Continuous Extrusion of Homogeneous and Heterogeneous Hydrogel 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

We present a platform that allows homogeneous and heterogeneous 3-D soft materials to be continuously defined in a single step. Biopolymer solutions are introduced to a microfluidic device and radially distributed to feed to a common outlet at the device center. This forms concentric sheaths of complex fluids and upon crosslinking, a hydrogel tube at the exit. This approach allows for the controlled and continuous extrusion of tubes with tailored diameters of 500 µm to 1500 µm, wall thicknesses of 20 µm to 120 µm, and compositions, as well as predictable mechanical and chemical properties. Using the same platform, single and multi-walled hydrogel tubes with defined heterogeneities and patterns of discrete spots of secondary biopolymer materials can be continuously extruded. A tube-hosting device is presented which can independently perfuse and superfuse isolated tube segments, allowing precise microenvironmental control without cannulation for up to an hour.
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1 Introduction

Soft materials with complex geometries and defined heterotypic composition are abundant in nature. These tissues often possess a hierarchical architecture at length scales ranging from large molecules to several millimeters that is closely related to the tissue’s biological function, and can often dynamically alter their structure and morphology. Examples of soft tissues in the body with similar composition and geometries include arteries, blood vessels, and capillaries, the intestinal mucosa and submucosa, and bronchioles. Very few approaches exist which allow the spatial organization of soft matter into 3D tissues, specifically perfusable tubes, in a scalable format. The continuous production of microscale fibers and tubes is of particular interest in the generation of vascular grafts and cell-encapsulation for soft tissue applications [1, 2].

The lack of scalable ways of achieving a heterotypic composition is particularly evident at the micrometer to millimeter length scales that are of key importance for nutrient transport, cell-cell and cell-matrix interactions. Previously employed top-down fabrication approaches start from planar substrates and employ a series of processing steps (e.g., lithography, printing, engraving or direct writing) to ultimately obtain the desired heterotypic characteristics [3]. Bottom-up approaches are also possible, where microscale zero-dimensional and one-dimensional building blocks are assembled to form planar and 3-D assemblies [4].

There exist both micro-scale and macro-scale methods to assemble tubes and fibers for many applications in materials science and tissue engineering, both using microfluidic platforms and traditional macro-scale approaches. Here, we present a fabrication method and microfluidic platform that allows homogeneous and heterogeneous soft tubular materials to be continuously defined in a single step with predictable mechanical and chemical properties.

1.1 Macroscale Approaches to the Formation of Tubes and Fibres

Traditional approaches of producing micro-scale fibers and tubes often involve scaled-down macro-scale processes. Electrospinning of extracellular matrix fibers (ie. collagen, elastin, fibrin) is a commonly used approach in the tissue engineering community to produce 3D fiber cell culture meshes with highly controlled porosity and mechanical properties for soft tissues [5, 6].
and hard tissues [7]. Many synthetic polymers are also used and can be biofunctionalized [8], but the obvious drawback to this method is the difficulty in creating tube structures, not fibers. Conductive or non-conductive fibers can also be formed by wet-spinning [9] and gel-spinning [10]. Larger scale tubes with outer diameters on the range of millimeters can be made with batch methods, such as applying centrifugal forces during polymerization in a cylindrical mold [11], rolling up of sheets [12] or simple wrapping of sheets around a cylindrical mold [13].

1.2 Microfluidic Approaches to the Formation of Tubes and Fibres

There has been significant interest in continuous formation of fibers and tubes using microfluidic platforms. Early work in miniaturizing classic coaxial sheath flows in horizontal configurations established that 3D microstructures could be built in polydimethylsiloxane (PDMS) substrates [14] to define sheath flows with down to 1 µm thickness, or similarly in glass and silicon [15] by varying the relative flow rate of the inner and outer flows. This was extended to the extrusion of microscale tubes and fibers in microfluidic devices using a variety of materials; poly(lactic-co-glycolic acid) (PLGA) microfibers [16], UV photopolymerizable acrylic acid tubes [17], polyacrylonitrile (PAN), polysulfone (PSF), and polystyrene (PS) tubes [18], and UV photopolymerizable poly(ethylene glycol) (PEG-DA) tubes [19]. Though tube thickness and diameter can be well controlled, many of these platforms rely on fixed structures (i.e. pulled glass pipettes) or have applications limited by the working materials and are limited to homogeneous compositions. A similar approach has recently produced tubes with heterogeneous “mosaicked” compositions [2] in UV photopolymerizable chemistries. Other microfluidic approaches for the formation of microscale tubes, fibers, and vesicles exploit microscale arrays to extrude microstructured material vertically: calcium alginate microfibers and “tubes” [20-22], and lipid tubes and vesicles [23]. These approaches rely on fixed outlet configurations, which limit the heterogeneities possible, the scale of the tubes and fibers produced, as well as their collection.

Discontinuous approaches have also been used to form microfibers and tubes, using both microfluidic platforms and macro-scale techniques. Hydrodynamic spinning [24] using a custom-made spinneret to produce three phase coaxial flow has been used to form solid fibres and hollow fibres of varying materials, including alginate, poly-(N-isopropyl acrylamide),
polysulfone, and cell seeded gelatin-hydroxyphenylpropionic acid. Self-assembly of alginate and alginate-PLL hydrogel “microstrands” [25] has been demonstrated by fabricating porous filters from SU-8 and suspending them over crosslinker baths. Drops of hydrogel pre-cursor solution are placed above the filter and capillary forces draw them into the crosslinker bath, forming solid fibres. Roller systems [26, 27] have been used to define polysaccharide microfibre dimensions and mechanical properties after simple extrusion of polysaccharides through single channels.

Table 1. Summary of tube and fibre properties

<table>
<thead>
<tr>
<th>Material</th>
<th>Wall Thickness</th>
<th>Outer Diameter</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA, PLGA+fibronectin</td>
<td>N/A</td>
<td>20-230 µm</td>
<td>[16]</td>
</tr>
<tr>
<td>Acrylic acid</td>
<td>N/A</td>
<td>20-90 µm</td>
<td>[17]</td>
</tr>
<tr>
<td>Polyacrylonitrile, polysulfone, and polystyrene</td>
<td>40-150 µm</td>
<td>300-900 µm</td>
<td>[18]</td>
</tr>
<tr>
<td>PEG-DA</td>
<td>10-15 µm</td>
<td>55-75 µm</td>
<td>[19]</td>
</tr>
<tr>
<td>PEG-DA</td>
<td>70-140 µm</td>
<td>100 µm, 200 µm, 500 µm</td>
<td>[2]</td>
</tr>
<tr>
<td>Alginate, alginate+PLL</td>
<td>N/A</td>
<td>Tubes- 230 µm Fibres- 120 µm</td>
<td>[20]</td>
</tr>
<tr>
<td>Alginate and gelatin</td>
<td>N/A</td>
<td>100-1200 µm</td>
<td>[21]</td>
</tr>
<tr>
<td>Alginate</td>
<td>N/A</td>
<td>250-500 µm</td>
<td>[22]</td>
</tr>
<tr>
<td>DLPC</td>
<td>N/A</td>
<td>3.5-20 µm</td>
<td>[23]</td>
</tr>
<tr>
<td>Gelatin-hydroxyphenylpropionic acid (Gtn-HPA), NIPAAM, alginate</td>
<td>NIPAAM ID: 69±10 µm-93±16 µm</td>
<td>111±4 µm -227±9 µm (Gtn-HPA)</td>
<td>[24]</td>
</tr>
<tr>
<td>Alginate, alginate+PLL</td>
<td>N/A</td>
<td>30 µm, 60 µm, 90 µm, 180 µm, 300 µm</td>
<td>[25]</td>
</tr>
<tr>
<td>Alginate</td>
<td>N/A</td>
<td>1-10 µm</td>
<td>[26]</td>
</tr>
<tr>
<td>Alginate, alginate+chitosan</td>
<td>N/A</td>
<td>28.6±1.7 µm-31.3±1.5 µm</td>
<td>[27]</td>
</tr>
</tbody>
</table>
1.3 Materials

In order to finely control the geometry and microstructure of the final solid tube or fibre after formation, a fast gelation process is required. Many materials can undergo a sol-gel transition from a colloidal precursor solution to a gel network through controlled polymerization or cross-linking. This transition from colloidal solution to solid can be irreversible or specifically reversible, initiated by a number of means, including photopolymerization, temperature, pH, electric or magnetic field, and ionic concentration gradients [28-30]. Sol-gel inorganic and organic composites and synthetic polymers are widely used to immobilize a wide range of biological materials and in the formation of biosensors [31, 32], as are hydrogels like agarose, collagen, and gelatin [30].

Ionically cross-linking sol-gel materials, such as alginate, are ideal candidates for continuous extrusion of tubes and fibres because of their rapid ion exchange kinetics leading to quick, controlled gelation [33]. The structure of alginate is a family of co-polymers with varying proportions of two constituent monomers; \( \alpha \)-L guluronic acid (G) and \( \beta \)-D-mannuronic acid (M) [34]. Alginate gelation relies on the selective ionic affinity of alginates to calcium ions (Ca\(^{2+} \)), and its ability to cooperatively bind these ions. During ionic exchange this binding occurs strictly between the G residues and Ca\(^{2+} \) ions, where the total Ca\(^{2+} \) content and alginate concentration are the main factors affecting gelation rate [35], as well as the frequency and distribution of the G residues in the bulk material [36].

