Three-dimensional bioprinting of aneurysm-bearing tissue structure for endovascular deployment of embolization coils

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Abstract
Various types of embolization devices have been developed for the treatment of cerebral aneurysms. However, it is challenging to properly evaluate device performance and train medical personnel for device deployment without the aid of functionally relevant models. Current in vitro aneurysm models suffer from a lack of key functional and morphological features of brain vasculature that limit their applicability for these purposes. These features include the physiologically relevant mechanical properties and the dynamic cellular environment of blood vessels subjected to constant fluid flow. Herein, we developed three-dimensionally (3D) printed aneurysm-bearing vascularized tissue structures using gelatin-fibrin hydrogel of which the inner vessel walls were seeded with human cerebral microvascular endothelial cells (hCMECs). The hCMECs readily exhibited cellular attachment, spreading, and confluency all around the vessel walls, including the aneurysm walls. Additionally, the in vitro platform was directly amenable to flow measurements via particle image velocimetry, enabling the direct assessment of the vascular flow dynamics for comparison to a 3D computational fluid dynamics model. Detachable coils were delivered into the printed aneurysm sac through the vessel using a microcatheter and static blood plasma clotting was monitored inside the aneurysm sac and around the coils. This biomimetic in vitro aneurysm model is a promising method for examining the biocompatibility and hemostatic efficiency of embolization devices and for providing hemodynamic information which would aid in predicting aneurysm rupture or healing response after treatment.

1. Introduction
Aneurysms, localized dilations or ‘ballooning’ of arterial vessel walls in the brain due to the abnormal weakening of the muscular layer of a blood vessel, affect 2%–3% of adults and their rupture leads to subarachnoid hemorrhage (SAH) [1]. Approximately 85% of all SAHs are due to the rupture of saccular aneurysms and most of the cases involve debilitating or fatal complications such as permanent neurological deficit or death [1, 2]. The highly detrimental effects which can arise from an aneurysm rupture highlight the critical need for the development of devices that can effectively prevent the rupture and for testing the safety and effectiveness of those devices before clinical use.

Surgical clipping has been a long-standing standard treatment method for aneurysms [3, 4]. During surgical clipping, a clip is placed over the neck of the aneurysm to isolate it from the main bloodstream. However, since surgical clipping involves highly invasive intracranial surgery and some aneurysms are located in inaccessible regions within the brain, endovascular treatment using platinum coils has become an increasingly employed alternative. Platinum coils are delivered into the aneurysm using a microcatheter. Once the aneurysm is packed with coils, a thrombus forms which leads to the...
occlusion of blood flow within the aneurysm. In highly successful endovascular treatments, regrowth of the endothelium across this thrombus is observed at the neck of the aneurysm [5–7]. Endovascular treatment using coils possesses several advantages over surgical clipping such as lower invasiveness, decreased expense and duration of hospitalization, shorter patient recovery times, and limited post-surgical complications both in ruptured and unruptured aneurysms [4, 8]. These attractive features of endovascular treatment led to the active research and development of various endovascular devices to further enhance treatment efficacy, such as hydrogel coated coils [9–11], growth factor coated coils [12, 13], and shape memory polymer foam coated coils [14]. However, in vitro evaluation of clotting performance and predicting outcomes for new devices remain challenging.

Engineering an in vitro aneurysm model is desirable to test these next-generation endovascular medical devices for training practitioners how to deploy these devices, to develop accurate computational simulations, and for understanding how effectively treatments induce clotting or lead to a healing response [15–20]. Previously, silicone or clear liquid resin molding was used to simulate human cerebral aneurysms using the lost wax technique. In this approach, silicone or resin is poured over wax models of human vascular pathologies inside a transparent plastic box to eventually create a silicone hollow vascular channel that is compatible with x-ray and magnetic resonance imaging (MRI) and amenable to a visualization of vascular flow [18]. Modero et al fabricated a patient-specific aneurysm model by three-dimensional (3D) printing aneurysm geometry obtained from 3D computed tomography (CT) angiogram using water-dissolvable material which was then submerged in silicone and the printed geometry was dissolved after the silicone was cured [21]. Ohta et al used poly-vinyl alcohol hydrogel instead of silicone to simulate the stiffness of the in vivo tissue which allowed for pulsations during flow [22]. However, these studies were confined to mimicking the anatomical aneurysm shape or the mechanical stiffness of vascular tissues, and not the physiological cellular structure of blood vessels or extracellular matrices, which is a fundamental aspect of a functional in vitro aneurysm model for testing biocompatibility and healing efficiency of endovascular devices.

