_0^* - 2Q_i^* + 2Q_i^* - 1}} \]  \hspace{1cm} (A30)

\[ V_M^* = 2\sqrt{Q_0^*(Q_0^* + Q_i^*)} - Q_i^* - Q_0^* + 1 \]  \hspace{1cm} (A31)

**Model 2 (Liquid Model)**

A second model describes slow gelation of biopolymers by considering fluids as liquid sheaths upon exit of the device. This model assumes (a) steady flow, (b) flow symmetry with respect to the center of the tube, (c) zero velocity at the confinement wall, (d) the uniform velocity \( V_M \) to be applied across the gelling biopolymer solution and the inner focusing fluid, (e) a parabolic velocity profile in the outer focusing fluid and (f) zero velocity gradient at the tube outer wall.

**Inner Focusing Fluid**

The volumetric flow rate of the inner focusing fluid can be written as:

\[ Q_I = \pi r_I^2 V_M \]  \hspace{1cm} (A32)

and in the dimensionless form:

\[ Q'_I = D_i^2 V'_M. \]  \hspace{1cm} (A33)

**Biopolymer Solution**

The flow rate of the biopolymer solution can be written as:

\[ Q_M = 0.25\pi(D_o^2 - D_i^2) V_M \]  \hspace{1cm} (A34)
and in the dimensionless form:

\[ Q'_M = 1 - Q'_o - Q'_i = \left( D_o'^2 - D_i'^2 \right) V'_m. \]  \hspace{1cm} (A35)

**Outer Focusing Fluid**

For the outer focusing fluid we can write:

\[ Q_o = 0.125 \pi \left( D_c'^2 - D_o'^2 \right) V_m. \]  \hspace{1cm} (A36)

and in the dimensionless form:

\[ Q'_o = 0.5 \left( 1 - D_o'^2 \right) V'_m. \]  \hspace{1cm} (A38)

**Dimensionless Forms of the Solid and Liquid Models**

**Liquid Model.** For slow gelling biopolymers, we assume steady flow, flow symmetry with respect to the confinement axis, zero velocity at the confinement wall, a uniform velocity \( V_m \) across the gelling biopolymer solution, and matching pressure gradients between the inner and the outer focusing fluids. Equations (A2, A4, A6) can be solved to obtain:

\[ D'_i = \sqrt{Q'_i / (1+Q'_o)}. \]  \hspace{1cm} (A39)

\[ D'_o = \sqrt{(1-Q'_o) / (1+Q'_o)}. \]  \hspace{1cm} (A40)

\[ V'_m = 1 + Q'_o. \]  \hspace{1cm} (A41)
**Solid Model.** For rapidly gelling constructs we assume steady flow, flow symmetry with respect to the confinement axis, zero velocity at the confinement wall and a constant velocity \( V_M \) across the gelling biopolymer solution and within the inner focusing fluid. Assuming a parabolic velocity profile in the outer focusing fluid, zero velocity gradient at the tube outer wall and considering the force balance and continuity equations allow \( Q_I, Q_M \) and \( Q_O \) to be written as:

\[
Q_I = 0.25 \pi D_I^2 V_M \left( 1 + D_I^2 (2(D_C^2 - D_O^2))^{-1} \right), \quad (A42)
\]

\[
Q_M = 2 \pi \int_0^\infty V_M r \, dr = 0.25 \pi (D_O^2 - D_I^2) V_M \text{and} \quad (A43)
\]

\[
Q_O = 0.125 \pi (D_C^2 - D_O^2) V_M \quad (A44)
\]

