Freestanding hydrogel lumens for biological assays

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Abstract

Lumen structures exist throughout the human body, and the vessels of the circulatory system are essential in carrying nutrients and oxygen and regulating inflammation. Vasodilation, the widening of the blood vessel lumen, is important to the immune response as it increases blood flow to a site of inflammation, raises local temperature, and enables optimal immune system function. A common method for studying vasodilation uses excised vessels from animals; major drawbacks include inconsistencies, time-consuming procedures, the need to sacrifice animals, and differences between model animal and human biology. We have developed a simple, user-friendly *in vitro* method to form freestanding cell-laden vessel-like structures from collagen and quantitatively measure the effects of vasodilators on the size of the structure. The hydrogel rings are composed of collagen I laden with human vascular smooth muscle cells, a major cellular and structural component of blood vessels; we also demonstrate the ability to line the lumen with endothelial cells. The methods presented include a 3D printed device (which is amenable to future fabrication by injection molding) and commercially available components (e.g., Teflon tubing or a syringe) used to form hydrogel rings between 2.6-4.6 mm outer diameter and 0.79-1.0 mm inner diameter. The rings enable the measurement of dynamic changes when vasodilators are added; here we demonstrated a significant difference in ring area in the presence of a known vasodilator, fasudil (p<0.0001). Our method is easy to implement and provides a medium-throughput solution to generating blood vessel-like model structures for future investigations of the fundamental mechanisms of vasodilation (e.g., studying uncharacterized endogenous molecules that may have vasoactivity) and testing vasoactive drugs.

Introduction

Lumen structures exist throughout the body including in the glandular organs such as the breast and prostate, and blood vessels of the circulatory system. The circulatory system is essential for regulating blood pressure and body temperature. Vasodilation, the widening of the blood vessel lumen, is important to the immune response as it increases blood flow to a site of inflammation, raises local temperature,

and enables optimal immune system function.[1] Over the last two decades, tissue engineers and cell biologists have been working towards performing cell culture experiments in a three-dimensional environment, as opposed to two-dimensional culture.[2-4] It is now well accepted that in the human body, cells are encapsulated in a 3D environment (extracellular matrix, ECM) and receive signals very differently than they would in a 2D polystyrene culture plate. Incorporation of relevant hydrogels, multi-culture of different cell types, or generation of specific architectures such as tubular structures have resulted in changes in cell behavior that more closely replicate *in vivo* cell function and morphology.[7,8] Such observations have been increasingly helpful in drug discovery and tissue engineering due to increased cell to cell contact, cell communication, and cell-ECM interactions.[2] Current methods for studying vasodilation and constriction involve time-consuming *in vivo* animal studies, ex vivo methods utilizing blood vessels excised from animals (e.g., rabbit and rodent models), and simplified *in vitro* methods that capture a portion of the biological response.[5-8] While excised animal vessels are valuable in understanding cell signaling in an *ex vivo* environment, procedures are time intensive, requiring processing of the excised tissue and then almost immediate testing of samples. An innovative model system is needed that is (1) simple to use and multiplex, enabling rapid adoption by biology laboratories, (2) incorporates multiple human cell types, and (3) can quantify the degree of dilation.

To offer an alternative to animal methods and clinical trials in humans, several groups have recently developed *in vitro* assays with primary human smooth muscle cells to study the effects of vasoactive compounds[12-16]. Alford *et al.* created what they called 'muscular thin filament', or MTFs, which are strips of polydimethylsiloxane (PDMS) with adhered vascular smooth muscle cells.[9] The MTFs were imaged and analyzed, measuring constriction or dilation by curvature of the MTF.[9-11] Alternatively, several groups have performed vasoactivity tests on tissue engineered blood vessels (TEBVs) as part of characterization of TEBVs to be used for drug testing[12] or to be potentially implanted as vascular grafts.[13] Recently, Tseng *et al.* developed a platform to study vasoactivity of vascular smooth muscle cells that were 3D printed into a ring configuration that fit within the well of a 96-well plate.[14] We sought to add to this body of work by developing a user-friendly method for generating blood vessel mimics that require only commercially available supplies and simple parts produced with an inexpensive resin 3D printer.

Here, we present a modular casting method to form a 3D hydrogel structure with a lumen (i.e., a ring) that is embedded with smooth muscle cells. These hydrogel rings are 2.6-4.6 mm in outer diameter and are free-standing and transferable between well plates. This lack of attachment to any surface enables the hydrogel to be remodeled by the cells within; thus, the addition of vasoactive compounds results in a visible and quantifiable readout. Our method enables both the use of smooth muscle cells embedded in the hydrogel as well as the incorporation of endothelial cells in the

lining of the hydrogel lumen, resulting in a co-culture system (cells embedded in the hydrogel and cells lining the lumen of the hydrogel) that can be used to study vasoactivity as well as cell signaling. Vascular smooth muscle cells interact intricately with ECM proteins like collagen, and these interactions play a part in the dilation or constriction. Chemical signals, often generated by or transmitted through the endothelial cells, trigger smooth muscle cells to dilate or constrict the blood vessel. Our method could be used as an assay to help determine how potential drug candidates can reduce harmful vasodilation in patients with various inflammatory diseases (e.g., asthma, rheumatoid arthritis, Raynaud's syndrome). The method is user friendly and amenable to medium throughput experiments (100 lumens per day).