Alginate is also a desirable material because it doesn’t require any external stimuli (ie. temperature change or pH change) to initiate cross-linking, which would require additional components and limit the scalability and applications of the tube and fibre formation process. It is commonly used as an immobilization matrix for cells [37-40], in tissue engineering [41-43], and in drug delivery [28, 44].
2 Experimental Procedures and Setup

2.1 Device Fabrication

Transparency mask designs were prepared in a CAD program (AutoCAD San Rafael, California, United States) and photomasks were printed at 20 000 DPI (CAD/ART Services, Oregon, United States). Using standard soft lithography techniques, transparency masks were transferred to slide masters for replica molding of each layer [45]. 3”×4” glass slides (Corning Inc., Corning, New York, United States) were rinsed with isopropanol, acetone, and then isopropanol and dehydrated on a hot plate (HP30A, Torrey Pines Scientific, San Marcos, California, United States) at 200°C for 30 minutes. Slides were allowed to cool to 65°C and then treated with oxygen plasma for 30 seconds (PDC-32G, Harrick Plasma, Ithaca, New York, United States).

Using a layer of SU-8 25 negative photoresist (Microchem, Newton, Massachusetts, United States), a seed layer was spun on each slide at 2000 rpm for 30 seconds using a SCS G3 spin coater (Specialty Coating Systems, Indianapolis, Indiana, United States) and soft baked at 65°C and 95°C for 4 and 6 minutes respectively. The seed layers were exposed to UV light (365 nm) for 13 seconds (Model 200, OAI, San Jose, California, United States), and then baked again for 8 minutes at 95°C. Feature heights of 150 µm were achieved by spinning two 75 µm layers of SU-8 2050 negative photoresist (Microchem, Newton, Massachusetts, United States) at 1900 RPM for 30 seconds and soft baking in between spins for 5 minutes at 65°C and 15 minutes at 95°C. After the second 75 µm layer, the slides were baked for 15 minutes at 65°C and 45 minutes at 95°C. Using the photomasks, the features were exposed on the slides by exposing with 365 nm UV light at 300 J. Post-exposure, the slides were hard baked for 20 minutes at 95°C and then developed in SU-8 Developer (Microchem, Newton, Massachusetts, United States) for 10 minutes. The masters were then rinsed with isopropanol, dried under N2, and baked for 15 minutes at 80°C.

To obtain reliably bonded multilayer devices, a multilayer partial curing and bonding technique was adopted from previously established protocols [46, 47]. PDMS pre-polymer and curing agent were mixed in a ratio of 10:1 and was spin coated onto masters at 400 rpm for 30 seconds, making a final layer thickness of 400 µm. The top layer was not spin coated but rather covered with ~0.5 cm layer of uncured PDMS in a dish, as this layer had to be partially baked and peeled off the mold to bond with the first spin coated partially cured layer during the device fabrication.
The PDMS-coated masters were degassed in -25 inHg vacuum at room temperature for 1 hour, and then the top layer was partially baked at 80°C for approximately 12 minutes. The second layer was baked at 80°C for approximately 9 minutes. When partially cured, the thicker top layer was aligned over the sticky second layer and air bubbles were carefully squeezed out of the device with the blunt edge of a scalpel. The edges were sealed with uncrosslinked PDMS and these layers were further baked for 12 minutes to ensure strong bonding. This process was repeated until all layers were bonded together. After bonding, inlet holes were punched for all layers and both top and bottom were sealed with partially cured PDMS sheets with no features.

2.2 Materials

In this work, alginate is selected as the primary biopolymer material due to its fast gelation in the presence of Ca$^{2+}$ ions. No further external stimuli is required to initiate polymerization, such as temperature change or focused UV light, which reduces the complexity of the experimental setup. For the biopolymer solution, an aqueous mixture of 2% w/w alginate in DI water was used. Sodium alginate (sodium salt) was purchased from Sigma Aldrich (Oakville, Canada) and was dissolved in DI water and glycerol and sonicated for 2 hours to ensure uniform mixing. Glycerol is added to increase the density of the alginate solution, which stabilizes the extrusion process. The inner and outer focusing fluids are the same solution of 150 mM calcium chloride dihydrate (CaCl$_2$) in water with a density of 1.169 g/cm$^3$. The alginate material is extruded into a crosslinking bath, which contains a mixture of 150 mM CaCl$_2$ in DI water with a density of 1.148 g/cm$^3$. CaCl$_2$ was purchased from BioShop (Burlington, Canada). The density is adjusted with the addition of glycerol to the biopolymer and focusing solutions, and is carefully controlled to minimize the effect of gravitational acceleration during the vertical extrusion process. It also reduces re-circulation near the device outlet, which reduces device clogging. Glycerol was purchased from BioShop (Burlington, Canada). Additional biopolymers, e.g. pectin, were used in combination with alginate and were purchased from Sigma Aldrich (Oakville, Canada).

Two types of fluorescent microbeads were used for post-extrusion fluorescence imaging of tube segments, nile red FluoSpheres carboxylate-modified microspheres (0.02 µm diameter) (excitation/emission 535/575 nm) and 0.1 µm blue fluorescent beads (excitation/emission 350/440 nm) from Life Technologies (Carlsbad, USA). Microbeads were sonicated with alginate solutions (10% v/v) for 1 hour to prevent aggregation.
2.3 Device Design

Multiple PDMS devices have been developed to continuously extrude homogeneous and heterogeneous single and multi-layer alginate tubes with microscale dimensions. They offer significant potential for scalability and the continuous production of predictable and geometrically defined 3D materials. The vertical extrusion principle is demonstrated in Figure 1, where the three layers of the coaxial flow that defines a homogeneous tube in-flow are shown. The inner void of the tube is created by the inner focusing fluid layer, shown in purple. The tube material is supplied in the biopolymer layer, shown in blue. The outer focusing layer, shown in pink, defines the outer surface of the tube.

![Figure 1. Vertical extrusion principle:](image)

The simplest device extrudes homogeneous single-layer tubes (Figure 2a). The inlet channel of each layer splits evenly and is arranged in a radial configuration around the common 1/8” outlet hole, ensuring that a connected tube is formed during extrusion (see Figure 2a). Each PDMS layer is 400 µm thick with 150 µm tall features, with every outlet channel having a width of 230
µm and spacing between channels of 130 µm. This device was created by aligning alternating vertical arrangements of biopolymer and focusing layers, as demonstrated in Figure 2b. Due to the arrangement of many thin layers in the device and large pressure drop in each layer due to the highly viscous solutions, large areas of overlapping channels often result in rupture and leaking between layers. This was addressed by alternating the two main layer designs to minimize the overlap of channels between layers. Both layers have the same pressure drop of 1 psi but a different footprint, which reduces overlap between adjacent layers to two points. At these points, reinforcing posts are added to reduce the risk of rupture and leakage between layers. So, any two adjacent layers will have different footprints, which increase the robustness and lifetime of the completed device shown in Figure 2c.

The basic vertical arrangement of layers is, from top to bottom: inner streaming, biopolymer, and outer streaming. The basic biopolymer distribution layer is shown in Figure 2d, which extrudes homogeneous tubes as shown in Figure 2e. Using alternate vertical configurations of repeating layers and by altering the configuration of biopolymer layer(s), devices can be produced using the same methodology that produce tubes with specific heterogeneities or multiple layers. For instance, by increasing the number of biopolymer layers in the homogeneous biopolymer layer design, the in-flow creation of \( n \)-layered tubes is possible with up to \( n \)-compatible matrix materials (see Section 6). Similarly, creating tubes with 1, 2…\( n \) compatible materials across the tube circumference is achieved by modifying the single biopolymer layer to have \( n \) equally distributed inlets. Designs for all device layers are shown in Appendix A.
Figure 2. **Device design:** (a) PDMS microfluidic device to extrude a single layer biopolymer tube. Inset, top: Close-up view of resistance channels that evenly distribute flow of biopolymers and focusing fluids in a radial pattern around the common extrusion outlet. Section A-A shows the internal cross-section at the extrusion outlet. Scale bar 0.75 inches. Inset, bottom: Photograph of resistance channels at extrusion outlet, scale bar 1 mm. (b) Schematic showing layer-by-layer device assembly to form multi-layered PDMS devices. (c) Photograph of device to make bilayered tubes with food dye to show channel locations. Scale bar 10 mm. (d) Matrix layer to produce homogeneous tube, shown in inset. (e) From left to right: confocal image showing homogeneous tube cross-section, brightfield image showing homogenous tube segment, 3-D confocal image showing perfusable homogeneous tube. Scale bars, 100 µm, 500 µm, 500 µm.
2.4 Experimental Setup

A schematic of the experimental setup is shown in Figure 3. The microfluidic device is contained in a polycarbonate case with an enclosed extrusion outlet having an internal diameter of 6.35 mm and a length of 25.4 mm. The device and enclosure, rests inside a fluid-filled reservoir, and the hydrogel tubes are continuously vertically extruded into this bath. Cross-linking begins as soon as the fluid streams meet, so the spatial organization defined by the fluid flows inside the device is retained in the final soft material. The biopolymer and focusing streams are introduced to the microfluidic device via standard fluidic connections, and are fed with separate syringe pumps. Upon exiting the device, polymerization begins by diffusive mixing at the inner and outer surface of the tube. The presence of this enclosed extrusion outlet reduces shear during extrusion and stabilizes the coaxial flow and tube formation. The extrusion velocity is affected by the density difference between the biopolymer matrix and focusing streams.