The three equations were converted to a dimensionless form using the above conventions and solved for the diameters \( D_I^*, D_O^* \) and the write speed \( V_M^* \) as a function of the flow rates \( Q_I^*, Q_O^*, \) and \( Q_M^* \):

\[
D_I^* = \sqrt{\frac{2Q_0^* + 2Q_0^*Q_I^* - 2Q_0^* \sqrt{Q_0^*(Q_0^* + Q_I^*) + 2Q_I^* \sqrt{Q_0^*(Q_0^* + Q_I^*) - 2 \sqrt{Q_0^*(Q_0^* + Q_I^*) + 2Q_0^*}}}}{3Q_0^* + 2Q_0^*Q_I^* + 2Q_0^* - Q_I^*^2 + 2Q_I^* - 1}}, \quad (A45)
\]

\[
D_O^* = \sqrt{\frac{4Q_0^* + 2Q_I^* - 4Q_0^* \sqrt{Q_0^*(Q_0^* + Q_I^*) + Q_0^*^2 - Q_I^*^2 - 1}}{3Q_0^* + 2Q_0^*Q_I^* + 2Q_0^* - Q_I^*^2 + 2Q_I^* - 1}}, \quad (A46)
\]

\[
V_M^* = 2 \sqrt{Q_0^*(Q_0^* + Q_I^*)} - Q_I^* - Q_0^* + 1. \quad (A47)
\]
Figure A5. Dimensionless Plot, $D_0^* (Q_0^*)$ for various $Q_I^*$. $Q_I^*$=0.1 (red), $Q_I^*$=0.2 (orange), $Q_I^*$=0.3 (yellow), $Q_I^*$=0.4 (green), $Q_I^*$=0.5 (cyan), $Q_I^*$=0.6 (blue), $Q_I^*$=0.7 (lavender) $Q_I^*$=0.8 (purple) $Q_I^*$=0.9 (grey) $Q_I^*$=0.95 (black). Solid lines indicate liquid model, dashed lines indicate solid model. Triangles represent alginate tubes formed at $Q_T$=15mL/min, Circles represent alginate tube data formed at $Q_T$=1.5mL/min.
Appendix A6-Dimensionless Plot, $D_1^*(Q_O^*)$ for various $Q_I^*$

**Figure A6. Dimensionless Plot, $D_1^*(Q_O^*)$ for various $Q_I^*$.** $Q_I^*=0.1$ (red), $Q_I^*=0.2$ (orange), $Q_I^*=0.3$ (yellow), $Q_I^*=0.4$ (green), $Q_I^*=0.5$ (cyan), $Q_I^*=0.6$ (blue), $Q_I^*=0.7$ (lavender) $Q_I^*=0.8$ (purple) $Q_I^*=0.9$ (grey) $Q_I^*=0.95$ (black). Solid lines indicate liquid model, dashed lines indicate solid model. Triangles represent alginate tubes formed at $Q_T=15$mL/min, Circles represent alginate tube data formed at $Q_T=1.5$mL/min.
Figure A7. PL Spectra for 0.1% v/v FITC in pH 2-8 solutions. Curves represent FITC emission collected between 488-620nm in varying solutions pH 2 (purple), pH 3 (blue), pH 4 (dark green), pH 5 (green), pH 6 (orange), pH 7 (red), pH 8 (black).
Appendix A8- Experimental setup for cannulation

Figure A8. Cannulation setup for tube pressurization. (1) Camera for top-view visualization of cannulated tubes. (2) Cannulated collagen tube (3) Cannulation stage (4) Fluidic line leading to perfusion reservoir.
Figure A9. Enclosed 3D Tube Hosting Device Design. (a) Top view of rendered tube hosting device design with (1) fixation inlets (2) direct-loading perfusion channel (3) superfusion inlets. Scale bar: 5mm. (b) Side view of rendered tube hosting device design. (c) Image of 3D printed tube hosting device. Scale bar: 5mm.
Figure A10. Open well 3D Tube Hosting Device Design. (a) Isometric view of rendered 6-well configured tube hosting device design. (b) Top view of rendered 6-well configured tube hosting device design with hosting trough to accommodate hosted tube. (c) Side view of rendered tube hosting device design with perfusion inlet, P and fixation/vacuum inlet, V. Scale bar: 34.8mm
Figure A11. SEM Images of Collagen Tubes. SEM images of collagen tubes (a) Scale bar: 10μm (b) Scale bar: 20μm (c) 2μm.
Appendix A12- Viscosity data at different shear rates and temperature

**Figure A12. Viscosity data at different shear rates and temperature.** Collagen, [5mg/mL] and PEG (10% w/v) viscosity data plotted with shear rates at room temperature (21°C) indicated by the red markers and physiological temperature (37°C) indicated by the blue markers.