Results

Our goal was to develop a user-friendly protocol for producing cell-laden hydrogel rings that can be used for medium-throughput biology experiments and drug screening experiments. Lumen structures are commonplace in the human body, and our area of focus in this work is modeling blood vessels to study vasodilation. Here we present two casting methods to produce cell-laden hydrogel rings; our methods are easy to implement with 3D printed components and low-cost commercially available materials (e.g.,Teflon tubing).

To use cell-laden hydrogel rings in vasoactivity experiments, it is important that the hydrogel is not attached to stiff materials that may prevent hydrogel deformation (i.e., dilation or constriction); therefore, our approach was to cast hydrogels into an easily removable mold. We use a simple 3D printed design and Teflon tubing to mold precursor hydrogel solution into a ring that can be removed and placed in a standard 96-well plate. Current 3D printed molds consist of 6 posts per device, enabling the generation of 6 hydrogel rings per mold. A PDMS spacer was applied to the device, and Teflon tubing was added onto each post (Figure 1Aii). The PDMS spacer holds up the Teflon tubing, and F127 (a polyethylene glycol (PEG)-based hydrogel) is added to seal any gaps between the tubing and the 3D printed post. We chose F127 because it is a dissolvable gel that is not toxic to cells. Once the F127 gels, collagen I is added to form the ring. When the collagen I polymerizes, the spacer is removed, and the device is placed in phosphate buffered saline (PBS). The tubing is pushed down to reveal the hydrogel rings, which are released into solution, and the PBS dissolves the F127 (Figure 1Aiii). Collagen laden with primary human umbilical artery smooth muscle cells (HUASMCs) was used to form hydrogel rings. A live/dead stain was performed with calcein AM (stains living cells) and ethidium homodimer-1 (stains dead cells), which showed the cells maintaining excellent viability in Figure 1Bi. Further, arrays of hydrogel rings can efficiently be made with this method (Figure 1Bii).