To study the cellular responses and healing effects of the endovascular devices, in vivo studies are generally performed in various animal models by artificially generating aneurysms using balloon inflation or elastase infusion techniques [23–25]. However, reproducing the same anatomy or the same hemodynamic forces of human cerebral aneurysms in animal models is not currently achievable [24]. In addition, the artificially created surgical wound induces cellular migration (e.g. fibroblasts) into the lumen which is not observed in human cerebral aneurysms [24]. Healing responses also vary significantly between species which makes it challenging to apply the animal testing results to human physiology.

In this work, we present the first-ever in vitro living 3D printed aneurysm possessing a fully-encapsulating extracellular matrix to interrogate treatment with an endovascular device. The model supports the growth of endothelial lined channels in a biocompatible matrix with physiologically relevant mechanical stiffness to brain tissue. The 3D printed platform is transparent and therefore allows for flow visualization at varying flow rates within the in vitro aneurysm through particle image velocimetry (PIV). We establish in this work the utility of this platform to provide critical data for 3D computational flow models and further demonstrate the successful deployment of detachable coils inside the cell-laden aneurysm sac using a microcatheter to observe the in vitro clotting response of plasma.

2. Methods

2.1. Materials

Thrombin from bovine plasma (T4648, Sigma), Pluronic F127 (P2443, Sigma), gelatin from porcine skin, ~300 g Bloom, type A (G1890, Sigma), fibrinogen from bovine plasma, type I-S, 65%–85% protein (F8630, Sigma), transglutaminase, TI formulation (TG) (Modernist Pantry), sodium hydroxide (NaOH) (S2770, Sigma), phosphate buffered saline without calcium and magnesium, pH 7.4, 1× (PBS) (10010-02, Gibco), Hank’s balanced salt solution with calcium and magnesium, 1× (HBSS) (14025-092, Gibco), Calcium chloride (CaCl₂) (C1016, Sigma), SE-1700 silicone, clear base, and SE-1700 catalyst (Dow Corning), Bovine plasma with sodium citrate (IGBOPLANAC500ML, Innovative Research), 1 µm 580/605 red fluorescent FluoSpheres (F13083, Thermofisher), 10% w/v neutral buffered formalin (VWR) info, Alexa Fluor 488 phalloidin (A12379), Alexa Fluor 594 conjugated fibrinogen (F13193, Thermofisher).

2.2. HCMEC/D3 cell culture and maintenance

Cultures of immortalized human cerebral microvascular endothelial cells (hCMEC/D3, Cedarlane) were initiated in T-75 flasks pre-coated with 150 µg/mL rat tail collagen-Type I (Corning). Cells were maintained in EndoGRO MV culture media (Millipore-Sigma) supplemented with 1% Penicillin-Streptomycin (Gibco) and 1 ng ml⁻¹ human bFGF (Sigma) in a humidity-controlled CO₂ incubator held at 37 °C and 5% CO₂. The media was changed every other day until cultures were confluent and then sub-cultured according to the vendor’s protocol.
2.3. 3D printing of silicone sidewalls for bioreactor

Bioreactor sidewalls for the aneurysm platform were designed in SolidWorks, converted to G-code using open source Slic3r software, and then modified to integrate pump calls for the Ultimus V dispenser (Nordson EFD) using a custom Matlab script. Bioreactor walls were fabricated using SE-1700 silicone, prepared at a 10:1 elastomer to catalyst ratio and mixed with an ARV-310 centrifugal mixer (Thinky) at 2000 rpm for 2.5 min followed by a 15 min cooling step. This was repeated twice before loading the silicone material into a 10 ml syringe (Nordson EFD) and 3–5 min centrifugation of the syringe at 3234 rcf to eliminate bubbles. The walls were then printed onto precleaned 3” × 5” glass slides (VWR) using a custom extrusion-based bioprinter composed of CNC motion stages and controllers (Aerotech) connected to an Ultimus V precision dispenser. Post-printing, SE-1700 walls were cured at 65 °C for at least 2 h, followed by autoclave sterilization.

2.4. Fugitive vascular ink preparation

Fugitive ink was prepared as previously described [26]. Pluronic F127 (34.1 wt%) and 100 U ml⁻¹ (final concentration) thrombin in deionized water were added into a cold syringe and centrifuged at 2000 rpm for 30 s for homogeneous mixing. The ink was cooled at −20 °C for 5 min and centrifuged again at 2000 rpm for 30 s. A piston was plunged into the syringe and the prepared ink was stored at 4 °C until use.