![Experimental setup diagram](image)

**Figure 3. Experimental setup:** Experimental set-up including fluidic control, the microfluidic device, confinement at extrusion outlet, and a liquid filled reservoir. Scale bar 1.5 inches.

2.5 Operating Parameters

Several device and experimental setup parameters are important to produce a stable flow for tube extrusion; the relative streaming and matrix flow rates, the density difference between the
reservoir and the extrusion fluids, and the gap size between subsequent layers in the device. For the devices presented here, stable extrusion is possible with inner and outer streaming flow rates from 0.3 ml/min to 2 ml/min, when both inner and outer streaming have the same flow rate, and a matrix flow rate of 150 µl/min to 250 µl/min. The difference in density between extrusion fluids and reservoir fluid is important to prevent the extruded tubes from floating and accumulating at the device exit; a density difference of 92 kg/m³ was experimentally determined to prevent flotation during extrusion without significantly accelerating the tube extrusion velocity. The gap size between layers is the final important parameter to routinely form tubes during extrusion; a too-large gap size will not form a coaxial flow and will ultimately form a continuous alginate fibre instead of a tube. Here, a layer thickness of 400 µm was selected such that the gap from channel to channel between layers was 250 µm. Smaller gap sizes are possible, but decreasing gap size will increase the difficulty of fabrication.

3 Material Characterization

The material and geometric properties of the extruded tubes are influenced by many factors, including alginate composition and concentration, crosslinking time and calcium concentration, and extrusion velocity. These properties ultimately have a strong effect on the geometry of the extruded tubes, as well as affecting quantitative imaging of extruded and crosslinked hydrogel tubes.

3.1 Characterization of Aqueous Alginate Solutions

The rheological behaviour of aqueous alginate solutions is pseudoplastic and so the viscosity of the uncrosslinked alginate at the device outlet is dynamic and depends on the fluid velocity. The viscosity is also affected by the amount of glycerol added to stabilize the solution. The viscosity of aqueous biopolymer solutions was measured using a 2.5 cm diameter spindle in a rotational rheometer (DV-III Programmable Rheometer, Brookfield, Middleboro, USA). Samples were continuously tested from 1-60 RPM in increments of 5 RPM, from 60-100 RPM in increments of 10 RPM, and from 100-200 RPM in increments of 20 RPM. The dynamic viscosity of 2% alginate solutions with glycerol added to densities of 1077.4 kg/m³ (red line), 1196 kg/m³ (blue line), and 1169.4 kg/m³ (green line) was measured (Figure 4).
Figure 4. Rheometric data of aqueous 2% alginate solutions: Rheological characterization of uncrosslinked 2% alginate solutions of different densities: $\rho=1169.4$ kg/m$^3$ (green dotted line), $\rho=1119.6$ kg/m$^3$ (solid blue line), $\rho=1077.4$ kg/m$^3$ (solid red line). Measurements performed at room temperature.

At a velocity above 8 cm/s, the viscosity curve of each solution collapses onto each other and the relative density differences no longer affect the solution viscosity. However, in the typical aqueous alginate flow rate range of 100-200 $\mu$l/min and extrusion channel cross-section of 230 $\mu$m $\times$ 150 $\mu$m, the peak velocity at the channel outlet is calculated to be between 4.8-9.7 cm/s. In this range of calculated velocities there is a significant difference in measured alginate viscosity, which affects the final geometric properties of the tubes. This is an important factor that allows us to dynamically change the extruded material properties and geometries by altering the extrusion conditions but not the working fluid. This is further explored in Section 4.4.2. These measurements were performed at room temperature, and at higher temperatures the viscosity is expected to decrease significantly, proportional to the decrease predicted by Arrhenius’ Law [48].
3.2 Characterization of Crosslinked Alginate

3.2.1 Elastic Modulus of Crosslinked Alginate

Once extruded, the alginate tubes further crosslink in the extrusion reservoir or storage baths. The final tensile properties of the extruded material depend on the amount of crosslinking time, the crosslinker solution composition and the crosslinker concentration. The modulus of elasticity of tube segments was measured over a span of 14 days under two conditions (Figure 5); 2% alginate tubes crosslinked in 100 mM CaCl$_2$ reservoir buffer and stored in 100 mM CaCl$_2$ in DI water (solid pink), and 2% alginate tubes crosslinked in 100 mM CaCl$_2$ reservoir buffer and stored in DI water (blue stripes).

![Figure 5. Average modulus of elasticity of 2% alginate:](image)

(a) Average 2% alginate tube modulus of elasticity as a function of time. Tubes crosslinked in 100 mM CaCl$_2$ reservoir buffer and stored in either 100 mM CaCl$_2$ in DI water (solid pink) or DI water (blue stripes). Error bars indicate one standard deviation, n=3 tubes measured at each time point. (b) Average 2% alginate tube modulus at Day 0 compared to that of a 2% alginate planar hydrogel sheet as previously measured [47]. Tubes and sheets crosslinked in 100 mM CaCl$_2$ reservoir buffer and stored in either 100 mM CaCl$_2$ in DI water (angled stripes-tube) or DI water (solid- tube, vertical stripes- sheet).

The modulus of elasticity of wet tube segments was measured using a custom tensile tester (840LE2, Test Resources, Shakopee, USA). Samples were cut to an average length of 1.5 cm and measured prior to clamping between vertical grips. The samples were clamped by sandwiching the ends between cardboard strips to prevent tearing at the grip edge. A pulling speed ramp of 0.1 mm/s was applied until failure with a 1000 g load cell.

There was a significant increase in average elastic modulus between day 1 and day 7 for the tubes stored in 100 mM CaCl$_2$ in DI water, increasing by a factor of 5. This increase in elastic
modulus was maintained at the 14 day mark, indicating that crosslinking is complete and the gels are saturated with Ca\(^{2+}\) ions. The tubes stored in DI water only did not experience similar crosslinker saturation, with no significant increase in elastic modulus over 14 days. This data reflects the elastic modulus over time at a single crosslinker concentration, but previous elastic modulus studies done on 2% alginate sheets [47] showed that tensile strength was also proportional to the concentration of crosslinker i.e. the modulus of elasticity of 2% alginate tubes crosslinked in 50 mM CaCl\(_2\) is lower than that crosslinked in 100 mM CaCl\(_2\). The elastic modulus of tubes on day 1 is not significantly different than those of planar sheets of the same composition (Figure 5b), which suggests that the elastic modulus is independent of hydrogel geometry for homogeneous materials.

3.2.2 UV-Visible Light Absorbance Spectra of Alginate

The absorption of light by alginate in the visible range, from approximately 400 nm to 800 nm, is an important factor when imaging fluorescent beads and dyes inside the crosslinked alginate matrix and flowing through cannulated tube segments. The absorption in the visible range of uncrosslinked alginate, alginate crosslinked for 1 hour and alginate crosslinked for 24 hours was measured using a Cary 50 Bio NIR Spectrophotometer (Agilent, Santa Clara, CA). Samples of crosslinked alginate were prepared by mixing 2 mL of uncrosslinked 2% alginate (\(\rho=1169.4\) kg/m\(^3\)) with 500 μl of 100 mM CaCl\(_2\) in DI (\(\rho=1169.4\) kg/m\(^3\)) inside cuvettes and crosslinking for 1 hour and 24 hours. The scanning baseline was set using DI water. Absorption curves of uncrosslinked and crosslinked alginate from 300 nm-1100 nm are shown in Figure 6.
Figure 6. UV-Vis absorbance spectra of crosslinked and uncrosslinked alginate: Absorbance of uncrosslinked 2% alginate (short pink dashed line), 1 hour crosslinked 2% alginate (solid blue line), and 24 hour crosslinked alginate (long red dashed line) measured between 300 nm and 1100 nm.

Imaging fluorescent beads flowing through tubes or encapsulated in the matrix occurs in the visible spectrum, typically with nile red beads which have an excitation/emission wavelength of 535/575 nm. There are no absorbance peaks in the visible range, and for tubes crosslinked for 24 hours or more, the absorbance is approximately 0.1 AU at this wavelength. Applying the Beer-Lambert law in a liquid medium:

\[ T = \frac{I}{I_0} \quad \text{and} \quad A = -\log_{10}\left(\frac{I}{I_0}\right) \]

where T is transmittance of light, I is measured intensity (W/cm²), \( I_0 \) is original light intensity (W/cm²), and A is the absorbance (AU). With a measured absorbance of 0.1 AU, the percent transmission is 79.4%. Though the transmission at near UV is much lower, at critical wavelengths for fluorescent and brightfield imaging there is not significant absorbance or reduction of transmission through the fully-crosslinked alginate.

3.2.3 Microstructure of Crosslinked Alginate

The microstructure of homogeneous alginate and alginate-pectin composite gels was visualized using scanning electron microscopy (SEM). The porosity or void fraction of the gels affects the diffusivity and tensile mechanical properties of the bulk hydrogel. Planar samples were prepared
for imaging using devices and methods previously described [47], and then gel samples were processed to render the outer surface of the gels electrically conductive. Gel samples were fixed in 2% glutaraldehyde in a 0.05M sodium cacodylate buffer at pH 7.4 for 1 hour at room temperature, followed by gradual replacement of the liquid phase with 100% ethanol. Samples were dehydrated with liquid CO$_2$ at 10°C in a critical point dryer. Samples were then heated to a supercritical temperature and pressure, 31°C at 7.2 MPa. Decreasing the pressure at a constant temperature directly transitions the liquid to gas phase, without unwanted liquid-gas phase transition interactions. The dehydrated sample was then transferred into a vacuum chamber and vapour-deposited with a thin film of gold to render the outer surface of the gel electrically conductive for imaging.