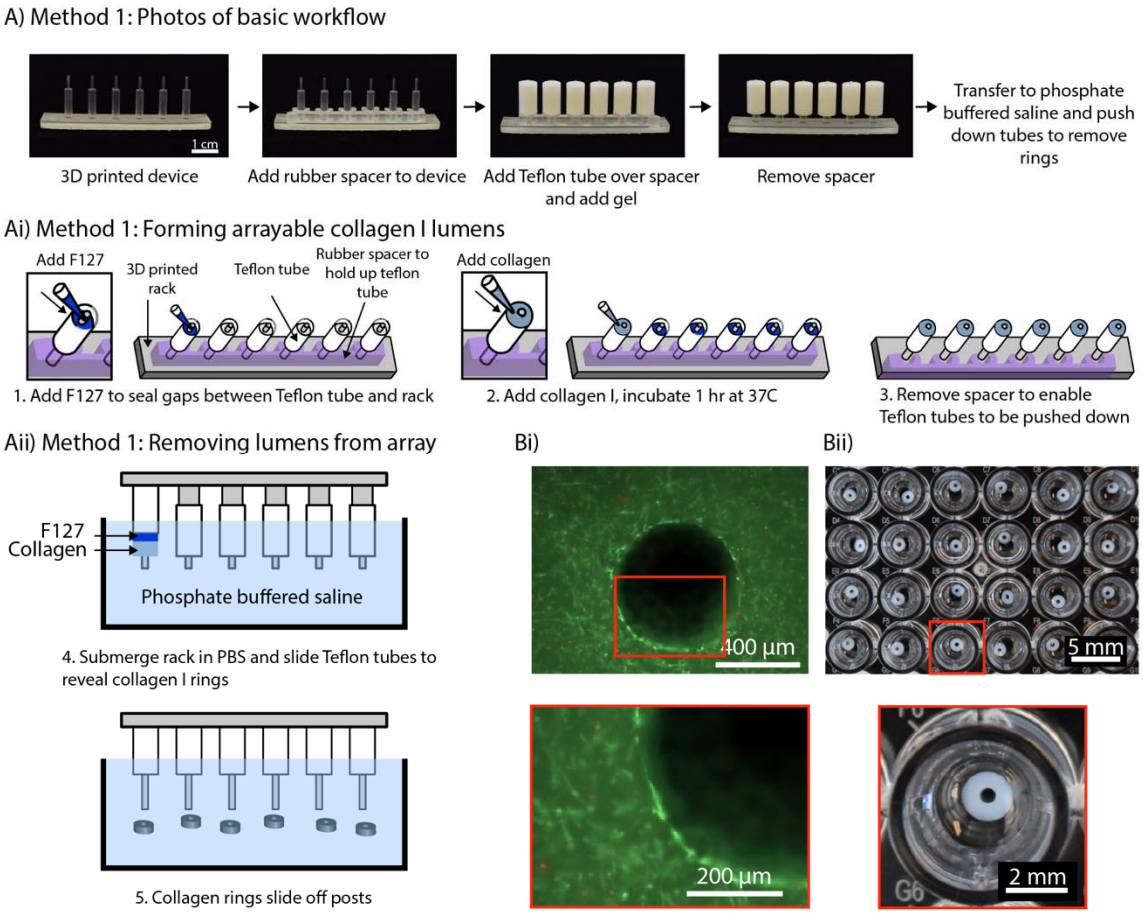

Figure 1. An arrayable method for fabricating cell-embedded free-standing collagen I lumens. (Ai) Photographs of device setup and basic workflow for Method 1. (Aii) A rubber spacer is added to the 3D printed device to hold up the Teflon tubes on each post. F127 hydrogel seals small gaps so the collagen I does not leak through. (Aiii) The rack is submerged in phosphate buffered saline, and the tubes are pushed down to remove the collagen rings and dissolve F127. (Bi) Fluorescence image of primary human umbilical artery smooth muscle cells embedded in a hydrogel ring, green indicates live cells and red indicates dead cells. (Bii) Image of an array of 3 mm OD and 1 mm ID hydrogel rings in a 96-well plate.

We evaluated the reproducibility of this method in two separate experiments with two devices of the same design (12 total collagen rings per experiment) in each experiment. The collagen rings were stored and imaged in a 24-well plate to eliminate glare-effects from the meniscus of the PBS (Figure 2A). While the rings can fit in a 96-well plate as shown in Figure 1B, the light from the imaging set up used to measure the rings reflects off the bottom of the concave meniscus, causing a glare over the collagen ring and impeding proper imaging. In a larger well plate, such as the 24 well plate, a glare is still present, however due to the larger diameter the bottom of the

concave meniscus is larger in diameter than the collagen ring, therefore the glare does not cover the ring.