2.5. Matrix preparation

Gelatin stock solution was prepared by dissolving 15 g of gelatin powder in 75 ml of PBS. The mixture was placed on a magnetic stir plate set to 150 rpm at 70 °C overnight until fully dissolved. The pH value of the solution was adjusted to 7.5–7.54 using 1 N NaOH and brought to a final volume of 100 ml using additional PBS. The solution was then filter sterilized through a 0.22 µm polyethersulfone (PES) filter, followed by aliquoting and storage at 4 °C until use. CaCl₂ stock solution (250 mM) was prepared by dissolving the salt in PBS on a magnetic stir plate set to 100 rpm at 90 °C until dissolved. The solution was then filter sterilized to remove any precipitate and stored at 4 °C until needed.

Fibrinogen (50 mg ml⁻¹, Sigma) was dissolved at 4 °C in PBS overnight, followed by warming to 37 °C before use. TG solution (60 mg ml⁻¹) was prepared by dissolving TG powder (Moo Glue Ti, Modern Pantry) in PBS less than 1 h before use. The fibrinogen and TG solutions were then sterile filtered before mixing steps were performed. All components were warmed to 37 °C before mixing steps. Fibrinogen, PBS, TG, and CaCl₂ solutions were mixed at 37 °C and held for 5 min before the addition of gelatin (15 wt%). After gelatin was added the mixture was held at 37 °C for 20 min. Finally, 200 U ml⁻¹ thrombin in PBS was added to the solution at a final concentration of 1 U ml⁻¹ and mixed. Final concentration of all components was 7.5 mg ml⁻¹ gelatin, 10 mg ml⁻¹ fibrinogen, 2 mg ml⁻¹ TG, and 2.5 mM CaCl₂, and 1 U ml⁻¹ thrombin. The solution (1 ml) was quickly cast into the bioreactor, which was followed by the removal of 800 µl of the solution to remove any bubbles and leave a thin base layer. This layer was allowed to cross-link and partially dry at room temperature for 35 min before the printing step.

2.6. Fabrication of vascular tissue structure

A 1.19 mm nozzle (Nordson EFD) was used to print the fugitive ink on the base layer, forming the basis for the resulting vessel channels and aneurysm dome. The same matrix formulation used for the base layer gel was used to make the encapsulating gel solution. After encapsulating the printed vascular ink with the matrix solution, the silicone walls were then sealed with an additional glass slide. The bioreactor was then incubated at 37 °C for an hour, followed by incubation at 4 °C for 20 min to liquify the printed fugitive ink. Fugitive ink was removed by pulling out the liquified solution with a syringe, followed by rinsing with 5 ml of cold EndoGRO MV culture media.

2.7. Perfusion fluids setup

The perfusion fluidics entry and exit reservoirs each consisted of GL45 glass bottles (Corning) with a two-port, 1/4-28 threaded, cap (Diba OmniFit). Each reservoir was connected to an air tubing (Tygon) in one port and a silicone media line in the other. Air tubing connected to the entry reservoir included two in-line PES, 0.2 µm membrane syringe filters (Whatman) to ensure that the reservoir was not contaminated by the compressed air used to pressurize the reservoir for flow. The air tubing connected to the exit reservoir had one PES syringe filter in-line in order to both equilibrate the contained media and to release the pressure within the reservoir as media is passed through the platform.

After fluidic tubing (IDEX) was connected to the reservoirs, 85 ml of Endogrow MV media (equilibrated with 5% CO₂ for at least 2 h) was then deposited in the entry reservoir, followed by 10 ml of media into the exit reservoir. Additionally, 5 ml of media was used to charge the fluidic tubing with media before flow connections were made. MFS3 flow rate sensors (Elveflow) were sterilized with a 10% bleach solution for 10 min followed by an eight times rinse with sterile dH₂O. The media line from the entry reservoir was then connected to one end of the flow rate sensor, with the other end of the flow sensor connected to the tubing extending from the bioreactor. The silicone tubing from the opposite side of the reactor was then connected via a union fitting to the exit reservoir’s media line, completing the perfusion path. The air line attached to the entry reservoir was then
connected to an OB1 microfluidic flow controller (Elveflow) to enable pressure control over the media within the reservoir. After the perfusion fluidics were constructed, the entire bioreactor was transferred to an incubator at 37 °C, 5% CO₂ and the flow rate was maintained at 10 µl min⁻¹ overnight.

