Perfusable Vessel-on-a-Chip for Antiangiogenic Drug Screening with Coaxial Bioprinting

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Abstract: Vessel-on-a-chips, which can be used to study microscale fluid dynamics, tissue-level biological molecules delivery and intercellular communication under favorable three-dimensional (3D) extracellular matrix microenvironment, are increasingly gaining traction. However, not many of them can allow for long-term perfusion and easy observation of angiogenesis process. Since angiogenesis is necessary for the expansion of tumor, antiangiogenic drugs play a significant role in cancer treatment. In this study, we established an innovative and reliable antiangiogenic drug screening chip that was highly modularly integrated for long-term perfusion (up to 10 days depending on the hydrogel formula) and real-time monitoring. To maintain an unobstructed flow of cell-laden tubes for subsequent perfusion culture on the premise of excellent bioactivities, a polycaprolactone stent inspired by coronary artery stents was introduced to hold up the tubular lumen from the inside, while the perfusion chip was also elaborately designed to allow for convenient observation. After 3 days of perfusion screening, distinct differences in human umbilical vein endothelial cell sprouting were observed for a gradient of concentrations of bevacizumab, which pointed to the effectiveness and reliability of the drug screening perfusion system. Overall, a perfusion system for antiangiogenic drug screening was developed, which can not only conduct drug evaluation, but also be potentially useful in other vessel-mimicking scenarios in the area of tissue engineering, drug screening, pharmacokinetics, and regenerative medicine.

Keywords: Coaxial bioprinting; Gelatin methacryloyl; Vessel-on-a-chip; Perfusion culture; Drug screening model

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1. Introduction

Angiogenesis, the generation of new blood vessels, plays a key role in various disease states, such as cancer, diabetic retinopathy, age-related macular degeneration, rheumatoid arthritis, psoriasis, atherosclerosis, and restenosis1,2. In the treatment of cancer, antiangiogenic drugs, which are often used to target angiogenesis in combination with other drugs3, play significant roles, since angiogenesis is necessary for the expansion of tumor. Many studies concerning antiangiogenic drug
screening adopted the use of animal model such as zebrafish\textsuperscript{24,45}, which has relatively low relevance to human microenvironment. Some researchers have attempted to establish antiangiogenic drug screening platform using microfluidics\textsuperscript{6,7}, which require high threshold and high cost; however, it is difficult to simulate the structure or texture of blood vessel with polydimethylsiloxane (PDMS) or polycarbonate, which are the most commonly used substrates. In contrast, hydrogels have been increasingly proposed for the construction of microfluidic chips\textsuperscript{80}.

Organ-on-a-chip, which was first reported by Donald Ingber for the development of a microfluidic chip to observe human lung organ-level functions in 2010\textsuperscript{9}, refers to a microfabricated biomimetic system combining the technologies of tissue engineering, microfluidics, and cytology, to simulate significant features of targeted tissue microenvironments and constructions\textsuperscript{10}. Among its numerous applications, 3D cell culture\textsuperscript{11-14}, drug screening\textsuperscript{15-18}, disease modeling\textsuperscript{19-22}, and tissue regeneration\textsuperscript{23-25} have received much attention. The bionic fabrication of blood vessels has also been rapidly developed in recent decades. In general speaking, there are four typical approaches to build a vessel-on-a-chip: microfluidics, sacrificial templating, 3D bioprinting, and self-organization\textsuperscript{26}. 3D bioprinting, which has attracted substantial attention in recent decades, is increasingly being applied for the creation of tissue models\textsuperscript{27-29}. 3D bioprinting, which is an inexpensive, fast, and controllable printing process and can utilize a wide range of bioink/biologics, can overcome many limitations of the other three techniques\textsuperscript{30,31}. Its ability to fabricate 3D freeform bioactive architectures brings new ideas for vessel-on-a-chip. Since our group developed a coaxial bioprinting approach to print alginate hollow filaments in 2015\textsuperscript{32}, coaxial bioprinting has become a popular research methodology with diverse applications\textsuperscript{33-37}. In simple terms, coaxial bioprinting utilizes different materials (or same material with different loads) that are extruded through a coaxial nozzle to form a fiber with core-shell structure. If the core material is sacrificial (e.g., gelatin, Pluronic F-127, etc.), the filament presents tubular structure. Based on our previous work\textsuperscript{38}, gelatin methacryloyl (GelMA)/gelatin is the ideal bioinks for bioprinting human umbilical vein endothelial cell (HUVEC)-laden hydrogel tubes.

In recent years, a few scholars have committed to the research in biomimetic vessel fabrication and perfusion culture. Using gelatin as sacrificial bioink, Lee et al. developed a functional in vitro vascular channel with perfused open lumen fully covered with endothelial cells in 2014, in which active angiogenic invasion of cells was observed\textsuperscript{39}. Gao et al. used coaxial bioprinting as a tool to construct a vascular model for the assessment of vascular physiological functions and pathological observation of airway inflammatory in 2018\textsuperscript{40}. In 2019, Cui et al. implanted 3D bioprinted vasculature, consisting of smooth muscle and endothelium, in immunodeficient mice to observe its development\textsuperscript{41}. Pennings et al. developed a bioreactor system to culture bi-layered vascular grafts under shear stress, with a compartmentalized exposure of the graft’s luminal and outer layer to cell-specific media\textsuperscript{42}. However, almost no studies concerning antiangiogenetic drug screening models fabricated using 3D bioprinting have been reported.