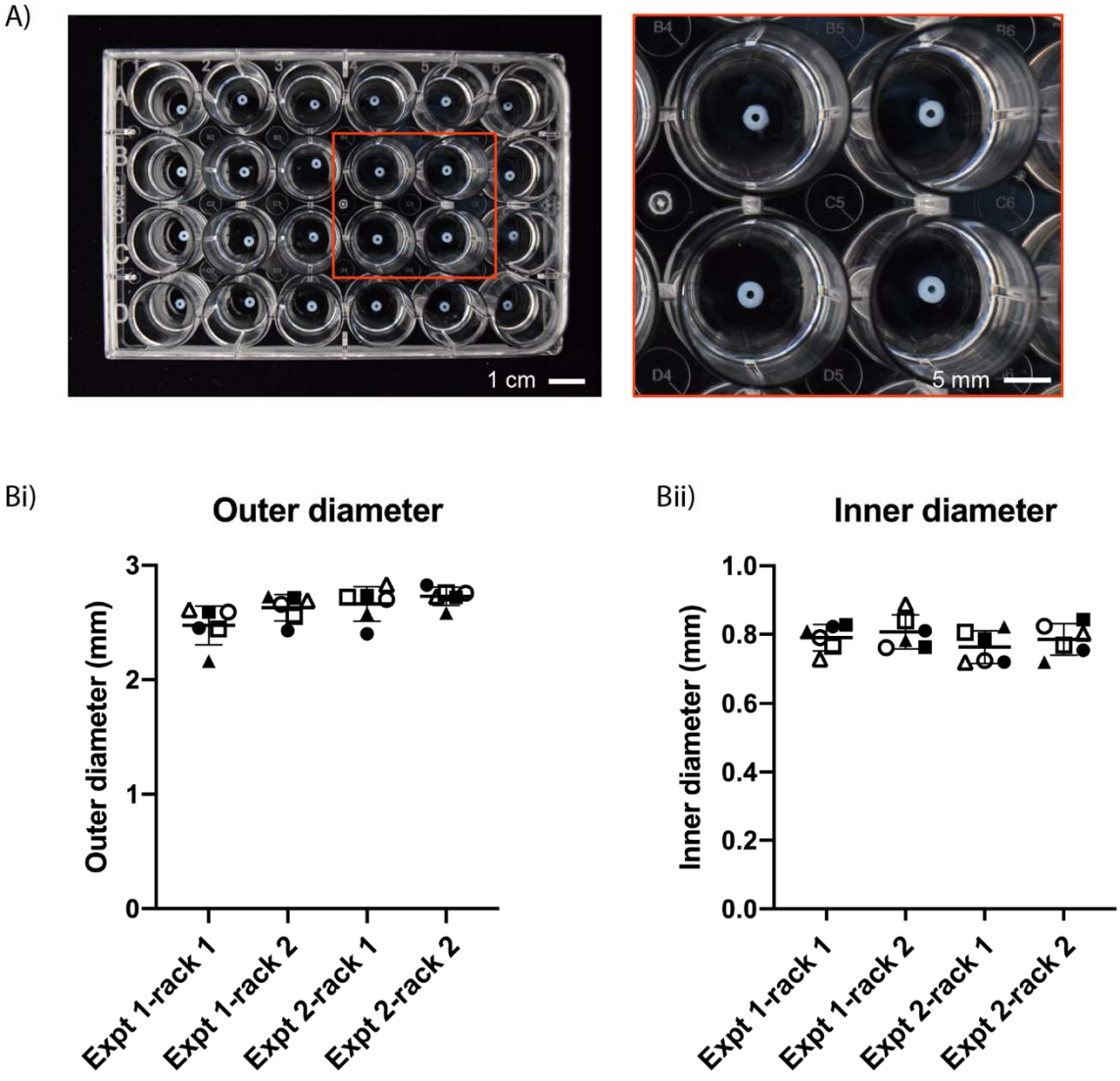

Figure 2. Reproducibility of collagen rings designed with an outer diameter of 3.0 mm and inner diameter of 1.0 mm. (A) Collagen rings of equal size in a 24-well plate. (Bi) The average outer diameter from each device was 2.48±0.17 mm, 2.63±0.12 mm, 2.66±0.15 mm, 2.73±0.08 mm and (Bii) their respective inner diameters were 0.79±0.04 mm, 0.81±0.05 mm, 0.76±0.05 mm, and 0.79±0.05 mm. Each symbol corresponds to the order in which each ring was imaged from a device. Results are plotted from two independent experiments, each with two racks (one rack is an array of 6 lumens). Error bars are mean ±SD.

The designed dimensions for the collagen rings were 3 mm outer diameter and 1 mm inner diameter. Overall, the actual inner diameter, outer diameter, and wall thickness were smaller than designed (Figure 2Bi and 2Bii). The average outer diameter for

each device ranged from 2.48 to 2.73 mm, a difference of 250 µm and the average inner diameter ranged from 0.76 to 0.81 mm, a difference of 40 µm. The difference can be attributed to the measurement technique (described in the methods section). Additionally, standard deviations can be attributed to the measurement technique and inherent differences that occur in 3D printed devices.

The addition of endothelial cells to line the lumen allows our method to further model the structure of a human blood vessel. Method 1 of creating collagen rings was adapted to make the post removable, enabling an endothelial cell suspension to be added to the lumen after gelation of the hydrogel ring (Figure 1). An endothelial cell suspension is pipetted on top of the removable post; when the post is retracted the cell suspension is drawn into the lumen, after which the device is incubated for 1 hour. During this incubation period, the device is rotated 180 degrees every 5 minutes for the first 20 minutes and then every 10 minutes for 40 minutes to ensure distribution of cells on all sides of the lumen. The spacer is removed and the Teflon tube is pushed down to reveal the ring with endothelial cells seeded in the lumen. The cells incubated in a well plate with media for 24 hours. A live/dead stain was performed with calcein AM (stains living cells) and ethidium homodimer-1 (stains dead cells). The images show that the cells are viable up to 24 hours and that the endothelial cells are evenly distributed around the entire lumen (Figure 3B).