2.8. Endothelialization of aneurysm-bearing vessels

The in vitro aneurysm bioreactors were perfused with media at 10 µl min⁻¹ for 24 h before hCMECs were seeded within the channel lumen. The hCMECs were harvested and resuspended to a concentration of 1 × 10⁶ cell ml⁻¹ and allowed to attach to the channel for 15 min at 37 °C. The bioreactor was then inverted and allowed to sit for an additional 15 min at 37 °C. This cycle was repeated three times total and followed by a waiting period of 4 h before the bioreactor was reconnected to flow at a rate of 10 µl min⁻¹ for long-term culture. Every 4 d, 60 ml of media was exchanged between the exit reservoir and the entry reservoir for continued culture.

2.9. Particle image velocimetry (PIV) analysis

PIV was performed on day 7 for all experiments involving endothelial cells. PIV was conducted using an Olympus IX83 inverted microscope equipped with LaVision FlowMaster 2-C components, including an Imager M-lite 2 M CMOS camera, a 1 W, 532 nm gateable DPSS laser, and a PTU-X HS timing unit. A solution of fluorescent beads was prepared containing 3.49% 1 µm 580/605 FluoSpheres (Thermofisher) suspended in HBSS with ions. The bead solution was perfused through the in vitro aneurysm using a KDS-210 syringe pump (KD Scientific) set to volumetric flowrates ranging from 300 µl min⁻¹ to 20 ml min⁻¹. PIV imaging was performed at predetermined positions within the artificial aneurysm and the Z-plane of imaging was selected by determining the focal point at the widest diameter of the vessel. PIV images were captured and processed using DaVis 10 software (LaVision) and exported as .dat files for comparison and incorporation into the fluid dynamics computational model.

2.10. Fluorescence staining and imaging

After PIV imaging, printed vessel geometries were fixed in formalin and stained for actin. Briefly, fixed samples were rinsed twice with PBS, permeabilized with 0.5% (v/v) Tween-20 and stained with 1:40 Alexa Fluor 488 phalloidin for at least 1 h at room temperature. Stained vessel geometries were imaged and reconstructed using tile-stitched Z-stacks gathered using a Zeiss LSM700 confocal microscope and Zen 2012 SP1 software (Carl Zeiss). To confirm the presence of an endothelial monolayer, even at extended culture times, one device was carried out to DIV 16, stained then imaged using confocal microscopy where Z-stack projection imaging and color-coded Z-depth analysis was performed to verify the presence of a single endothelial layer.

2.11. Computational flow modeling

The 3D surface model was reconstructed from segmented images of the aneurysm-bearing tissue structure, obtained by confocal microscopy, using the thresholding technique in the commercial image processing software Materialise Mimics. The 3D reconstructed surface model was exported as a mesh file in stereolithography (STL) file format. The open source modeling software Blender was used for mesh editing and converting the mesh file from STL to object file format (OFF), which can be imported into the computational fluid dynamics (CFD) solver. Volume measurements can be automatically obtained by the 3D printing toolbox, available as an add-on in Blender.

To computationally model the flow, we employed HARVEY, the in-house developed massively parallel CFD solver [27]. HARVEY is based on the lattice Boltzmann method (LBM), a mesoscopic modeling approach. In the LBM framework, distribution functions \( f_i(\vec{x}, t) \) representing the density of particles at position \( \vec{x} \) and time \( t \) with velocity \( \vec{c}_i \) along the \( i \)-th lattice direction are tracked. The evolution of the distribution functions is governed by the lattice Boltzmann equation:

\[
f_i(\vec{x} + \vec{c}_i \Delta t, t + \Delta t) = f_i(\vec{x}, t) + \Omega_i(\vec{x}, t).
\]

Here, the Bhatnagar–Gross–Krook collision operator is considered, \( \Omega_i(f) = -\frac{\Delta t (\vec{c}_i - \vec{u})}{\tau} \) [28]. The equilibrium distribution function \( f_i^{eq} \) is given by the Maxwell–Boltzmann distribution function. The relaxation time \( \tau \) is determined such that \( f_i \) relax to \( f_i^{eq} \), it is related to the fluid kinematic viscosity by \( \nu = \frac{\sqrt{c_i}}{(\Delta x \Delta t)} \). \( c_i = \frac{\Delta t}{\sqrt{\Delta x \Delta t}} \) denotes the lattice speed of sound, with \( \Delta x \) and \( \Delta t \) being respectively the lattice spacing and time step. The macroscopic quantities of the fluid, namely the density \( \rho \) and velocity \( \vec{u} \), are given by the 0-th and 1-st moments of the distribution function:

\[
\rho(\vec{x}, t) = \sum_i f_i(\vec{x}, t),
\]

\[
\vec{u}(\vec{x}, t) = \frac{\sum_i \vec{c}_i f_i(\vec{x}, t)}{\rho(\vec{x}, t)}.
\]