Figure 7. SEM Images of 1% alginate, 2% alginate, and alginate-pectin gel microstructure: (a) Cross-section of 1% alginate gel, scale bar 2 µm. (b) Cross-section of 2% alginate gel, scale bar 5 µm. (c) Cross-section of 0.5% alginate-0.25% pectin gel, scale bar 2 µm. (d) Cross-section of 0.75% alginate-0.75% pectin gel, scale bar 2 µm.
The porosity of homogeneous alginate gels and alginate-pectin composite gels can be seen in Figure 7. As expected, increasing the weight percent of alginate in homogenous gels increased the mesh density (e.g. 1% alginate vs. 2% alginate, Figure 7a vs. Figure 7b). This increase in mesh density directly relates to the decrease in diffusivity of different species through the matrix material [47]. The addition of pectin, a plant polysaccharide with the same gelation mechanism as alginate, greatly increases the mesh density at relatively low concentrations of alginate and pectin (Figure 7c and Figure 7d). The addition of low weight percent pectin and the increase in alginate concentration also greatly increases the measured tensile strength of the gels (Section 3.2.1 and [47]), suggesting that the relative mesh density is directly related to the tensile strength of the gel. Though the addition of secondary biopolymers (i.e. pectin) or other additives have been demonstrated to modify the resultant hydrogel and tube properties, for the majority of this work 2% alginate is used for experiments to validate the on-chip extrusion method.

4 Characterization of Formation of Homogeneous Soft Material Tubes

The final geometry of extruded tubes depends on many factors, including: the relative flow rates of all coaxial flows, the viscosity of the working fluids, the diameter of the extrusion hole, and the presence or absence of confinement during polymerization. To determine the relative importance of these factors, numerical and analytical models were developed to predict tube formation and compared to experimental data.

4.1 Alginate Crosslinking Kinetics

Macroscopically homogeneous or inhomogeneous alginate gels are formed by the internal or external addition of calcium ions, respectively [49]. The internal addition of a calcium ion source reduces the crosslinking rate and produces a more homogenous gel because there is no large concentration gradient driving the crosslinking process, which creates a gradient of polymer crosslink density in the gel from the outside surface towards the gel centre [50]. Here, we use an external crosslinking process during the extrusion process; at the moment of tube formation, the inner and outer tube surface is instantly crosslinked and then further diffusive flux of calcium ions from the reservoir solution into the gel completes crosslinking. The maximum growth rate of the alginate gel crosslink density occurs within the first 15 minutes of crosslinking [33]. Crosslinking continues inside the gel until either the ion source is or the uncrosslinked positions
in the gel are depleted. Due to the stabilizing instantaneous crosslinking at the inner and outer surfaces, extruded tubes can be cut and handled after approximately one to two minutes of incubation in crosslinking solution. Generally, increasing the alginate concentration will increase the time required for crosslinking due to the increased number of binding sites in the same volume of gel and increasing the concentration of calcium ions in solution will increase the initial crosslinking rate due to the larger ionic gradient.

Since the binding kinetics of calcium and alginate are so rapid compared to the diffusion of calcium, the diffusion of calcium ions is the rate-limiting step in the crosslinking process. The diffusivity of calcium ions in porous gels and alginate beads is generally considered to be the same as that in water, with a rate reduction of no more than 10% [50]. This is generally applicable to any molecule, where no significant diffusive resistance is present if the molecular weight is less than 20000 Da [51]. In the case of calcium ions, the molecular weight of calcium chloride is 110.98 Da, which is one order of magnitude larger than that of water (18 Da) and well below the size-based diffusive limit.

The diffusive flux of calcium ions through the tube wall can be calculated using Fick’s first law, which describes the diffusive flux of a species due to a concentration gradient from regions of high concentration to low concentration under steady state conditions. In molar form, the law is written as

\[ J_{Ca} = -D_{Ca} \nabla C_{Ca}, \]

where \( J_{Ca} \) is the diffusive molar flux of calcium ions in kmol/s⋅m\(^2\), \( C \) is the total molar concentration of calcium ions and alginate in kmol/m\(^3\), \( D_{Ca} \) is the mass diffusivity constant of calcium ions in alginate gel in m\(^2\)/s, and \( \nabla C_{Ca} \) is the gradient in the calcium mole fraction. Using Fick’s law, the diffusive molar flux of calcium ions through the alginate gel was calculated to be \(-5\times10^{-7}\) kmol/s⋅m\(^2\) using an experimentally-determined diffusivity value for calcium ions in water at room temperature, \( D_{Ca}=1\times10^{-9}\) m\(^2\)/s [50], and assuming: a calcium ion reservoir with a volume of 2L and a concentration of 100 mM CaCl\(_2\), a 20 cm long tube segment with a wall thickness of 100 \( \mu \)m and outer diameter of 1 mm, an alginate concentration of 2% w/w in 50 mL, and an average alginate molecular weight of 176.12 g/mol.
4.2 Stability of Coaxial Flow Conditions during Extrusion

When interfaces of different fluids are present in immiscible coaxial pipe flows, the difference in viscosity between layers is the most important factor which decreases flow stability compared with other effects such as surface tension or density stratification. Classical studies of coaxial flows in fluid mechanics have proven that in the case of viscosity stratification between fluids, the flow is always unstable to small perturbations which grow with time at an exponential rate and regardless of the Reynolds number [52]. Specifically considering the case of two immiscible fluids with different viscosities but equal density throughout a pipe; the configuration with the thin fluid at the core is always unstable, and the stability of the thick fluid in the core depends on the ratio of radii of the inner and outer fluid regions (stability if $\frac{R_1}{R_2} \geq 0.7$ and $\frac{\mu_1}{\mu_2} \geq 1$) [53]. The instability that occurs due to viscosity stratification in parallel flows is termed the Kelvin-Helmholtz instability. It occurs due to the buildup of kinetic energy generated by the relative motion between fluid layers, and is unaffected by the magnitude of the velocity difference between layers. Well-documented analytical solutions determine the criteria for stability or instability across viscosity stratifications in fluids [54].

Considering the coaxial flows generated during the extrusion process, the experimental setup here appears to be unstable for all extrusion cases due to the presence of viscosity stratification and the unstable configuration of low viscosity core and outer fluid with an intermediate high viscosity fluid layer. However, extrusion is possible in a known operating range and stable flow has been observed on the scale of hours. The increased stability that allows the coaxial flow to overcome the Kelvin-Helmholtz instability and also the highly unstable coaxial configuration is created by the instant crosslinking that occurs at the fluid interfaces. After surface crosslinking, the interface is no longer that of two fluids with different viscosities, but rather a pseudo-solid traveling through a low viscosity fluid which dampens the waves induced by the instability.

4.3 Numerical Modeling of Tube Formation

A numerical simulation of the flow profile inside a three-layer device was performed in Comsol Multiphysics 4.1 assuming no crosslinking and shown in Figure 8a. The geometry, boundary conditions, and validation of mesh independence are shown in Appendix C.i. Numerical modeling and streamline overlays inside the device validated several experimental observations;
• Re-circulation occurs between the top of the inner streaming inlet and the roof of the device, often experimentally observed as bubbles being trapped.

• The formation of a void space inside the tube is reflected in the velocity surface map, where the peak fluid velocity in the intersection region of all three streams is observed with the closest proximity to the tube centerline.

Two additional numerical simulations were performed in COMSOL Multiphysics 4.1 to estimate the effect of confinement on tube formation, shown in Figure 8b-c. Without confinement, tubes can be extruded directly into the reservoir but this process was observed to be much less stable than confined extrusion and tube geometry was unpredictable. The geometry, boundary conditions, and validation of mesh independence for these models are shown in Appendix C.ii and C.iii. Numerical modeling and streamline overlays demonstrate the focusing effect of confinement, and the loss of focusing and large recirculation of fluid when extruding into an open reservoir. This confirms the experimental observation that the presence of confinement during extrusion increases the flow focusing effect of the inner and outer streaming. These models also suggest that a smaller confinement would increase the hydrodynamic flow focusing effect even further, which could extend the range of tube geometries possible from a given device.
Figure 8. COMSOL Numerical Modeling Results: (a) Velocity map with streamline overlay of the flow profile inside a three-layer device. Inner streaming (IS), matrix (M), and outer streaming (OS) channel inlets indicated. (b) Velocity map with streamline overlay of the flow profile outside of an extruded alginate tube inside a confining tube, wall of alginate tube shown in black. (c) Velocity map with streamline overlay of the flow profile outside of an extruded alginate tube without a confining tube, wall of alginate tube shown in black.
4.4 Experimental Characterization and Prediction of Tube Geometry with an Analytical Model

Using the presented platform, homogeneous and heterogeneous tubes of varying composition, diameter, wall thickness and length were routinely produced. Dynamic control of the relative flow rates of the inner core flow \( Q_C \), the biopolymer flow rate \( Q_M \), and the outer focusing flow \( Q_F \) allows for the dynamic control of the tube diameter \( D \) and wall thickness \( t \), by altering the pressure drop across the tube wall during formation. Another important factor which affects tube formation is the diameter of the extrusion hole that is punched during fabrication. After extrusion, geometry was characterized by measuring wall thickness and outer diameter of wet tube segments in a focusing liquid-filled, glass-bottom Petri dish. Depending on the size of the tube outer diameter, photographs were taken either with a stereomicroscope or bright-field images with an inverted fluorescent microscope. Quantitative measurements were taken from these photographs using ImageJ, and compared to results predicted from an analytical model.