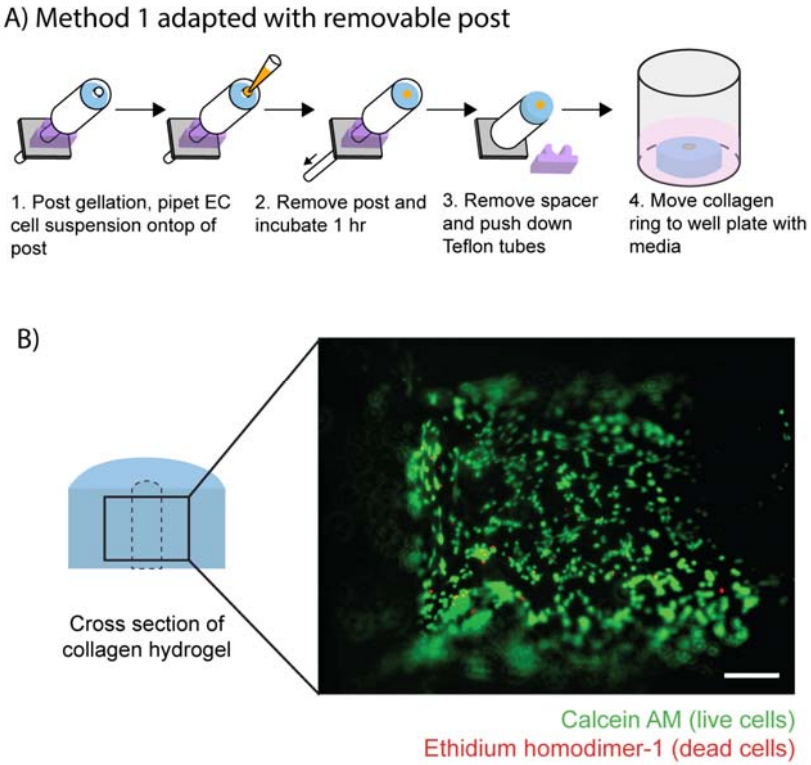

Figure 3. Addition of endothelial cells to the lumen of collagen rings. (A) Workflow for adding the endothelial cell suspension to the gelled collagen rings after adapting Method 1. (B) Cross section image of the lumen was taken after 24 hours of endothelial cell seeding (rings were stored in media and incubated in a cell culture

incubator) to show the viability of the endothelial cells. Green indicates live cells, red indicates dead cells. Scale bar = 100 µm.

While Method 1 (Figure 1) works well for arrayed generation of hydrogel rings, we also sought to develop a simple method to make individual hydrogel rings with a simpler setup, avoiding the need to use F127 and multiple pieces (spacer, Teflon tubes, etc.). Method 2 uses a syringe that has been adapted to form collagen rings by cutting off the tip and inserting a 3D printed core. Collagen laden with smooth muscle cells was then pipetted into the syringe. The syringe and collagen were incubated to allow the collagen to gel. The 3D printed core can then be pushed upwards, revealing the collagen ring, and the ring can be moved to a cell plate with media for storage. Following 5 days in culture, cell-laden hydrogel rings were treated with a known vasodilator, fasudil. Hydrogel rings were submerged in Tyrode's Solution and treated with either a control (additional Tyrode's Solution) or the vasodilator (fasudil). The collagen rings were recorded for 20 minutes using a stereoscope to monitor any change in geometry and size. Images obtained from these recordings were analyzed using ImageJ to

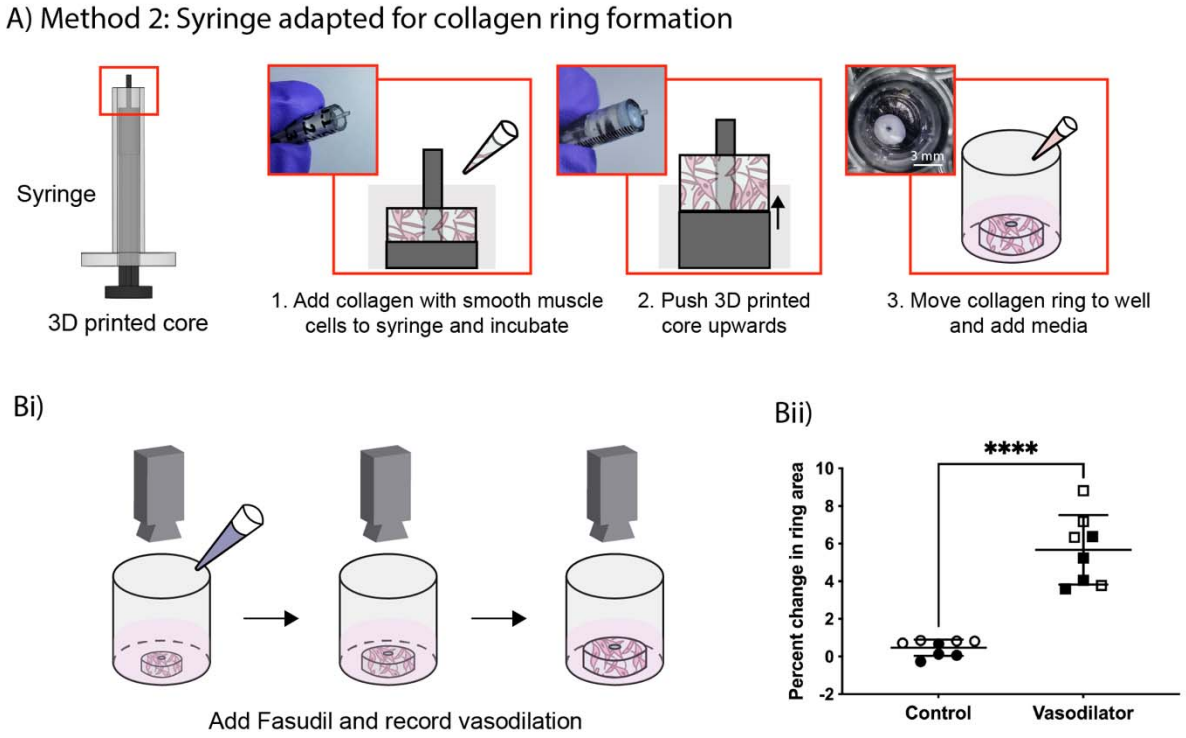

Figure 4. Vasoactivity data of human umbilical artery smooth muscle cells seeded in hydrogel rings when a vasodilator (fasudil) is added. (A) Method 2 workflow. A syringe with a 3D printed insertable core that has been adapted to make cell laden collagen rings. (Bi) The hydrogel rings were recorded for 20 minutes after addition of fasudil, and their percent change in area was calculated using ImageJ. (Bii) Percent change in ring area data for hydrogel rings treated with buffer (control) or vasodilator (fasudil).