In this study, utilizing 3D bioprinting, an innovative perfusion system for antiangiogenic drug screening, was established. In this work, process optimization and printability of coaxial bioprinting are discussed. Since the GelMA tube with excellent cell activity is too soft to maintain its shape, a polycaprolactone (PCL) stent is introduced to hold up the tubular lumen from the inside, which is inspired by coronary artery stents used to keep arteries open. Then, a comparison between hydrogel structures with/without a stent was conducted. Afterward, diffusion analysis verified the barrier function. Next, bioactivity characterization of cell-laden constructs was measured throughout the perfusion system. A perfusion chip was also elaborately designed with three main advantages: (i) Long-term perfusion with no leaking; (ii) cells surrounded by nutrients; and (iii) convenient observation.

The U.S. Food and Drug Administration has approved 14 kinds of angiogenesis inhibitors to treat cancer in humans thus far\textsuperscript{43}. As the first approved agent to target tumor angiogenesis in 2004, bevacizumab, which is the most common antiangiogenic drug, is a recombinant, humanized monoclonal antibody that binds to vascular endothelial growth factor (VEGF) and prevents it from binding to its receptors, VEGF receptor (VEGFR)-1 and VEGFR-2 on the surface of vascular endothelial cells, thereby inhibiting tumor angiogenesis\textsuperscript{44} (Figure 1). Based on the ingenious perfusion system, the application of antiangiogenic drug screening model was finally assessed by different spraying levels corresponding to bevacizumab at a gradient of concentrations. From the results, the perfusing of HUVECs was observed, which demonstrated the effectiveness of this perfusion system, and the differences in the bevacizumab gradient-screening test provided evidence that this antiangiogenic drug screening system is reliable. This study contributes to advancing basic scientific research and clinical applications related to not only antiangiogenic drug evaluation, but also vascular disease drug assessment in the areas of tissue engineering, drug screening, pharmacokinetics, and regenerative medicine.
2. Experimental section

2.1. Reagents and materials

3D bioprinter (EFL-BP6601, EFL-BP5800) and photocuring light source (EFL-LS-1601, 405 nm, 25 mW/cm²) were manufactured by Engineering for Life (EFL), Suzhou, China.

PCL (CAPA6800, Perstorp Ltd., Sweden) was used to 3D print the stents. Its molecular weight is 80,000 g/mol and its melting temperature is 60°C.

GelMA (EFL-GM-30, EFL, China) used for bioprinting and casting in this study was 5% (w/v). It was prepared by dissolving GelMA in endothelial cell medium (ECM; ScienCell Research Laboratories, US), containing 0.5% (w/v) photoinitiator, lithium phenyl-2,4,6-trimethylbenzoylphosphinate (EFL-LAP, EFL) at 37°C for 2 h, before being 0.22 μm-filter-sterilized.

Gelatin (Sigma-Aldrich, Shanghai, China) for bioprinting was 5% (w/v). It was prepared by dissolving gelatin in phosphate-buffered saline (PBS) (Qizhenhu Biological Technology Co., Ltd, Hangzhou, China) at 37°C for 2 h, and the solution was then 0.22 μm-filter-sterilized.

2.2. Manufacturing process of perfusion system

2.2.1. Specifications of coaxial nozzle

Three specifications of coaxial nozzles were applied in this study. Nozzle-18G/25G was manufactured by welding 18G and 25G needles together. Nozzle-1-2.5 and nozzle-1-3 were designed using 3D modeling software Solidworks 2016, and 3D printed by a commercial ceramic printer (Carmel 1400C, XJet, Israel) afterward (Figure S1).

2.2.2. Preparation of prebioinks and bioprinting of tubular structure

Before bioprinting, GelMA/gelatin solutions were sterilized and kept at 37°C. GelMA solution was mixed with HUVECs at the concentration of 3×10⁶ cells/ml. 3 ml of GelMA/gelatin solutions were pipetted to 10-ml syringes separately and kept at 37°C in water bath. GelMA/gelatin solutions were then turned into gelled prebioinks by cooling them in the refrigerator.

A 3D bioprinter (EFL-BP6601) was used for coaxial bioprinting. A syringe containing GelMA prebioink was connected to the outer channel of coaxial nozzle, and another syringe containing gelatin prebioink the inner one. GelMA and gelatin were extruded from the nozzle driven by syringe pumps at expected flow rates. Extruded fibers were deposited onto a cooling platform (2°C) and photocured for 20 s afterward. After 30 min of culture at 37°C, fibers became tubes and were cut into segments of 4.5 mm long. On the 1st day, culture medium was changed twice, and then the medium changed every 2 days.