4.4.1 Model Parameters

An analytical model was derived from a force balance of the axisymmetric annular flow inside the microfluidic device. It considers three coaxial layered fluid streams with a varying change in composition in the radial direction, passing through a cylindrical flow conduit. The cross sectional geometry in proximity to the outflow hole is illustrated in Figure 9. We assume fully developed viscous flow with a constant streamwise pressure gradient. The subscripts c, m, and f denote core flow, matrix flow, and outer focusing flow respectively. This flow satisfies the following equations:

\[
- \frac{dP}{dz} + \rho_c g + \mu_c \frac{1}{r} \frac{d}{dr} \left( r \frac{dV_{z,c}}{dr} \right) = 0 \quad [1]
\]

\[
- \frac{dP}{dz} + \rho_m g + \mu_m \frac{1}{r} \frac{d}{dr} \left( r \frac{dV_{z,m}}{dr} \right) = 0 \quad [2]
\]

\[
- \frac{dP}{dz} + \rho_f g + \mu_f \frac{1}{r} \frac{d}{dr} \left( r \frac{dV_{z,f}}{dr} \right) = 0 \quad [3]
\]
with the boundary conditions:

\[ \frac{dV_{Z,C}}{dr}(r) = 0 \]  

\[ \mu_c \frac{dV_{Z,C}}{dr}(\delta_1) = \mu_m \frac{dV_{Z,M}}{dr}(\delta_1) \]  

\[ V_{Z,C}(\delta_1) = V_{Z,M}(\delta_1) \]  

\[ \mu_m \frac{dV_{Z,M}}{dr}(\delta_2) = \mu_f \frac{dV_{Z,F}}{dr}(\delta_2) \]  

\[ V_{Z,M}(\delta_2) = V_{Z,F}(\delta_2) \]  

\[ V_{Z,F}(R) = 0 \]  

Where symbols \( P, \rho, g, \mu, r, V_Z, \delta_1, \delta_2 \) represent pressure, density, gravitational acceleration, viscosity, radial position, axial velocity, radial position of first interface, and radial position of second interface. Using equations [1-3], analytical solutions for \( Q_C, Q_M, \) and \( Q_F \) were obtained (equations [10-12]) which predict the value of the \( \delta_1 \) and \( \delta_2 \) interfaces, as well as the pressure drop, \( \frac{dP}{dz} \) as a function of \( Q_M \) and \( Q_F \) (see Appendix B for derivation):

\[ Q_C = \frac{\pi A}{2} \left( \frac{-\delta_1^4 + \delta_1^2 - \delta_2^2 \delta_2^2}{2\mu_m} + \frac{\delta_2^2 \delta_1^2 - R^2 \delta_2^2}{2\mu_f} \right) \]  

\[ Q_M = \frac{\pi A}{2} \left( -\delta_1^4 + \delta_2^2 \left( \frac{\mu_f \delta_2^2 - \mu_m \delta_2^2 + R^2 \mu_m}{2\mu_f \mu_m} \right) + \frac{-\delta_2^2 \mu_f + 2\mu_m (\delta_2^4 - \delta_2^2 R^2)}{4\mu_f \mu_m} \right) \]  

\[ Q_F = \frac{\pi A}{8\mu_f} \left( -R^4 + 2R^2\delta_2^2 - \delta_2^4 \right) \]
Figure 9. Analytical Model Parameters: (a) Typical device geometry in the extrusion outlet. (b) Left: Schematic of analytical model parameters. Right: Normalized velocity profile from centerline to extrusion outer radius, calculated from analytical model.

The normalized flow profile (shown in Figure 9b) shows the relative velocities of inner streaming, matrix, and outer steaming in a three layer coaxial flow. A comparison of predicted values and experimentally determined wall thickness and outer diameter for two size ranges is shown in Figure 10, accompanied by a visual comparison of the size of tube produced from devices with different extrusion hole diameters. The predicted wall thickness and outer diameter display the same trend as the experimentally determined values but have uniformly different magnitudes. The difference in magnitude can be attributed to the effects of polymerization during extrusion; polymerization begins as soon as the focusing and biopolymer matrix streams
meet inside the device which creates a gradient of stiffness in the tube inside the device during extrusion as well as in time. This increasing material stiffness decreases the effect of focusing on the wall thickness of the tube, which leads to a predicted wall thickness that is lower than the actual wall thickness. It also reduces the focusing effects of both the inner and outer streaming, leading to a different tube diameter. The model considers an ideal entrance length such that there is fully developed flow as the coaxial flows meet. However, as demonstrated with the numerical models, the flow is not fully developed inside the device as it begins to crosslink. The model also considers all the fluids to be non-polymerizing Newtonian fluids, but uncrosslinked alginate is non-Newtonian and shear thinning, which also contributes to the difference in magnitude.

The analytical model also assumes perfect coaxial symmetry, but in reality there is the possibility of misalignment between layers during device fabrication. The alignment of each layer of the device with all other layers has an effect on the wall thickness of the extruded tubes, as misalignment near the extrusion channels leads to the outlet on one or more layers to shift with respect to the extrusion hole punching location. This changes the resistance of one side of a layer compared to the other side of the extrusion hole, as the length of channel that gets “gained” or “lost” is the smallest width (and therefore the highest resistance). When this happens, tubes with uneven wall thickness are produced. To minimize this effect, alignment guides for each layer should be used and a guide for the hole punch can be used to ensure completely vertical punching.
Figure 10. Experimental and Model Results: (a) Experimental tube data and predicted data from analytical model with an extrusion radius of 1.59 mm. Actual tube outer diameter (red circular symbols) and wall thickness (blue square symbols) as a function of streaming flow rate with \( Q_M = 190 \mu l/min \). Error bars indicate standard deviation, 10 measurements in center plane per tube for \( n=5 \) tubes, plotted with line of best fit. Analytical model predictions shown in black. (b) Comparison of the outer diameter of tubes from (a) and (c), with the diameter of the extrusion hole indicated in the figure. Scale bar 2.5 mm. (c) Experimental tube data and predicted data from analytical model with an extrusion radius of 1.34 mm. Predicted tube outer diameter (red circular symbols) and wall thickness (blue square symbols) with \( Q_M = 190 \mu l/min \). Error bars indicate standard deviation, 10 measurements in center plane per tube for \( n=5 \) tubes plotted with line of best fit. Analytical model predictions shown in black.
4.4.2 Parametric Studies

The analytical model was used to determine the effect and importance of different parameters on the wall thickness and inner or outer diameter of extruded tubes. The effect of alginate viscosity was determined by comparing the outer diameter and wall thickness at the same streaming and matrix conditions as shown in Figure 11a and Figure 11c. For both the wall thickness and diameter, the most pronounced effects are seen at the lower end of the viscosity range (see Figure 11b and Figure 11d). Above a matrix viscosity of 0.25 Pa-s, the outer diameter varies by 10 µm or less in a range of 40 µm. Similarly, above a matrix viscosity of 0.25 Pa-s, the wall thickness varies by 20 µm or less in a range of 120 µm. The effect of viscosity on wall thickness and outer diameter is relevant because of the non-Newtonian and shear-thinning behavior of alginate. Considering an extrusion velocity \( \leq 10 \text{ mm/s} \), the range in viscosity for 2% alginate with a density of 1196 kg/m\(^3\) is significant (see Figure 4). In this range, changing the uncrosslinked biopolymer flow rate can have a significant effect on the wall thickness and diameter of the extruded tube.

The effect of the extrusion hole diameter on the geometry of extruded tubes was investigated in a similar way. The extrusion radius was varied from 0.25 mm - 2 mm at a constant set of flow rates \((Q_{M}=210 \mu l/min, Q_{C}=Q_{F}=0.25 \text{ ml/min}-2 \text{ ml/min})\). As shown in Figure 12b and Figure 12d, at a constant flow rate the outer diameter and wall thickness scales linearly with increasing extrusion hole size. However, considering a range of flow rates we can observe that at inner and outer streaming flow rates of less than 0.25 ml/min, the relationship is non-linear (Figure 12a). This is likely due to the decreased effect of fluid focusing at lower streaming flow rates. Above 0.25 ml/min, the outer diameter is almost constant as a function of extrusion hole radius, suggesting that the outer diameter of the tube is predominately a function of extrusion radius. Considering the same set of parameters, the wall thickness is highly variable throughout the entire range of inner and outer streaming flow rates (Figure 12c). At a single extrusion radius value, the wall thickness can vary from 100 µm-600 µm depending on inner and outer focusing flow rate. This demonstrates the effects of flow focusing on tube wall thickness; the maximum tube wall thickness here is found at the lowest focusing flow rates and in the largest extrusion hole. As extrusion hole diameter decreases and flow rate increases, the wall thickness uniformly
decreases. At high inner and outer streaming flow rates $\geq 1.5$ mm, the tube wall thickness reaches a terminal value that is constant between all extrusion hole sizes $\pm 100 \mu$m.