Data points are from 8 rings across 2 independent experiments; error bars are mean ± SD. A standard two-sample unpaired t-test (two-tailed) was used. ****p<0.0001.

determine the change in percent area of the rings over time (Figure 4). The data showed a significant difference when hydrogel rings were treated with a control (buffer) compared to the vasodilator (fasudil).

Methods

Fabrication of device to mold hydrogel rings

Method 1 (Figures 1-3): Molds were designed using Solidworks (Dassault Systems, Waltham, MA). Hydrogel ring molds were fabricated using a Form 2 SLA 3D printer (Formlabs, Somerville, MA) using Clear V4 resin (Formlabs) with a Z resolution of 0.05 mm. After printing, molds were cleaned in a FormWash with isopropyl alcohol (IPA) for 10 min, followed by a second wash with fresh IPA for 10 min. Molds were then dried using compressed air and cured under UV (FormCure, Formlabs) for 30 min at 60°C. A 3.5 mm thick polydimethylsiloxane (PDMS) spacer was made by milling a polystyrene mold (Datron Neo) and pouring PDMS (Sylgard™ 184, Dow) in a 1:8 ratio. The PDMS was left to cure at room temperature for 48 hrs. Prior to use for cell culture, molds were sprayed with 70% ethanol, air dried in a biosafety cabinet, and irradiated with UV light for 15 minutes.

Method 2 (Figure 4): Cores were prepared using the same printing and sterilization protocols as Method 1. After posts were UV sterilized in the biosafety cabinet (for 20 min), posts were soaked in 1% bovine serum albumin (BSA) for 40 minutes (to prevent the collagen from sticking to the post and enabling easier removal of the hydrogel ring) and left to air dry before use. 1 mL syringes were trimmed at the 0.8 mL mark and the core was replaced with the 3D-printed posts.

Cell culture of human umbilical artery smooth muscle cells (HUASMCs) and human umbilical vein endothelial cells (HUVECs)

Human umbilical artery smooth muscle cells (HUASMCs; Cell Applications) were cultured in human smooth muscle cell growth medium (SmGM; Cell Applications) supplemented with penicillin (100 units mL^{-1}) and streptomycin (100 µg mL^{-1}). Human umbilical vein endothelial cells (HUVECs; Lonza) were cultured in endothelial cell growth medium (EGM-2; Lonza) supplemented with penicillin (100 units mL^{-1}) and streptomycin (100 µg mL^{-1}). Culture flasks were maintained at 37 °C with 5% CO_2. HUASMCs between passages 4 and 8 were used; HUVECs between passages 5 and 11 were used.

Encapsulation of HUASMCs in collagen I hydrogel

HUASMCs were trypsinized and resuspended at a concentration of 5 x 10^6 cells mL^{-1} in growth medium (SmGM). The cell suspension was then added to the neutralized collagen solution for a final concentration of 1 x 10^6 cells mL^{-1} and 6 mg mL^{-1} collagen I.

Hydrogel ring fabrication

Method 1 (Figure 2 and 3)

Teflon tubing (McMaster Carr) with an inner diameter of 3.175 mm and an outer diameter of 6.35 mm was cut to 1 cm, sonicated in with 70% ethanol for 30 min, and air dried then transferred onto the 3D printed molds with PDMS spacers. A 20% solution of Pluronic® F-127 (P2443–1KG; referred to as F127) (Sigma Aldrich) was pipetted into the mold for the rings and then removed, leaving a thin layer of F127 and filling any gaps between the 3D printed device and the Teflon tubing. The devices were placed in a box with kimwipes saturated in 1X Phosphate-buffered saline (PBS) to prevent evaporation of any gel. The box was put in a 37C incubator for 5 minutes to fully set the F127. To make the collagen, HEPES buffer was prepared as 500 mM in 10X PBS and adjusted to a pH of 7.4 using NaOH pellets. HEPES buffer was then thoroughly mixed with high concentration collagen I (8 – 10 mg mL^{-1}; Corning) in a 1:9 ratio of HEPES to collage. The tube of collagen was warmed up to room temperature to prevent it from dissolving the F127. The collagen was then pipetted into the molds, placed back in the box with PBS, and incubated for 1 hr. To remove the collagen rings the PDMS spacer was removed and the device was submerged in PBS. The Teflon tube was pushed down and the collagen rings were gently removed from the 3D printed post using tweezers or by gently shaking the device. They were then transferred to a 24 well plate with 500uL of PBS in each well.