2.2.3. 3D printing of PCL stents

A direct writing device (EFL-BP5800), which contained two heaters (a pneumatic system and a motor-driven rotating shaft), was used to print stents by the method of fused deposition of PCL on the rotating shaft; the diameter of which was 1 mm. The temperature of syringe and nozzle was both set to 115°C. Air pressure supplied to the polymer melt was 200 kPa. The nozzle-to-shaft-distance was set to 2 mm. After printing, PCL stents were soaked in 75% ethanol for 30 min on account of demolding and sterilization. They were cut into segments of 5 mm long afterward (Figure S2).
2.2.4. Perfusion chip establishment and perfusion system assembly

After 5–7 days of culture, the cell-laden tube was inserted with a PCL stent to be cast into bulk. First of all, tube with stent was placed into a PLA frame, which was printed by an FDM printer (CR-10 MAX, Creality 3D Technology Co., Ltd., China). Secondly, 75 μl of GelMA solution containing VEGF (PeproTech, US) at the concentration of 200 ng/ml was pipetted into the frame. Photocuring for 25 s was applied afterward. Subsequently, the PLA frame with hydrogel bulk inside was fitted onto a PLA subplate. Next, it was mounted in a PDMS (Sylgard 184, Dow Corning, US) wall and surrounded by two PET films; two cover plates of stainless steel were pressed on both sides and fixed with bolts and nuts subsequently. After that, needles of inlet and outlet were inserted into the tube through PDMS. Finally, a medium container, a peristaltic pump (BT300-2J, Longer Precision Pump Co., Ltd., China), a bubble remover (FluidicLab, China), and the perfusion chip were connected by silicone tube to form a perfusion circulation.

2.3. Property characterization

2.3.1. Rheology

Rheological properties of GelMA and gelatin were measured by a rheometer (MCR 102, Anton Paar, Austria) equipped with a 50 mm-diameter plate-plate in all measurements. All hydrogel samples were placed on the plate at the beginning to completely fill the gap (1 mm) between the two plates. The measure of storage/loss modulus and temperature was performed by varying temperature from 37 to 10°C, or from 10 to 37°C at the rate of 2°C/min, while the hydrogel samples were equilibrated at 37°C/10°C, respectively. For the measure of viscosity as a function of shear rate and storage/loss modulus as a function of amplitude sweep, the initial temperature of hydrogels samples was 10°C, and then, the samples were warmed to 20°C before the two tests. The measure of viscosity and shear rate was performed by changing shear rate from 0.1 to 1000. The measure of storage/loss modulus toward periodic amplitude sweep was performed by setting the amplitude of shear strain as 1% and 200%, which alternated every 30 s for three loops.

2.3.2. Mechanical properties

The mechanical properties of hydrogel bulks with/without stent were characterized by compression tests using a dynamic mechanical analysis instrument (ElectroForce, TA Instruments, US) at 25°C. Each hydrogel sample was cast as the same size as the one in the perfusion chip (4.5 × 4.5 × 4 mm³), enveloping the same hydrogel tube. Samples were placed between two plates and compressed at a displacement rate of 1 mm/min. Stresses at the strain of 50% and 60% were inspected particularly.

2.3.3. Perfusion performance

Perfusion performance was measured by inserting an 18G needle into a 3-cm long tube with/without stent under the flow rate of 6 μl/min, 60 μl/min, 600 μl/min, and 6000 μl/min from a peristaltic pump (BT300-2J, Longer Precision Pump Co., Ltd.).

2.3.4. Swelling

Hydrogel tubes with/without stent reached equilibrium swelling after being immersed in culture medium for 24 h. After that, bright field images were taken by microscope (WMF-3690, Shanghai Wumo Optical Instrument Co., Ltd., China) to measure the inner diameters of tubes.

2.3.5. Diffusion

To examine the barrier function of the endothelialized hydrogel tube, moistened tube was placed on the platform of fluorescent microscope (WMF-3690, Shanghai Wumo Optical Instrument Co., Ltd.) at first, and 6 μl fluorescein isothiocyanate (FITC)-dextran with a molecular weight of 40 kDa at the concentration of 500 μg/ml was injected into the tube. Fluorescent images were captured every 15 min under the same parameters. Images were transformed into grayscale images and analyzed with ImageJ software.

2.3.6. Scanning electron microscopy (SEM) analysis

Hydrogel bulks with/without stent were treated by graded ethanol dehydration. Afterward, the constructs were coated with platinum in a sputter coater (Ion Sputter E-1045, Hitachi, Japan), and then imaged by an SEM system (SU-6600, Hitachi, Japan).

2.4. Bioactivity characterization

2.4.1. Cell culture

HUVECs were cultured in ECM with 10% fetal bovine serum (Gibco, US), 1% penicillin (100 units/ml), and streptomycin (100 μg/ml) (Qizhenhu Biological Technology Co., Ltd.) at 37°C and 5% CO₂. Cells were passaged every 4 days and culture medium was changed every 2 days.