Figure 11. Effect of Viscosity on Extruded Tube Geometry. (a) The effect of variable biopolymer matrix viscosity on tube outer diameter at fixed flow rates ($Q_U = 210 \ \mu$l/min, $Q_C = Q_F = 0.75$ ml/min-1.25 ml/min) with fixed inner and outer streaming viscosity of 0.05 Pa-s. Matrix viscosity varied between 0.05 Pa-s-0.7 Pa-s (arrow indicates direction). (b) The effect of matrix viscosity on tube outer diameter at $Q_C = Q_F = 0.75$ ml/min. (c) The effect of variable biopolymer matrix viscosity on tube wall thickness at fixed flow rates ($Q_U = 210 \ \mu$l/min, $Q_C = Q_F = 0.75$ ml/min-1.25 ml/min) with fixed inner and outer streaming viscosity of 0.05 Pa-s. Matrix viscosity varied between 0.05 Pa-s-0.7 Pa-s (arrow indicates direction). (d) The effect of matrix viscosity on tube wall thickness at $Q_C = Q_F = 0.75$ ml/min. (a), (b), (c), (d) are calculated from analytical model.
Figure 12. Effect of Device Extrusion Hole Radius on Extruded Tube Geometry. (a) The effect of variable extrusion hole radius on tube outer diameter at fixed flow rates ($Q_M=210$ µl/min, $Q_c=Q_f=0.25$ ml/min-2 ml/min). Hole radius varied between 0.25 mm- 2 mm (arrow indicates direction). (b) The effect of extrusion hole radius on tube outer diameter at $Q_c=Q_f=0.25$ ml/min. (c) The effect of variable extrusion hole radius on tube wall thickness at fixed flow rates ($Q_M=210$ µl/min, $Q_c=Q_f=0.25$ ml/min-2 ml/min). Hole radius varied between 0.25 mm- 2 mm (arrow indicates direction). (d) The effect of extrusion hole radius on tube wall thickness at $Q_c=Q_f=0.25$ ml/min. (a), (b), (c), (d) are calculated from analytical model.

5 Tube Perfusion

Once extruded, both homogeneous and heterogeneous tubes can be readily perfused off-chip by cannulation or on-chip in a specially designed and reversibly sealing microfluidic device. Using this device, tubes can be perfused and superfused in isolation, providing local microenvironmental control. The ability to perfuse and pressurize hydrogel tubes is important to isolate and deduce mechanical properties in the circumferential direction, as well as locally control the dissolved gas and fluid composition.
5.1 Tube Perfusion Off-Chip

The conventional macro-scale technique to perfuse vessels or tubular constructs is cannulation, where glass capillaries are placed inside both ends of the hydrogel tube and tied shut with suture wire or otherwise sealed. This provides a fluidic connection inside the tube and allows for gentle pressurization. Using a modification of this technique, 2% alginate tubes were cannulated and perfused with fluorescent dye either with syringe-driven flow or hydrostatic head. Cannulation was routinely performed on hydrogel tubes with 360 µm OD silicon capillaries (Polymicro Technologies, Phoenix, AZ) permanently bonded inside PEEK tubes with epoxy. The PEEK tubes could then be connected to standard fluidic connections for hydrogel tube perfusion. As an alternative to cannulation with suture wires, which can quickly cut through hydrogels while being tied, the cannulated hydrogel tubes are permanently bonded to the capillaries using Loctite 4541 adhesive. Using this technique, 2% alginate tubes suspended in DI water and were perfused with DI water by syringe pump at 1-2 ml/hr and hydrostatic head at 0-60 mmHg. This is illustrated in Figure 13a. Figure 13b shows a fluorescent image of a 2% alginate tube segment that has been cannulated and perfused with 1 mM fluorescein dye. Brightness in the tube walls is due to the rapid diffusion of fluorescein through the walls during perfusion; the molecular weight of fluorescein is 376 Da, which is comparable to the molecular weight of water (18 Da).

5.2 Tube Perfusion On-Chip

Though it was possible to routinely perfuse cannulated tubes as described above, it is difficult to pressurize cannulated tubes due to the adhesive bond between the tube and capillary. This technique is also not suitable for long-term experiments and does not offer microenvironmental control. Similar to a previously developed artery hosting microfluidic device; a reversibly sealing microfluidic device was designed to host tube segments and reliably perfuse and superfuse in isolation, as well as pressurize the tubes in a non-destructive manner (shown in Figure 13c-f). The devices were micro-milled in cyclic olefin polymer (COP), a clear plastic with excellent solvent resistance and optical properties in the near UV range. These devices were designed to accommodate a range of tube outer diameters and reversibly seal against a glass slide for imaging with a selectively applied vacuum. A ridge of 500 µm separates fluid lines from the vacuum region, which prevents crosstalk and leak between channels and into the vacuum lines up to -25 psi of vacuum. To enhance sealing and minimize the effects of milling defects on the
sealing surface (see Appendix D), 2” x 3” glass slides were coated with a layer 500 µm thick of soft PDMS mixed to a ratio of 1:20 (crosslinker:pre-polymer). This layer provides a robust and highly compressible surface to seal against in the case of surface defects and roughness and creates a leak-free seal at 8 psi. An alternate design is shown in Figure 13f, which uses a single vacuum source to apply fixation vacuum to the tube as well as seal the device.

Fluidic connections are made on-chip with PEEK tubes, which are permanently bonded to the inlets and outlets of each channel and can be interfaced with standard fluidic connections. Prior to use, all channels were flushed with DI water to remove bubbles. To minimize the leak of fluids from the superfusion and perfusion to the fixation lines, tube segments were cut to a length of 1 cm (the distance from outer fixation fork to outer fixation fork). Tubes were placed into the liquid-filled well and aligned with the fixation forks before sealing the device with a PDMS coated glass slide. The thin layer of PDMS acts as a gasket and allows for a robust seal. Vacuum pressure to seal the device is produced by a micro diaphragm pump (Parker Hannifin, Milton, ON), which is separated from the device by a liquid trap and a 0.2 µm filter to prevent damage to the pump. Fixation on chip, which holds the tube open during active perfusion and superfusion, is achieved with fixation forks at the top and bottom of each end of the tube. These fixation lines are liquid-filled and connected to reservoirs at 45 mmHg below atmospheric pressure. The quality of fixation depends on the pressure applied and the size of the tube; a more robust seal is achieved when the tube outer diameter closely matches or is larger than the channel width. Superfusing flow was maintained at 2-10 ml/hr with syringe pumps, and perfusing flow was either supplied by syringe pumps or hydrostatic head at 2-5 ml/hr or 60 mmHg, respectively. Tube segments were individually perfused (Figure 13h) and superfused (Figure 13i) with fluorescent beads suspended in DI water, to demonstrate spatiotemporal control around the tube microenvironment. When perfusing tube segments with hydrostatic head, pressurization can be noted; this indicates an acceptable seal at the tube fixation locations, shown in Figure 13g. Tubes were successfully perfused and superfused for up to an hour, with failure caused by the introduction of bubbles into the device.
Figure 13. Tube Perfusion Off-Chip and On-Chip (a) Schematic of tube perfusion with defined hydrostatic head on one side of a cannulated, liquid-immersed tube and outlet to atmospheric pressure. (b) Fluorescent image showing perfusion of cannulated tube segment with 1mM fluorescein dye. Scale bar 100 μm. (c) Schematic of tube hosting device showing; (1) vacuum inlets, (2) fixation lines, (3) superfusion lines, (4) perfusion lines. Scale bar 5 mm. (d) Photograph of milled thermoplastic device for tube hosting. Scale bar 1.5 mm. (e) 3-D rendering of tube hosting device with isolated fixation and vacuum channels. Scale bar 5 mm. (f) 3-D rendering of tube hosting device with connected fixation and vacuum channels. Scale bar 5 mm. (g) Photograph of a device-hosted 2% alginate tube during active perfusion of DI water from a hydrostatic reservoir at 60 mmHg and a superfusion flow rate of 2 ml/hr. Scale bar 600 μm. (h) Photograph of a device-hosted 2% alginate tube during active perfusion of 2.5% v/v 1μm fluorescent beads from a hydrostatic reservoir at 60 mmHg and a superfusion flow rate of 2 ml/hr. Scale bar 600 μm. (i) Photograph of a device-hosted 2% alginate tube during active superfusion of 2.5% v/v 1μm fluorescent beads at a superfusion flow rate of 2 ml/hr and perfusion from a hydrostatic reservoir at 60 mmHg. Scale bar 600 μm.
6  Formation of Heterogeneous Soft Material Tubes

3-D heterogeneous soft materials with controllably non-uniform cross-section and properties can be very powerful experimental tools, allowing customized material properties through the continuous material and time. Using the same basic vertical arrangement of layers as shown in Section 2.3, heterogeneous tubes can be produced in a flow-focusing format by repeating or modifying the biopolymer distribution layers.

Using the basic vertical extrusion design principle with two identical biopolymer layers stacked in between the inner and outer streaming layers (see Figure 14a), the continuous formation of bilayered tubes was demonstrated. Using a total matrix flow rate of 200 µl/min and inner and outer streaming flow rates of 0.5 ml/min to obtain comparable layer thicknesses as homogeneous tubes, 2% alginate bilayered tubes were created with uniform and densely crosslinked connections between layers. The result is a single continuous alginate material with two distinct coaxial regions, demonstrated by the inclusion of distinct fluorescent beads in each region of the matrix. By further increasing the number of biopolymer layers, we predict that the in-flow creation of \( n \)-layered tubes is possible with up to \( n \)-compatible matrix materials. Here, compatibility means similarity in crosslinking mechanism, but further non-compatible matrix materials can be included if alginate is used as a rapidly-crosslinking sheath [58] prior to further time- or temperature-dependent crosslinking processes. This could be used to extrude continuous tubes of complex biomaterials with sacrificial alginate layers, which could prevent diffusion of other materials before crosslinking is complete.