The simulations are performed using the D3Q19 lattice arrangement [29]. The fluid kinematic viscosity and density are taken equal to those of the bead solution in HBSS (with ions), that is \( \nu = 0.9565 \text{ mm}^2 \text{s}^{-1} \) and \( \rho = 995.609 \text{ kg m}^{-3} \). To ensure the stability and accuracy of the numerical solutions, we consider a relaxation time of \( \tau = 1 \) and 0.6 and fluid resolutions of 10 and 5 µm at the 0.3 and 20 ml min⁻¹ flowrates, respectively. Finally, the no-slip boundary
condition on the structure walls is applied by the half-way bounce-back scheme [30], while the finite difference boundary scheme [31] is employed for enforcing the desired velocity and pressure profiles at the structure’s inlet and outlets.

2.12. Deployment of detachable coils into the aneurysm platform
The aneurysm system used for coil deployment testing was cultured for 9 d before testing. A microcatheter (Vasco+, Balt Extrusion) was used to deliver two detachable coils (Barricade Complex Finish, 2 mm × 3 cm and 3 mm × 6 cm, Blockade Medical) into the printed aneurysm sac under static conditions. The microcatheter was carefully inserted into the metal pin and navigated to the aneurysm sac using brightfield microscopy. After placing the tip of the microcatheter at the neck of the aneurysm sac, the coil was dispensed from the microcatheter using the push wire until the entire coil was deployed in the aneurysm. The coil was detached from the push wire using an electrolytic detachment controller (Shape Memory Medical). The entirety of the insertion and detachment processes were monitored in real-time using brightfield microscopy.

2.13. Coil packing density calculation
The packing density of coils was calculated using the equation below.

\[
\text{Packing density} = \frac{\text{Coil volume}}{\text{Aneurysm sac volume}} \times 100\%
\]

The coil volume was calculated by assuming the bare platinum coil is a simple cylinder. The diameters of all the Barricade coils used in this study were 0.011”. The length of coils used for the deployment testing was 9 cm and the coil length for the plasma clotting test was 8 cm. The aneurysm volume was measured from the reconstructed model in the computer simulation which provided accurate volumes of non-spherical aneurysms.

2.14. Plasma clotting within the coil-treated aneurysm sac
For clotting test, the aneurysm platform was cultured with hCMECs for 9 d and a detachable coil (3 mm × 8 cm) was delivered into the aneurysm sac using a microcatheter as mentioned above. Bovine plasma anticoagulated with sodium citrate was used for the clotting experiment. To initiate clotting, a stock solution of 800 mM CaCl₂ was added to the plasma to a final concentration of 10 mM. One hundred forty-seven microliters of 1.5 mg ml⁻¹ fluorescent fibrinogen stock was added to 10 ml of plasma which was then perfused through the channels using a syringe. The channels filled with plasma were held for 30 min at room temperature under static conditions. After 30 min, the channels were thoroughly rinsed via injection with 10 ml of HBSS to remove any uncoagulated plasma from inside the vessel channels. Clot formation within the aneurysm platform was then imaged using confocal microscopy.

3. Results

3.1. Fabrication of aneurysm-bearing tissue structure
The geometry of the printed aneurysm at the branching vessels is representative of an aneurysm at the basilar artery bifurcation. The elemental geometries used were based on previous publications where an idealized 2D computational model was developed to mimic an aneurysm at the basilar artery bifurcation [32, 33]. A 3.6 mm diameter aneurysm dome was created with an aneurysm neck width of 1.9 mm. The parent vessel was 2.4 mm wide while the branching vessels were 1.8 mm wide from the top point of view. The afferent and efferent vessels were connected to stainless steel tubes which were connected via fluidic tubing to media reservoirs with a pressurized entry reservoir and a vented exit reservoir, respectively. This bioreactor system allowed a constant flow of cell media through the vessels in the bioreactor (figure 1(a)). Fluorescent beads were perfused through the channels to confirm the complete evacuation of the fugitive ink from the complex aneurysm-bearing vessel structure. Precise and clear vessel structure embedded in the transparent gelatin-fibrin hydrogel were observed without showing any leakage from the vessels (figure 1(b)).