2.4.2. Cell morphological analysis

Morphologies of HUVECs were visualized by cell cytoskeleton staining, including F-actin and nucleus staining utilizing HUVECs-laden hydrogel tube with inner/outer diameters of 500 μm/1200 μm. F-actin staining was applied using TRITC phalloidin (Yeasen Biological Technology Co., Ltd., China), and nucleus
staining was performed using 2-(4-aminophenyl)-6-indolecarbamidine dihydrochloride (DAPI) staining solution (Yeasen Biological Technology Co., Ltd.). Samples were firstly washed by PBS and fixed with 4% paraformaldehyde for 30 min. After that, they were washed by PBS again and permeabilized with 0.5% Triton X-100 (Solarbio Co., Ltd., China) for 5 min. Then, the samples were washed by PBS again, and stained with TRITC phallodin (0.1 μM) for 30 min in the dark. Subsequently, they were washed by PBS again and stained with DAPI (10 μg/ml) in the dark. Finally, the samples were washed by PBS and imaged by a confocal fluorescence microscope (LSM880, ZEISS, Germany).

2.4.3. Cell activity analysis

Cell activity was measured using a cell counting kit-8 (CCK-8; Dojindo Chemical Technology Co., Ltd., China). The cell-laden hydrogel tubes were separately cultured in a 24-well plate for 1, 4, and 7 days. At first, tubes were washed with PBS 3 times. Then, a mixture of 50 μl CCK-8 reagent and 1450 μl ECM was added to each well. After 3 h of culture, the solutions were transferred to a 96-well plate to test the optical density values using a microplate absorbance reader (iMark, Bio-Rad, US).

2.4.4. Immunostaining of HUVECs

After 3 days of perfusion culture, vinculin antibody (Abcam, UK) and ZO-1 antibody (Invitrogen, US) immunostaining was performed on the samples to investigate the intercellular connection and functionalization of HUVECs. The samples were fixed with paraformaldehyde for 30 min, and permeabilized with 0.5% Triton X-100 for 5 min. Subsequently, samples were blocked in 5% bovine serum albumin (BSA; Sigma-Aldrich) for 1 h and incubated overnight in vinculin primary antibody in 1:200 dilution and ZO-1 primary antibody in 1:50 dilution according to instructions. Then, samples were incubated in 1/500 dilution of Alexa Fluor 488 goat anti-rabbit immunoglobulin (Ig)G (H+L) secondary antibody (Beyotime, China) or Alexa Fluor 488 goat anti-mouse IgG (H+L) secondary antibody (Beyotime) for 2 h at room temperature. Next, samples were stained with TRITC phallodin for 30 min and DAPI for 10 min. They were finally imaged by a confocal fluorescence microscope (LSM880, ZEISS). All reagents were injected into the tubular lumen for better contact and reaction.

2.4.5. Real-time monitoring of HUVEC sprouting

An incubation monitoring system (CM20, Olympus, Japan) was used for real-time observation of HUVEC sprouting during perfusion culture in 48 h. Time-lapse images of the sprouting area were taken every 20 min.

2.5. Statistical analysis

Unless otherwise stated, all characterizations were processed by data analysis software Origin 2018 and image analysis software ImageJ, and all data are presented as mean ± standard deviation. Differences between groups were conducted by one-way analysis of variance followed by Student’s t-tests. Single asterisk (*), double asterisks (**), and triple asterisks (****) indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

3. Results

3.1. Fabrication of a perfusable vessel-on-a-chip

According to the bioprinting strategy we proposed[38], cell-laden tubular constructs could be efficiently produced by coaxial bioprinting of GelMA/gelatin prebioinks. Based on the thermosensitivity of GelMA/gelatin, gelled gelatin and EC-laden GelMA prebioinks were simultaneously printed through a coaxial nozzle to form a core-shell structure. Then, it was exposed to 405 nm blue light for photocuring of GelMA (Figure 2Ai). After that, the printed fibers were transferred into an incubator for 30 min, in which the temperature of 37°C led to the liquefication of gelatin and the formation of tubular constructs. These tubes were cut into segments for better nutrient supply and subsequent use, and they were cultured for 5–7 days, allowing endothelial cells to proliferate and form an endothelialized vessel (Figure 2Aii). Inspired by coronary artery stents, a PCL stent was adopted to support the hydrogel vessel. It was achieved by fused deposition of PCL on a rotating shaft (Figure 2Aiii). Stents were subsequently demolded and sterilized by soaking in ethanol (Figure 2Aiv). Next, the construction of the vessel-on-a-chip can be divided into three steps: (i) The PCL stent was inserted into the cell-laden vessel (Figure 2Av); (ii) GelMA containing VEGF was utilized for casting the hydrogel bulk restricted by a prepared poly lactide (PLA) frame (Figure 2Avi); (iii) the perfusion chip was established by assembling two cover plates, two polyester (PET) films, the hydrogel bulk, a PLA subplate and a PDMS wall with four sets of bolts and nuts (Figure 2Avii, 2B and C).

When biofabricating a large-scale hydrogel vessel, it is quite difficult to obtain excellent biological activities and adequate structural fidelity at the same time. The GelMA (EFL-GM-30) used in this study is a soft and biologically friendly hydrogel that has outstanding cell viability but relatively poor mechanical properties. The introduction of a PCL stent solved the problem of vessel collapse or jamming during perfusion when liquid flowed through a soft hydrogel tube.