Similarly, using a modified biopolymer layer with two equally distributed inlets; the continuous formation of Janus tubes was demonstrated (see Figure 14b). A total matrix flow rate of 200 µl/min and inner and outer streaming flow rates of 0.5 ml/min were used to extrude continuous tubes with different materials in each half of the tube cross-section. As a case study, 2% alginate with two distinct fluorescent beads was used to demonstrate the ability to form different regions in the tube cross-section. These formed uniform and densely crosslinked connections between regions, creating a continuous material with well-defined regions across the cross-section. This can be extended to extrude tubes with 1, 2…\( n \) compatible materials across the tube cross-section by increasing the number of inlets to \( n \) equally distributed inlets.
Figure 14. Heterogeneous Tubes: (a) Left: confocal image of bilayered tube, scale bar 100 µm. Right: matrix layers to produce homogeneous bilayer tube, shown in inset. (b) Left: confocal image showing cross-section of Janus tube at region where two materials meet, scale bar 100 µm. Right: matrix layer to produce Janus tube, shown in inset. (c) Left: confocal image of patterned bilayered tube, scale bar 100 µm. Right: matrix layer and distribution layer to produce spotted or striped tube, shown in inset.

The techniques to form bilayer and Janus tubes can be combined to form complex patterned bilayer tubes (see Figure 14c). This is achieved by stacking single inlet biopolymer distribution layers with multiple inlet distribution layers, which greatly increases the complexity possible in a continuous format in the extruded tubes. An extension of this technology is the capability to create discrete spots or patterns within a continuously extruded hydrogel matrix. Using modified
biopolymer distribution layers with computer-controlled pneumatic valves that control the pressure head in on-chip reservoirs, series of discrete spots can be predictably incorporated around the circumference of the continuously extruded tube and along its length. The on-chip reservoirs are connected to uniformly distributed 300 μm-wide spotting channels around the circumference of the extrusion hole, a device with three spotting locations is shown in Figure 15. During extrusion of the continuous matrix phase and the inner and outer streaming, these reservoirs are transiently pressurized to initiate outflow and create spots that are incorporated within the continuous tube matrix using a custom Labview computer interface. The head pressure in the on-chip reservoirs is controlled by a series of solenoid valves, which are opened or closed to apply or remove the pressure head.

The size of the spots is affected by the open time of the valve and the head pressure that is applied, and the gap between continuous spots is controlled by the closed time of the valve. If a valve is continuously kept open, a stripe pattern can be produced with any length. As a demonstration of this capability, 0.75% w/w alginate solution was prepared and used as the secondary biopolymer to be spotted in a continuous matrix of 2% alginate. Inner and outer streaming flow rates were set to a constant rate of 0.5 ml/min, and the continuous matrix was set to a constant rate of 180 μl/min. Two pressurized reservoirs were controllably pressurized to 1 psi to form spots concurrently, to form offset spots, and to form spot and stripe patterns. With a head pressure of 1 psi and δt₀=100 ms, the average spot length was 1080 μm. With a head pressure of 1 psi and δt₀=200 ms, the average spot length was 1550 μm. When the valve opening time was increased to 500 ms, the average spot length was increased to 2950 μm. Confocal imaging confirmed that the spots penetrated the entire volume of the continuous matrix. The spot length does not linearly relate to valve open time because multiple factors influence the spot length, including the microchannel resistance and the magnitude of the pressure head.
Figure 15. **Spot Patterns and Valve Control.** (a) Photograph of microfluidic device to create discrete spots and patterns in continuously extruded tubes. The secondary spotting material is stored in the on-chip reservoirs, which are actuated by computer-controlled solenoid valves which control the pressure inside the wells. Inset: schematic showing valve actuation and mask design illustrating on-chip reservoirs and continuous matrix inlets. (b) Fluorescence image of tube with two actuated on-chip reservoirs, $dt_o=200$ ms, $dt_c=200$ ms. Outline of tube outer diameter shown in red. (c) Fluorescence image of tube with two actuated on-chip reservoirs, $dt_o=200$ ms, $dt_c=600$ ms. Outline of tube outer diameter shown in red. (d) Fluorescence image of tube with two offset actuated on-chip reservoirs, $dt_o=200$ ms, $dt_c=800$ ms. Outline of tube outer diameter shown in red. (e) Fluorescence image of tube with two actuated on-chip reservoirs, one open continuously to produce a stripe and the other producing spots with $dt_o=200$ ms, $dt_c=200$ ms. Outline of tube outer diameter shown in red. All scale bars 1.5 mm.
7 Conclusion

The work described in this thesis presents a platform to continuously extrude homogeneous and heterogeneous alginate hydrogel tubes. An in-flow vertical assembly approach was developed using a multilayered microfluidic device to distribute working fluids in plane around an extrusion hole. A multi-layered coaxial flow was developed inside the device that contains at a minimum, an inner focusing stream, a biopolymer matrix, and an outer focusing stream. After in-flow formation, the biopolymer matrix material was rapidly crosslinked through an ionic exchange mechanism. This device offers significant potential for scalability and the continuous production of predictable and geometrically defined 3D materials. The tube wall thickness and outer diameter are a function of the inner and outer focusing flow rates, as well as the matrix flow rate and the geometry of the constriction that they flow through during formation.

Using the same vertical extrusion principle, heterogeneous and multi-layered tubes can also be created in a continuous process. Demonstrated here are Janus tubes, bilayer tubes, and patterned bilayer tubes. Using an alternate device design with on-chip reservoirs, patterned tubes with discrete spots of a secondary biopolymer were demonstrated. The microstructure and material properties of the base biopolymer were characterized, and their effects on tube formation were quantitatively estimated using an analytical model. Using homogeneous 2% alginate tubes, the ability to perfuse and pressurize tube segments with cannulation and using a specially designed device were demonstrated. The 3-D hydrogels produce here demonstrated the ability to form continuous and spatially heterogeneous microstructured material with dynamically changing material and chemical composition.
8 Future Directions

In this thesis, the basic functionality of creating bilayer tubes and patterning hydrogel tubes was demonstrated in an acellular context. Future work to extend this patterning capability to pattern cells into continuous single and bilayer tubes could enable the creation of 3-D and organ scale living tissues with spatially-dependent properties and composition. Similarly, an exciting application to create cell-laden and physiologically relevant tubes is the creation of single-layered tubes from matrix materials with long crosslinking times (i.e. collagen, fibrinogen) using sacrificial rapidly-crosslinking inner and outer alginate layers.

Using a continuous vertical extrusion process, it is simple to create large volumes of soft material tubes in a short amount of time. Using continuous segments of tubes as a building block in a larger assembly could quickly create a large-scale bulk perfusable material. If a template is used to guide the tube location in plane, a moldable layer can be created by casting the tube and template in a secondary biopolymer. Stacking up and further casting of these layers could produce a centimeter-scale bulk material with spatially dependent properties and material composition.
9 References


Figure 16. **Mask designs:** Mask designs for inner streaming, outer streaming, and monolayer or multilayer matrix layers (a-h), Janus tube matrix layer (i), spotted or striped tubes matrix layer (j), and spot/stripe fluid distribution layer (k).
B. Derivation of Analytical Model

Assume axisymmetric annular flow that is laminar and fully developed with a constant streamwise pressure gradient. The subscripts c, m, and f denote core flow, matrix flow, and outer focusing flow respectively.

\[ -\frac{dP}{dz} + \rho_c g + \mu_c \frac{1}{r} \frac{d}{dr} \left( r \frac{dV_{z,c}}{dr} \right) = 0 \]  
\[ -\frac{dP}{dz} + \rho_m g + \mu_m \frac{1}{r} \frac{d}{dr} \left( r \frac{dV_{z,m}}{dr} \right) = 0 \]  
\[ -\frac{dP}{dz} + \rho_f g + \mu_f \frac{1}{r} \frac{d}{dr} \left( r \frac{dV_{z,f}}{dr} \right) = 0 \]

General solution for velocity of each layer [52]:

\[ V = \frac{1}{4\mu} \left( \frac{dP}{dz} - \rho g \right) r^2 + B \ln r + C \]  

Let \( A = \frac{dP}{dz} - \rho g \)

\[ V = \frac{A}{4\mu} r^2 + B \ln r + C \]  

\[ \frac{dV}{dr} = \frac{A}{2\mu} r + \frac{B}{r} \]

With boundary conditions [59]:
Where $P$, $\rho$, $g$, $\mu$, $r$, $V_z$, $\delta_1$, $\delta_2$ represent pressure, density, gravitational acceleration, viscosity, radial position, axial velocity, radial position of first interface, and radial position of second interface.