3.2. Particle image velocimetry (PIV) measurement
Analyzing the hemodynamics around an aneurysm is very critical in understanding the mechanisms of aneurysm pathogenesis, progression, and rupture [34, 35], evaluating the treatment efficacy [36], and predicting the occlusion time after the treatment [37]. PIV is a strong analyzing tool to quantitatively measure the flow dynamics within cerebral aneurysms and to validate computational simulation or clinical results [38]. Figures 2(a) and (b) demonstrate the flow dynamics within the channels of the in vitro living aneurysm platform. A flow rate of 300 μl min⁻¹ was supplied to the channels which led to the average flow velocity (Uavg) of 1.18 mm s⁻¹ for the parent vessel, 1.86 and 1.71 mm s⁻¹ for the left and right daughter vessels respectively, 0.19 mm s⁻¹ at the neck of the aneurysm, and no detectable flow inside the aneurysm sac (figure 2(a) and supplementary information table S1 (available online at stacks.iop.org/BF/13/015006/mmedia)). As the flow rate increased, the fluid motion within the aneurysm dome also increased and began exhibiting intraneurysmal circular flow at 20 ml min⁻¹ flow rate showing Uavg of 0.88 mm s⁻¹ (figure 2(b) and supplementary information table S2). The high flow velocities of fluorescent beads in the parent and daughter vessels at 20 ml min⁻¹ flow rate, however,
Figure 1. The *in vitro* living cerebral aneurysm. (a) Illustration of the 3D printed aneurysm bioreactor. (b) The *in vitro* aneurysm vessel structure perfused with red fluorescent beads, demonstrating the formation of patent vessels post-evacuation of sacrificial ink.

Figure 2. Particle image velocimetry (PIV) analysis and 3D computational flow model simulations. (a) (Above) PIV measurement at the back of the aneurysm dome showing no detectable flow at a 300 µl min⁻¹ flow rate. (Middle) A 3D flow simulation of the same geometry and flow rate at z = −0.66 mm. (Below) PIV measurements within the parent and daughter vessels captured with a 2× objective at the same flow rate. (b) (Above) PIV measurement gathered at the back of the aneurysm dome showing circular flow patterns and captured with a 4× objective at a 20 ml min⁻¹ flow rate. (Below) Simulation of the same geometry and flow rate demonstrating that fluid motion only occurs within the dome at high flow rates. (c) High-fidelity geometric reconstruction of the printed living aneurysm constructed from image stacks gathered via confocal microscopy.

could not be measured using PIV due to the low frame rate of the camera. The observed flow patterns were consistent with previous computational simulation results where the flow velocity in the aneurysm sac was more stagnant at the lower flow rate and at least one order of magnitude smaller than in the bifurcation channels [39–41]. In addition, many computational studies, as well as clinical studies, reported similar circular flow happening inside the bifurcation aneurysm sac at higher flow
rates [40–43]. This demonstrates that the in vitro aneurysm platform we developed can be used to simulate and analyze flow dynamics in cerebral aneurysms.

3.3. 3D computational flow model simulation
The 3D printed aneurysm vessel was reconstructed as a 3D computational model using the image stacks from confocal microscopy (figure 2(c)). The flow simulations were demonstrated at flow rates of 300 µl min⁻¹ and 20 ml min⁻¹ to compare the flow patterns and velocities of the simulated models to those in the in vitro bioreactor at the given flow rates. The reconstructed model and the in vitro system displayed similar flow patterns, showing almost no flow inside the aneurysm sac at 300 µl min⁻¹ and high flow at the starting points of two daughter vessels (figure 2(a)). The high-speed flow at the daughter vessels was due to the daughter vessels being printed with smaller vessel lumens at the branching point as shown in (figure 2(c)). The $U_{\text{avg}}$’s for the parent vessel, daughter vessels, and the aneurysm neck region in the 3D computational model (at $z = -0.66$ mm) showed less than 50% relative error compared to those in PIV result (supplementary information table S1). At 20 ml min⁻¹ flow simulation, more active fluid motion was observed in the aneurysm sac which was consistent with the PIV result that showed active intra-aneurysmal circular flow (figure 2(b) and supplementary information table S2). However, the relative error of $U_{\text{avg}}$ between PIV ($U_{\text{avg}}$ of 0.88 mm s⁻¹) and the computational model ($U_{\text{avg}}$ of 1.70 mm s⁻¹) in the aneurysm sac was quite high (93.2%) which could be attributed to the vessel smoothing process during the reconstruction of the computational model slightly decreasing the vessel diameters and resulting in higher flow velocities in the vessels and in the aneurysm dome. Although the reconstruction process needs to be improved to decrease the flow velocity discrepancies between the simulation models and the in vitro models, the general consistency between those models supports the flow dynamic information obtained in the in vitro platform.