Maintaining perfusion without leakage has always been a challenge for vessel-on-a-chip. The employment of a PDMS wall and bolt-on mounting mode effectively achieved long-term perfusion culture. In addition, the
design of the perfusion chamber ensured sufficient contact between the hydrogel bulk and culture medium, which provided abundant surrounding nutrient supply for the cells inside. As shown in Figure 2A, B, and C, the PLA frame and adequate cavity limited by the PDMS wall contributed to enough room for the hydrogel bulk contacting culture medium omnidirectionally. PDMS is a biological friendly polymer that has been widely applied in microfluidics. The PDMS wall Figure 2C used here supported the entire chip and prevented culture medium leaking when the bolts were tight. Moreover, the use of hollow cover plates and transparent PET films Figure 2B offered an observable window of the chip, which made real-time observation possible. Using an incubation monitoring system, real-time observation of the sprouting area during perfusion culture is feasible. As long as proper focal distance and range were set in advance, the monitoring system would send back real-time images of sprouting area from the incubator. Besides, with the help of several quickly-separable connectors, this modularized perfusion chip could also be conveniently separated from the perfusion system. It could be easily taken out of incubator and observed under a microscope, if needed, without introducing contamination.

3.2. Printability analysis of GelMA/gelatin bioinks

3.2.1. Rheological analysis of GelMA/gelatin

To optimize printing parameters during coaxial bioprinting for precisely constructing cell-laden tubes, the rheological properties of gelatin and GelMA bioink were measured. First, the variation of the storage modulus (G') and loss modulus (G'') of GelMA/gelatin with changing temperature was tested during both cooling and warming processes, as shown in Figure 3A and 3B. GelMA and gelatin exhibited very similar gelation temperatures (G' = G''). During the cooling process, GelMA/
gelatin showed a typical liquid-like behavior with $G'$ close to zero when the temperature was over $20^\circ$C. The gelation temperatures of GelMA and gelatin were approximately $18.5^\circ$C and $16.5^\circ$C, respectively. Below the gel temperature, $G' > G''$, indicating characteristics of a gel-like status of GelMA and gelatin. In contrast, during the warming process, GelMA/gelatin possessed higher gelation temperatures. The gelation temperatures of GelMA and gelatin were approximately $29^\circ$C and $28^\circ$C, respectively. These results guided the preparation of prebioinks. Prebioinks were cooled from $37^\circ$C to reach approximately $15^\circ$C, and then bioprinted at an ambient temperature of approximately $20^\circ$C, during which the bioinks were at relatively stabilized conditions for extrusion according to the behaviors in the process of warming.

Regarding the premise of cooling-preparation of bioinks, shear rate-dependent behaviors and thixotropic

Figure 3. Printability analysis of GelMA/gelatin bioinks. (A) Rheological analysis of GelMA/gelatin: (i) effect of temperature on the storage modulus ($G'$) and loss modulus ($G''$) of gelatin; (ii) effect of temperature on the storage modulus ($G'$) and loss modulus ($G''$) of GelMA; (iii) viscosity as a function of the shear rate of GelMA and gelatin; (iv) storage modulus ($G'$) and loss modulus ($G''$) as a function of periodic amplitude sweep of GelMA and gelatin. (B) Printing of unicusural pattern: (i) a horse; (ii) a snowflake; (iii) an Archimedes curve (inset scale bar: $500 \mu$m); (iv) mascot of Zhejiang University, the Qiushi eagle; (v) abbreviation of Zhejiang University in Chinese. Scale bar: $10 \, \text{mm}$. (C) Schematic diagram of outer and inner diameters of the fiber. (D) Printability window of tubular constructs: (i) nozzle-1-2.5; (ii) nozzle-1-3. (E) Outer and inner diameter of fibers as a function of $Q_{\text{outer}}/Q_{\text{inner}}$: (i) nozzle-1-2.5; (ii) nozzle-1-3.
properties of gelatin and GelMA were then demonstrated. Shear-thinning behaviors of GelMA/gelatin were observed, with excellent extrusion properties (Figure 3Aii). When facing periodic amplitude sweep of shear strain, gelatin showed good response-to-deformation capability with fast switching: In the case of small amplitude (1%), $G' > G''$, representing a gel-like status; in the case of large amplitude (200%), $G' < G''$, representing a liquid-like status. However, GelMA had a relatively poor response capability (Figure 3Aiv). That is why a 2°C cooling platform was introduced for printed fiber deposition, as shown in Figure 2A, to ensure better structural fidelity.

3.2.2 Printing of unicursal patterns

To mimic the complicated structural features of organoids, freeform biofabrication of coaxial bioprinting GelMA/gelatin bioinks was desirable. The versatility of bioprinting was well exhibited through several unicursal patterns. When using a coaxial nozzle-18G/25G, all printed patterns that consisted of one continuous hollow filament closely resembled the morphology of targets (Figure 3B and Figure S3).