Applying boundary condition (7):

$$
\frac{dV_{z,c}}{dr}(0) = 0 \quad (7)
$$

$$
\mu_c \frac{dV_{z,c}}{dr}(\delta_1) = \mu_m \frac{dV_{z,m}}{dr}(\delta_1) \quad (8)
$$

$$
V_{z,c}(\delta_1) = V_{z,m}(\delta_1) \quad (9)
$$

$$
\mu_m \frac{dV_{z,m}}{dr}(\delta_2) = \mu_f \frac{dV_{z,f}}{dr}(\delta_2) \quad (10)
$$

$$
V_{z,m}(\delta_2) = V_{z,f}(\delta_2) \quad (11)
$$

$$
V_{z,f}(R) = 0 \quad (12)
$$

Substituting $B_c$ into boundary condition (8):

$$
\frac{dV_{z,c}}{dr}(0) = 0
$$

$$
\frac{1}{2\mu_c}(0) + \frac{B_c}{0} = 0
$$

$$
B_c = 0
$$
Substituting $B_M$ into boundary condition (10):

$$
\mu_M \frac{dV_{z,M}}{dr}(\delta_1) = \mu_F \frac{dV_{z,F}}{dr}(\delta_2)
$$

$$
\mu_M \left(\frac{A}{2\mu_M} \delta_2 + \frac{B_M}{\delta_2}\right) = \mu_F \left(\frac{A}{2\mu_F} \delta_2 + \frac{B_F}{\delta_2}\right)
$$

$$
\frac{A}{2} \delta_2 + \mu_M \frac{B_M}{\delta_2} = \frac{A}{2} \delta_2 + \mu_F \frac{B_F}{\delta_2}
$$

$B_F = 0$

Substituting $B_M$ and $B_C$ into boundary condition (9):

$$V_{z,C}(\delta_1) = V_{z,M}(\delta_1)$$

$$
\frac{A}{4\mu_C} \delta_1^2 + B_C \ln \delta_1 + C_C = \frac{A}{4\mu_M} \delta_1^2 + B_M \ln \delta_1 + C_M
$$

$$
\frac{A}{4\mu_C} \delta_1^2 + C_C = \frac{A}{4\mu_M} \delta_1^2 + C_M
$$

(9*)

Similarly, by substituting $B_M$ and $B_F$ into (11):

$$
\frac{A}{4\mu_M} \delta_2^2 + C_M = \frac{A}{4\mu_F} \delta_2^2 + C_F
$$

(11*)

Substituting $B_F$ into (12):
\[ V_{z,p}(R) = 0 \]
\[ \frac{A}{4\mu_F} R^2 + B_F \ln R + C_F = 0 \]
\[ C_F = -\frac{A}{4\mu_F} R^2 \]

Substituting \( C_F \) into (9*) and (11*), we obtain:
\[ C_M = \frac{A}{4} \left( \frac{\delta_2^2}{\mu_F} - \frac{R^2}{\mu_F} - \frac{\delta_2^2}{\mu_M} \right) \]
\[ C_C = \frac{A}{4} \left( \frac{\delta_1^2}{\mu_M} - \frac{\delta_1^2}{\mu_C} + \frac{\delta_2^2}{\mu_F} - \frac{R^2}{\mu_F} - \frac{\delta_2^2}{\mu_M} \right) \]

Substituting coefficients into (5), we obtain equations for the velocity in each fluid layer:
\[ \begin{align*}
Q_C &= \frac{\pi A}{2} \left( \frac{-\delta_1^4}{4\mu_C} + \frac{\delta_1^4 - \delta_2^2 \delta_1^2 + \delta_2^2 \delta_1^2 - R^2 \delta_2^2}{2\mu_M} \right) \\
Q_M &= \frac{\pi A}{2} \left( \frac{-\delta_1^4}{4\mu_M} + \frac{\mu_F \delta_2^2 - \mu_M \delta_2^2 + R^2 \mu_M}{2\mu_F \mu_M} \right) + \frac{-\delta_2^2 \mu_F + 2 \mu_M \left( \delta_2^2 - \delta_2^2 R^2 \right)}{4\mu_F \mu_M} \\
Q_F &= \frac{\pi A}{8\mu_F} \left( -R^4 + 2R^2 \delta_2^2 - \delta_2^4 \right)
\end{align*} \] (13) (14) (15)

These systems of equations were solved for various conditions in MATLAB.
C. COMSOL Numerical Modeling

i. Model 1: Flow inside a Three-Layer Device

A COMSOL numerical model was made to visualize the flow profile inside a three-layer device, as shown in Figure 17. The geometry of the model represents typical device measurements. All internal regions are connected with a continuity relation. Other than symmetry boundaries and inlets or outlets, all bounding edges are walls with a no slip assumption. Using the laminar flow module, the inner and outer streaming inlet boundary conditions were set to a laminar inflow of 0.5 ml/min, a density of 1169 kg/m$^3$, and a viscosity of 0.05 Pa-s. The matrix inlet was a laminar inflow of 120 µl/min with a density of 1169 kg/m$^3$ and a viscosity of 0.5 Pa-s. The entrance length for all inlets was 6.5 mm, to create a fully-developed flow. Region 1 was given a density of 1074 kg/m$^3$ and a viscosity of 0.05 Pa-s. A volume force of $-\rho g$ was added to regions 2, 3, and 4 to add the effect of gravity to the laminar flow model inside the device. The outlet boundary condition was a laminar outlet with a normal stress of 0 N/m$^2$. Table 2 shown below summarizes a mesh independence study for this model.

To further verify the results of the analytical model, a simulation was run with identical conditions using both the analytical and numerical model and compared. The velocity profile from the centerline to extrusion radius $R$ was plotted for the following conditions: $R = 3.175$ mm $Q_{IS} = Q_{OS} = 0.75$ ml/min, and $Q_M = 0.211$ ml/min. The results are in very good agreement and are shown in Figure 18.
Figure 17. COMSOL Model 1 schematic.

Table 2. Mesh independence study- COMSOL Model 1

<table>
<thead>
<tr>
<th>Property/Calculated Value</th>
<th>Mesh 1</th>
<th>Mesh 2</th>
<th>Percent Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mesh elements</td>
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<td>112 302</td>
<td>N/A</td>
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<td>Average velocity at outlet (mm/s)</td>
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<td>1.751</td>
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<td>Peak velocity at outlet (mm/s)</td>
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<td>Average shear rate at outlet (s(^{-1}))</td>
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Figure 18. Comparison of analytical model and numerical model results: Velocity profile from centerline to outer extrusion radius R as calculated by the analytical model (red solid line) and the COMSOL numerical model (blue dashed line) for $R = 3.175$ mm $Q_{ts} = Q_{os} = 0.75$ ml/min, and $Q_m = 0.211$ ml/min.

ii. Model 2: Flow outside an Extruded Tube with Confinement

A COMSOL numerical model was made to visualize the flow profile on the outside of an extruded alginate tube inside a confining tube, as shown in Figure 19. Region 1, representing the moving crosslinked alginate tube, was modeled as a moving wall with a defined velocity of 3 mm/s. Region 2 represents the reservoir fluid outside of the tube in the confinement. Using the laminar flow module, the inlet was set to a laminar inflow of 0.5 ml/min, a density of 1169 kg/m$^3$ and a viscosity of 0.05 Pa-s. Other than inlets or outlets, all bounding edges are walls with a no slip assumption. The outlet boundary condition was a laminar outlet with no viscous stress at 0 Pa. Table 3 shown below summarizes a mesh independence study for this model.
Table 3. Mesh independence study- COMSOL Model 2

<table>
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<td>Average velocity at outlet (mm/s)</td>
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<td>Peak velocity at outlet (mm/s)</td>
<td>2.67613</td>
<td>2.75201</td>
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<td>Average shear rate at outlet (s⁻¹)</td>
<td>2.69539</td>
<td>2.69444</td>
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</table>

Figure 19. COMSOL Model 2 schematic.
iii. Model 3: Flow outside an Extruded Tube without Confinement

A COMSOL numerical model was made to visualize the flow profile on the outside of an extruded alginate tube into an open reservoir, as shown in Figure 20. Region 1, representing the moving crosslinked alginate tube, was modeled as a moving wall with a defined velocity of 3 mm/s. Region 2 represents the outer streaming fluid flowing outside of the tube and into the reservoir. Region 3 represents the open reservoir with a density of 1077.4 kg/m$^3$. Using the laminar flow module, the inlet was set to a laminar inflow of 0.5 ml/min, a density of 1169 kg/m$^3$ and a viscosity of 0.05 Pa-s. Other than inlets or outlets and internal boundaries, all bounding edges are walls with a no slip assumption. The internal boundary between region 2 and 3 is joined by flow continuity. The outlet boundary condition was a laminar outlet with no viscous stress at 0 Pa. Table 4 shown below summarizes a mesh independence study for this model.

Figure 20. COMSOL Model 3 schematic.
Table 4. Mesh independence study- COMSOL Model 3

<table>
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</table>
D. Profilometer Measurement of Milling Defects

Figure 21. Profilometer Measurement of 1.5 mm Wide Device.

A 3 cm × 2 cm scan of the feature region of a 1.5 mm width device was performed with a Contour GT-K optical profilometer (Bruker, Tucson, AZ). Scan was performed in 200 µm × 200 µm regions and stitched together in post-processing with 10% overlap between regions and a scan depth of 1900 µm. The surface map is shown in Figure 21. Volume analysis was performed to calculate the net missing volume from the measured surface and a plane parallel to the reference plane of the surface which intersects with the maximum height of the surface. The net missing volume was calculated to be 177.513 mm³, which accounts for surface roughness as well as bowing and uneven milling depths. This is evident in the black regions on the surface map, where the depth is lower than the sampling range. Some sealing defects are evident on the sealing surface of the device, requiring the use of the PDMS as a gasket to seal against a glass slide.