3.4. Endothelialization of aneurysm-bearing vessels
The innermost layer of cerebral arteries is composed of a single layer of endothelial cells which plays an important role in maintaining the vessel patency [44]. The endothelial cells are responsible for recanalization after the arterial occlusion [6], or endothelium formation at the occluded aneurysm neck [45]. The hCMEC line has been investigated extensively because they retain most of the morphological characteristics of human blood–brain barrier endothelial cells in vitro [46, 47]. In this study, hCMECs were seeded on the inner wall of the vessel structures to mimic the innermost cerebral blood vessel wall [48]. After seeding hCMECs, they were cultured for seven days to form a confluent single layer. They formed a uniform single layer along the walls of the channels and inside the aneurysm dome with actin filaments and closely associated phenotype (figure 3 and supplementary information figure S2). It should be noted that endothelial cells which are present at the seam which is formed between the encapsulation and base-layer gels tend to prefer to grow along the seam, leading to higher intensity actin staining in these regions. This demonstrates the biocompatibility of the gelatin-fibrin hydrogel with hCMECs as well as its feasibility to serve as a 3D vascularized tissue scaffold for in vitro aneurysm structures.

3.5. Deployment of detachable coils into the aneurysm sac
The approximate volume of the printed aneurysm dome was calculated to choose the right dimensions of coils to be inserted. Two detachable coils
Figure 4. Deployment of endovascular bare platinum coil (BPC) intervention treatment within the in vitro aneurysm. (a) Image of dual coil deployment with the aneurysm dome. (b) Micrographs of brightfield monitoring during BPC insertions from the endovascular microcatheter (1st BPC: 3 mm × 6 cm, 2nd BPC: 2 mm × 3 cm). (c) Maximum projection confocal image stacks of an artificial aneurysm filled with 1 μm red fluorescent beads before and (d) after BPC (2 mm × 3 cm) deployment and retraction, demonstrating no damage to the aneurysm sac during BPC insertion.

(3 mm × 6 cm and 2 mm × 3 cm) were delivered inside the aneurysm using a microcatheter as shown in (figure 4(a)), resulting in a volumetric filling percentage of 36.5%. The coils were densely packed inside the aneurysm without protruding out to the parent vessel or piercing into the gelatin-fibrin hydrogel matrix (figure 4(b)). As the coils were being delivered, the tip and the side of the coils collided with the aneurysm walls, slightly, but reversibly, deforming the walls and scraping some endothelial cells off the aneurysm wall (supplementary information videos 1 and 2). This endothelial denudation was previously reported in dense coil packing of aneurysms with Guglielmi detachable coils [49]. The removal of endothelial layer is considered necessary to promote thrombus formation, prevent recanalization, and improve the healing of endovascularly treated aneurysms [45, 50]. To evaluate whether the aneurysm dome was damaged or deformed during coil deployment, bare gel aneurysm channels were filled with fluorescent polystyrene beads (1 μm diameter) before and after the deployment and retraction of a 2 mm × 3 cm detachable coil (figures 4(c) and (d)). Confocal imaging of the aneurysm dome after the coil retraction showed the walls were fully intact and did not show any difference compared to the walls before the deployment, despite the deformation of gel seen during coiling. This demonstrates that while the vessels are highly compliant relative to silicone-based models, the sturdiness of the gelatin-fibrin hydrogel channels is robust enough to be readily used as a training platform for surgeons or as an in vitro testing system for embolization devices.

3.6. Plasma clot formation inside the aneurysm sac

The aneurysm platform used for the clotting test was endothelialized and packed with a detachable coil (3 mm × 8 cm) which filled 32.5% of the aneurysm sac volume. Coagulation-activated bovine plasma including fluorescent fibrinogen was injected through the endothelialized in vitro aneurysm with coils deployed in the aneurysm sac. Clot formation in the channels was observed using confocal microscopy under static conditions. After a 30 min static dwell time, a significant clot was formed around the deployed coils in the aneurysm sac, but not at the branching point or outside the aneurysm sac (figure 5). A clot formation only around the occlusion device indicates that the in vitro aneurysm which is made of gelatin-fibrin gel does not trigger the clotting cascade of plasma through the contact pathway. This also shows that the deployed coils were densely packed in the aneurysm sac and had sufficient surface area to initiate clotting around the coils when performed under static conditions within the device. Overall, this proves the feasibility of the in vitro aneurysm platform to compare the coagulation efficiency of diverse embolization devices, to observe clotting behaviors and to understand how clotting leads to a healing response.
4. Discussions

This study presents for the first time the development of a printed in vitro living aneurysm platform with potential use in surgical intervention simulations and for analysis of clotting responses post-treatment. The system is made of compliant and biocompatible extracellular matrix (ECM) material, gelatin-fibrin hydrogel, which has similar stiffness to the brain tissue, compared to the previous aneurysm models [17, 18, 22, 51]. The brain tissue without vasculature has reported elastic modulus of 0.1–1 kPa [52, 53], while the existence of vasculature is likely to increase the mechanical modulus of the brain [54, 55]. Since the aim of the study was to analyze the hemodynamics and cellular responses inside the brain vessel that is embedded in the brain tissue, the elastic modulus of the gelatin-fibrin gel (1.1 kPa) [56] was considered suitable for this investigation.