3.2.3. Flow rate analysis

The outer diameter of vessel-like structures biofabricated through nozzle-18G/25G was approximately 1200 μm. To biofabricate large-scale tubular constructs mimicking human blood vessels for perfusion culture, the use of nozzles with larger diameters was inevitable. Nozzle-1-2.5 (inner/outer diameters of the nozzle were 1 mm/2.5 mm) and nozzle-1-3 (inner/outer diameters of the nozzle were 1 mm/3 mm) were adopted for the experiments below. The printability windows of nozzle-1-2.5 and nozzle-1-3 are shown in Figure 3D as the inner/outer flow rates varied. If the difference between the flow rate of the outer channel ($Q_{outer}$) and the flow rate of the inner channel ($Q_{inner}$) was too large, the tubular lumen could not be maintained; if $Q_{outer}$ and $Q_{inner}$ were too close, the shell layer could not completely enfold the core layer. These two conditions were considered not printable since no clear hollow tube was achieved.

Apparently, the construction of tunable tubular structures could be achieved by regulating the flow rate of GelMA/gelatin or changing the size of nozzles. It was easy to understand that the fiber extrusion speed was affected by the flow rate, and the inner/outer diameters of the printed fibers were affected by the ratio of the outer/inner flow rate ($Q_{outer}/Q_{inner}$). Figure 3E displays the variation of printed fiber diameters corresponding to the modulation of $Q_{outer}/Q_{inner}$ while using nozzle-1-2.5/nozzle-1-3. It is worth noting that the outer diameter of the fiber remained almost the same as a little bit larger than the outer diameter of the nozzle. Along with the decrease in $Q_{outer}/Q_{inner}$, the inner diameter of the fiber increased gradually.

In consideration of a good nutrient supply and subsequent unobstructed perfusion, the tubular constructs with outer/inner diameters of approximately 3 mm/1 mm were ideal. Hence, nozzle-1-3 was selected for follow-up bioprinting. The relationship between the outer/inner flow rate and outer/inner fiber diameter could be calculated according to the formula $Q_{outer}/Q_{inner} = \frac{S_{outer}}{S_{inner}} = \frac{D^2 - d^2}{d^2}$, where $S_{outer}$ and $S_{inner}$ were the cross-sectional area of the outer/inner channel of the fiber, and $D$ and $d$ were the outer/inner diameters of the fiber. Putting $D = 3$ mm, and $d = 1$ mm into the formula, $Q_{outer}/Q_{inner} = 8$, which was identical to the result presented in Figure 3Eii. Therefore, $Q_{outer}$ of 0.3 ml/min and $Q_{inner}$ of 0.0375 ml/min were employed in subsequent experiments. Under these bioprinting parameters, the average outer/inner diameters of hydrogel tubes were 3116 μm and 1063 μm, respectively.

3.3. Comparison of hydrogel constructs with/without a PCL stent

3.3.1. Mechanical properties of the hydrogel bulk with/without a stent

To better understand the advantage of PCL stents during perfusion culture, a mechanical property test was first applied to measure the improvement of the structural deformation-resistance capacity that a stent would provide, as shown in Figure 4Ai. The hydrogel bulks containing tube with/without stent were exhibited vividly in lateral and cross sections of SEM images in Figure S4. Figure 4Aii shows the compressive stress-strain curves of the bulks. In the early stage of the curves (strain <20%), it was mainly the hydrogel part that resisted deformation by force, whereas the two curves were almost indistinguishable. As pressure increased (strain >20%), the tubular lumen of the bulk began to be squeezed. The hydrogel bulk with a stent presented increasing superiority with ascending force. The higher the strain was, the greater the stress differed between bulks with/without a stent. When facing the same stress of 30 kPa for instance, the hydrogel bulk with a stent sustained a strain of approximately 60% while the bulk without a stent reached 70%. The stresses the hydrogel bulk experienced at the same strain (50% and 60%) were also studied, as shown in Figure 4Aiii. At a strain of 50%, the stress the bulk with a stent sustained was 1.53 times greater than that of the bulk without a stent. At the strain of 60%, this value increased to 2.2 times. These results demonstrated that the PCL stent offered greater resistance to deformation.
3.3.2. Patency of hydrogel tubes with/without a stent after swelling

Not only the mechanical properties, but also the swelling characteristics of hydrogels need to be considered during perfusion. In general, hydrogel tubes reach swelling equilibrium after being immersed in culture medium for 24 h, leading to the expansion of the tube wall and the narrowing of the inner channel. After reaching swelling equilibrium, the introduction of a PCL stent guaranteed that the inner diameter of the hydrogel tube would be 1212 μm, while the one without a stent possessed a channel diameter of 879 μm (Figure 4B).

3.3.3. Perfusion performance of hydrogel tubes with/without a stent

As stated above, GelMA (EFL-GM-30) is a soft hydrogel that may lead to tube collapse during perfusion. A perfusion performance test was conducted to assess whether liquid could flow through the hydrogel tubes with/without a stent at different flow rates (Figure 4C, Videoclip S1). Four flow rates from 6 μl/min to 6000 μl/min were selected. At all flow rates, liquid could flow through smoothly with the help of the PCL stent, while the tube without a stent faced the problem of wall collapse resulting in liquid backflow. These findings showed that a PCL stent served was a key factor in the long-term perfusion process.