Method 2 (Figure 4)

For Method 2 the trimmed syringes and BSA soaked 3D printed cores were combined to form the mold. The cell-laden collagen solution was then carefully pipetted into the mold so as to avoid bubbles; depending on the tubing size, roughly 20 – 30 µL of cell-laden collagen solution is used per post. After addition of hydrogel mixture, the molds were placed in a BioAssay Dish that is lined with Kimwipes soaked in 1X PBS. The BioAssay Dish containing molds was then transferred to an incubator to allow the collagen to gel. For the first 10 minutes, the BioAssay Dish was carefully flipped 180° every 2 minutes to ensure distribution of cells throughout the collagen.

After 1.5 h of incubation, the BioAssay Dish containing hydrogel ring molds was removed from the incubator and brought into the biosafety cabinet. The Teflon tubing was lowered and the hydrogel rings were transferred to individual wells in a 96-well plate. 100 µL of cell culture media was added to each well, and the surrounding wells were filled with 1X PBS to prevent evaporation of media. The hydrogel rings were then

incubated at 37 °C with 5% CO_2 until experimentation, with growth media replaced every 24 h.

Measurement of hydrogel rings

Images were obtained using an Amscope MU1403B High Speed Microscope Camera mounted on an Amscope SM-3TZ-80S stereoscope and processed in FIJI (ImageJ). All images were thresholded between 40 and 255 prior to obtaining measurements. The outer diameter was measured manually by drawing a line across the image at 0, 45, 90, and 135 degrees and averaging those values; the same procedure was used for the inner diameter.

Addition of endothelial cells to hydrogel rings

Using Method I, a 3D printed mold was generated with a hole rather than a post. Teflon tubing (McMaster Carr) was run through each mold to act as a retractable post. Collagen I was added to the mold to avoid bubbles and incubated for 1 h, as outlined above. After 1 h of incubation, the BioAssay Dish containing hydrogel ring molds was removed from the incubator and brought into the biosafety cabinet. The retractable Teflon tubing was then drawn down to reveal a lumen while the hydrogel rings remained in the mold. Approximately 5 µL solution of HUVECs ($1x10^6$ cells mL^{-1}) was carefully pipetted into the lumen for each ring. The devices were rotated 180 degrees every 5 minutes for the first 20 minutes and then 180 degrees every 10 minutes for the following 40 minutes to ensure even distribution of HUVECs within the lumen. After 1 h of incubation and rotation, hydrogel rings were removed in a solution of PBS and transferred to a well-plate as described above. Each ring was incubated in 60 µL of EGM-2 media for 24 hour. At the 24 hour mark rings were washed with PBS and stained with calcein AM and ethidium-homodimer 1 (Sigma Aldrich) for 30 minutes at 37 °C, rinsed with PBS, and imaged using a fluorescence microscope (Zeiss Axiovert 200 and an Axiocam 503 mono camera; Carl Zeiss AG, Oberkochen, Germany).

Dilation experiments

After 6 to 8 days in culture, cell-laden hydrogel rings were used for vasodilation experiments. Two hydrogel rings were transferred to individual wells of a new 96-well plate, and submerged in 60 µL Tyrode's Solution. After 5 minutes of equilibration the solution was removed and replaced with fresh Tyrode's Solution. 6 µL of either additional Tyrode's Solution (control) or fasudil (vasodilator) was then added. The well plate was then placed under a stereoscope and recorded for 20 minutes after which hydrogel rings were transferred to a new well plate containing cell media for staining and imaging. This process was repeated with additional hydrogel rings until the desired number of replicates had been tested.

Imaging of vasodilation experiments and processing

Top-view images of hydrogel rings were recorded using an Amscope MU1403B High Speed Microscope Camera mounted on an Amscope SM-3TZ-80S stereoscope. Stills were obtained from video recordings for every 60 s (i.e., 21 stills were obtained for a 20 minute recording). These images were then processed identically using FIJI (ImageJ) to calculate the total hydrogel area.

Conclusion

We demonstrate that we have created a simple method for forming freestanding hydrogel rings that model vessel-like structures and can be used in a quantifiable assay for measuring the dynamic change after treatment with a known vasodilator. Our focus was to create a method to form freestanding hydrogel lumens that does not rely on animal models or rigid devices to stabilize the lumen. Our device takes the unique approach to being free standing which allows unrestricted movement in all three dimensions in response to vasoactive compounds. This method enables researchers to study the response of a vessel model using human cells to different inflammatory drugs or molecules, as well as study the cellular communication that is important to understanding the pathways that are activated during inflammation. We envision that our model will be important for studying a range of diseases such as asthma, cancer, rheumatoid arthritis, and other diseases that have periods of induced inflammation, vasodilation, or constriction. The present model uses vascular smooth muscle cells and endothelial cells. In future work we will integrate these freestanding lumens with other co- and multiculture platforms to incorporate epithelial cells, immune cells, fibroblasts, and other stromal cells important for modeling disease-specific vasodilation.