3.4. Diffusion analysis of the hydrogel tube

For the purpose of characterizing the diffusional permeability of hydrogel materials and the barrier function of the endothelialized fabricated vessel, FITC labeled dextran with a molecular weight of 40 kDa was utilized. As illustrated in Figure 5G, after injecting FITC-dextran solution into the hydrogel tube, fluorescence microscopy images were captured at 5–80 min, at intervals of 15 min. Hydrogel tube without cells (Figure 5A-C) and endothelialized hydrogel tube, which was cultured 7 days after printing (Figure 5D-F), were tested under the same condition. In terms of general trends, dextran was gradually diffused from the central channels to the surrounding hydrogel tube (Figure 5A and D); the gray level distribution (Figure 5B and E) became broader and more dispersed with time, and the curve of which was flattened; moreover, as illustrated in the gray level distribution histograms in Figure 5C and F, the gray levels mainly concentrated on high values at the beginning of the test, and the peak of the gray levels moved toward lower values as time passed. Obviously, the diffusion rates in HUVEC-laden tube were much slower than that in the cell-free tube. Even in the beginning of the test, without the barrier function of endothelial cells, diffusion
area of cell-free tubes was much larger than that of cell-laden tube, and this gap in area widened over time, as shown in Figure 5H. It is quite obvious that after 80 min of diffusion, dextran in cell-free tube spread much further. All of that proved a tight connection among HUVECs realized in endothelialized fabricated vessel.

3.5. Bioactivity characterization of cell-laden constructs

3.5.1. Bioactivity of fabricated vessels

To guarantee that HUVECs were viable and functionalized in the following period of perfusion culture, the endothelialization of HUVEC-laden hydrogel tubes was a primary precondition. Confocal fluorescence microscopy images of cell morphologies were captured on day 1, day 4, and day 7 to prove marked cellular spreading and proliferation (Figure 6A). HUVECs reproduced rapidly inside the hydrogel tube within 7 days of culture. The quantitative analysis of cell activity also verified this process (Figure 6B). It can be found that after 7 days of culture, the HUVEC layer could be visualized through bright field of microscope, covering the entire surface of the tube, meaning a HUVEC-well-spread hydrogel tube was achieved (Figure 6C). To facilitate the observation of subsequent experiments, green fluorescent protein-labeled HUVECs (GFP-HUVECs) were utilized. Confocal images of three views and cross-section view of this endothelialized hydrogel tube displayed the uniform distribution of endothelial cells in the fabricated vessel and the tight intercellular conjunction among HUVECs (Figure 6D).

3.5.2. Functionalization of HUVECs after perfusion culture

After the endothelialization of HUVECs, the hydrogel tube was inserted by a PCL stent and cast into bulk with
GelMA for perfusion culture afterward. To transport nutrients effectively without injuring the inner wall of the vessel, the flow rate of perfusion culture was set to 600 μl/min. After perfusion culture for 3 days, immunostaining with vinculin antibody and zona occludens 1 (ZO-1) antibody was applied to evaluate the functionalization of HUVECs. The appearance of vinculin, a focal adhesion protein among the cell-extracellular matrix, confirmed a firm adhesion between HUVECs and hydrogel was generated. The expression of ZO-1, which is an important protein marker to intercellular junction, demonstrated a fairly tight cell-cell connection. The whole perfusion system was shown in Figure 6G. Each end of the silicone tube was connected to a needle, which was pierced into the injection stopper (Figure S5) of perfusion chip inlet/outlet separately. To maintain proper pH value during perfusion culture, culture medium container was equipped with a channel coupled with a filter for gas exchange without causing contamination.

### 3.6. HUVEC sprouting and antiangiogenic drug screening model

To establish an antiangiogenic drug screening model, observable HUVEC sprouting based on the perfusion system was realized. As demonstrated in Figure 2Avi, the introduction of VEGF (200 ng/ml) in hydrogel bulk created directional guidance for the HUVECs encapsulated in endothelialized vessels in subsequent continuous perfusion culture, during which cells sensed gradient concentrations of VEGF and began to sprout outward. After 3 days of perfusion culture, spouting of HUVECs was distinctly observed by confocal fluorescence microscopy images (Figure 7Ai) and optical images (Figure 7Bi). With the help of a live cell monitoring system, the sprouting process of HUVECs was clearly recorded in the first 48 h of perfusion (Videoclip S2).

Bevacizumab, a recombinant humanized monoclonal antibody directed against VEGF, has been proven to be effective in tumor therapy, during which it binds to VEGF...
and inhibits VEGFR binding, thereby preventing tumor blood vessels from being maintained or developing. Three concentrations of bevacizumab (MedChemExpress, US) were employed for drug screening through the perfusion system: 10 ng/ml, 50 ng/ml, and 100 ng/ml. After perfusion culture for 3 days, the levels of HUVEC sprouting differed varying concentrations of bevacizumab, and these factors were negatively correlated (Figure 7A). For the control groups perfused with no bevacizumab, HUVECs sprouted prosperously (Figure 7Ai), whereas the samples perfused with 100 ng/ml had almost no spouting (Figure 7Aiv). Optical images of the antiangiogenic drug screening model were also taken to evaluate the effect of different bevacizumab concentrations on HUVEC sprouting (Figure 7B). Based on these images, a quantitative analysis of HUVEC sprouting with perfusion of varying drug concentrations was conducted. The differences in sprouting numbers in the same view were tremendous (Figure 7Ci), which could also be easily found from optical images. Nevertheless, the differences in the average lengths of sprouting were not very large (Figure 7Cii). Finally, the relative areas of sprouting in the same view were evaluated. Due to the large gaps in sprouting numbers, relative areas exhibited significant variances with varying concentrations of bevacizumab (Figure 7Ciii).