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Conflicts of Interest

ABT has ownership in Stacks to the Future, LLC and EB has ownership in Stacks to the Future, LLC, Tasso, Inc., and Salus Discovery, LLC. However, the work presented in this publication was not related to these companies.

References

1. Horvath, G.; Wanner, A., Inhaled corticosteroids: effects on the airway vasculature in bronchial asthma. *Eur Respir J* **2006,** *27* (1), 172-87.
2. Edmondson, R.; Broglie, J. J.; Adcock, A. F.; Yang, L., Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors. *Assay Drug Dev Technol* **2014,** *12* (4), 207-18.
3. Tibbitt, M. W.; Anseth, K. S., Hydrogels as extracellular matrix mimics for 3D cell culture. *Biotechnol Bioeng* **2009,** *103* (4), 655-63.
4. Duval, K.; Grover, H.; Han, L.-H.; Mou, Y.; Pegoraro, A. F.; Fredberg, J.; Chen, Z., Modeling Physiological Events in 2D vs. 3D Cell Culture. *Physiology* **2017,** *32* (4), 266-277.
5. Marceau, F.; deBlois, D.; Petitclerc, E.; Levesque, L.; Drapeau, G.; Audet, R.; Godin, D.; Larrivée, J. F.; Houle, S.; Sabourin, T.; Fortin, J. P.; Morissette, G.; Gera, L.; Bawolak, M. T.; Koumbadinga, G. A.; Bouthillier, J., Vascular smooth muscle contractility assays for inflammatory and immunological mediators. *Int Immunopharmacol* **2010,** *10* (11), 1344-53.
6. Zhang, Y.; Yu, Y.; Akkouch, A.; Dababneh, A.; Dolati, F.; Ozbolat, I. T., In Vitro Study of Directly Bioprinted Perfusable Vasculature Conduits. *Biomater Sci* **2015,** *3* (1), 134-43.
7. Pi, Q.; Maharjan, S.; Yan, X.; Liu, X.; Singh, B.; van Genderen, A. M.; Robledo-Padilla, F.; Parra-Saldivar, R.; Hu, N.; Jia, W.; Xu, C.; Kang, J.; Hassan, S.; Cheng, H.; Hou, X.; Khademhosseini, A.; Zhang, Y. S., Digitally Tunable Microfluidic Bioprinting of Multilayered Cannular Tissues. *Advanced Materials* **2018,** *30* (43), 1706913.
8. Jia, W.; Gungor-Ozkerim, P. S.; Zhang, Y. S.; Yue, K.; Zhu, K.; Liu, W.; Pi, Q.; Byambaa, B.; Dokmeci, M. R.; Shin, S. R.; Khademhosseini, A., Direct 3D bioprinting of perfusable vascular constructs using a blend bioink. *Biomaterials* **2016,** *106*, 58-68.
9. Alford, P. W.; Nesmith, A. P.; Seywerd, J. N.; Grosberg, A.; Parker, K. K., Vascular smooth muscle contractility depends on cell shape. *Integr Biol (Camb)* **2011,** *3* (11), 1063-1070.
10. Hald, E. S.; Steucke, K. E.; Reeves, J. A.; Win, Z.; Alford, P. W., Long-term vascular contractility assay using genipin-modified muscular thin films. *Biofabrication* **2014,** *6* (4), 045005.
11. Steucke, K. E.; Tracy, P. V.; Hald, E. S.; Hall, J. L.; Alford, P. W., Vascular smooth muscle cell functional contractility depends on extracellular mechanical properties. *J Biomech* **2015,** *48* (12), 3044-51.

12. Fernandez, C. E.; Yen, R. W.; Perez, S. M.; Bedell, H. W.; Povsic, T. J.; Reichert, W. M.; Truskey, G. A., Human Vascular Microphysiological System for in vitro Drug Screening. *Scientific Reports* **2016,** *6* (1), 21579.
13. Jung, Y.; Ji, H.; Chen, Z.; Fai Chan, H.; Atchison, L.; Klitzman, B.; Truskey, G.; Leong, K. W., Scaffold-free, Human Mesenchymal Stem Cell-Based Tissue Engineered Blood Vessels. *Scientific Reports* **2015,** *5* (1), 15116.
14. Tseng, H.; Gage, J. A.; Haisler, W. L.; Neeley, S. K.; Shen, T.; Hebel, C.; Barthlow, H. G.; Wagoner, M.; Souza, G. R., A high-throughput in vitro ring assay for vasoactivity using magnetic 3D bioprinting. *Scientific Reports* **2016,** *6* (1), 30640.