4. Discussion

In addition to bevacizumab, other drugs could either share the same or use different antiangiogenic mechanisms. These drugs include: Ranibizumab, which is a monoclonal antibody that binds to all active forms of VEGF-A and inhibits its activity; aflibercept, which is a recombinant fusion protein that functions as a decoy VEGFR with a propensity to bind VEGF-A, VEGF-B, placental growth factor (PIGF)-1, and PIGF-2 and prevents the binding and activation of their cognate receptors; and regorafenib, which is an orally active diphenylurea multikinase inhibitor of VEGFR1-3, protooncogene c-KIT, tyrosine-protein kinase receptor TIE-2, platelet-derived growth factor receptor-b, fibroblast growth factor receptor-1, and tyrosine-protein kinase receptor RET, etc. Although the antiangiogenic mechanisms of these drugs are multifarious, there are no contradictions in principle when screening them with this perfusion system. More works should be done to verify the universality of our antiangiogenic drug screening model.

As we mentioned above, the vessel-on-a-chip we use in this work satisfies the need for both long-term perfusion (up to 10 days according to different hydrogel formulas) and easy observation. The elaborate design of the perfusion system ensures that all components are sterilizable at high temperature and recyclable (except for the PDMS wall, for which we cannot guarantee the absence of leaks upon reuse). The observable window of our perfusion chip makes real-time monitoring feasible. Moreover, it is highly modularly integrated with the injection stopper in both the inlet and outlet, which offers a quickly-separable option for observation under a microscope in the middle of perfusion days (Figure S5). As shown in Figure 6G, there are needles connected to the silicone tube both from/to the peristaltic pump. To
pause perfusion culture, one needs to: (i) Turn off the peristaltic pump; (ii) fasten the Robert clamps of inlet and outlet separately; (iii) pull out the needles from the injection stoppers at both ends, and vice versa. A quickly-separable perfusion chip is then obtained without the risk of bacterial contamination. Side needles are applied at the very start of perfusion to fill the cavity with medium and exhaust gas. In addition, compared to microfluidic chips, our chip more closely mimics the vascular environment in the human body with a larger-scale and higher flow rates, which greatly expands its possible applications.

Nevertheless, the present study still has room for improvement. Even though this system provided a long-term perfusion capability, the hydrogel bulk containing VEGF limited the subsequent culture duration. Since VEGF would certainly permeate into the culture medium gradually, the stimulus to HUVECs sprouting toward the bulk would dissipate after several days. Introducing tumor cells, which can secrete VEGF persistently, might be a good solution. Instead of VEGF-containing, applying tumor-cell-laden GelMA to cast into a hydrogel bulk enveloping endothelialized vessels would realize a tumor-cell-HUVEC coculture system, which would transform this antiangiogenic drug screening model into a more specific tumor model. In addition, the improvement of coaxial bioprinting process can bring deeper significance to biological applications. For example, three-layer coaxial bioprinting of endothelial cells layer and smooth muscle cells layer would make these vessels more biologically representative.

Furthermore, the drug screening model we established in this study has great potential for the future studies. Based on the vessel-on-a-chip approach, our perfusion system cannot only be regarded as an antiangiogenic drug screening model, but also serve as an effective tool to model interactions between various cells/organoids by changing the perfusion liquid, encapsulated cells in the tube, or bulk containing substances/biological reagents. For example, to explore the effects of anti-inflammatory compounds on coagulation and thrombosis could be another application scenario of this model. In addition, based on the advantage of coaxial bioprinting process, biofabrication of hydrogel tubes with variable-diameter or complex shapes would simulate a particular vascular microenvironment or a disease model. Adopting more than one vessel in one chip with different perfusion liquids would also meet the requirements of some in vitro models. Moreover, connecting several perfusion chips in series with one-way flow would further provide a platform for investigating the effects of upstream secretions on downstream components.

5. Conclusion

In summary, we coaxially bioprinted HUVEC-laden GelMA tubes and cultured them to form endothelialized biomimetic blood vessels. With the assistance of a PCL stent, a perfusion system was then set up for antiangiogenic drug screening. Guided by rheological tests, the printability of coaxial bioprinting is discussed. The significance of the inserted stent was also demonstrated by the mechanical, swelling, and perfusion tests. Afterward, diffusion analysis proved the barrier function of the endothelialized vessel. As a proof of concept, HUVECs encapsulated in hydrogel constructs showed an excellent proliferation rate, good cell morphology, and expression of certain functional markers. Finally, images and videoclips of HUVEC sprouting and bevacizumab-screening test revealed the reliability of this perfusion system. We believe that this proposed biofabrication process and its application may open up a new approach for the construction of vessel-on-a-chip and offer an accessible tool for vascularization and pharmaceutical research.

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Conflict of interest

The authors declare no conflicts of interest.

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