Our platform allows for 3D printing complex vascular structure with variable sizes and geometries of vessel lumens and aneurysm domes. Many previous aneurysm in vitro models use stiff materials such as liquid resin [18], silicon [17, 57, 58], and polydimethylsiloxane [59] due to the ease of fabricating complex anatomical shapes. Although these systems are able to reproduce accurate shapes of aneurysms and are amenable for the testing of flow dynamics, they do not simulate the mechanical modulus of brain tissue which can derive inaccurate results for hemodynamic studies and are non-physiological environments for cell culture or endovascular device training. To address this limitation, some research groups have fabricated softer in vitro aneurysm models to address the problems using poly-vinyl alcohol hydrogel [22] or electrospun poly(D,L-lactide-co-glycolide) (PLGA) scaffolds [60], however these approaches still are not comparable to the elastic modulus of brain tissue. The PLGA vessel scaffold from Cardinal group was cultured with human umbilical vein endothelial cells (HUVECs) and human umbilical artery smooth muscle cells (HUASMCs) and flow diverters were deployed to evaluate the cellular responses to this method of aneurysm treatment. However, the electrospun PLGA was not transparent which limited the visibility of cultured cells and the deployed devices inside the scaffold. Additionally, the method of electrospinning PLGA upon a stainless-steel mandrel limits the vascular structure to that of a simple straight channel. Our in vitro platform is able to pattern a complex vascular in soft, biocompatible, and transparent fibrin-gelatin hydrogel which can provide a brain tissue-like environment for cells and allows for straightforward visualization of cells or devices in the vessels. This in vitro model can be used for training surgeons or medical students to deploy neurovascular devices into a complex vascular structure and can be readily utilized in investigations related to analyzing clotting responses of embolization devices, healing responses.

Figure 5. Plasma clot formation in response to BPC deployment within in vitro living aneurysm dome. Maximum projection confocal image stack of the complete in vitro aneurysm after BPC deployment and injection with bovine plasma. Clot formation is visualized via accumulation of trace fluorescently labeled red human fibrinogen included with the plasma mixture. Endothelial cells are fluorescently stained green for actin. Imaging reveals clot formation and occlusion of the aneurysm sac, with no major clot formation present elsewhere in the vessel structures.
after treatment, and the biophysical mechanisms of aneurysm formation or rupture using the resulting hemodynamic data.

Intracranial aneurysms are usually formed at places where abnormal hemodynamic forces are exerted upon the vascular wall [61]. Wall shear stress (WSS), which is a tangential force applied by blood flow against the endothelial cell lining is the key determinant of vascular remodeling [62]. Thus, matching the WSS’s of the printed vessels to the physiological values is critical in developing the in vitro aneurysm platform. The assumptions we used to calculate the WSS’s of the in vitro vessel structures are as follows: the flow splits into both daughter vessels equally after passing through the intersection, the printed vessels are in cylindrical shapes, and there is no contribution of compliance to vessel diameter under flow. At 20 ml min$^{-1}$ flow rate, the calculated WSS was ~2.22 dyne cm$^{-2}$ for the parent vessel and ~2.63 dyne cm$^{-2}$ for the daughter vessel. These values were low compared to some literature values for arteries in vivo (10–70 dyne cm$^{-2}$) but were inline with that of veins (1–6 dyne cm$^{-2}$) [63]. Since the platform is modeling an aneurysm on the basilar artery, the WSS of our printed vessels is an order of magnitude lower. However, this is primarily due to the limitations of the PIV imaging rate, and the syringe pump perfusion rate. These limitations can be addressed by using a faster PIV imaging rate and faster perfusion rate to achieve clinically relevant WSS values. In addition, the similar trend was observed in the flow velocities obtained from the computational model at 20 ml min$^{-1}$ flow rate. The average velocity in the parent vessel was 13.4 cm s$^{-1}$ which is smaller than the velocity at basilar arteries in vivo (~32.8 cm s$^{-1}$) [64] but within the range of that of cerebral veins (4–17 cm s$^{-1}$) [65]. The computational 3D vascular flow model used here to recapitulate the experimental aneurysm flow velocities can also be used to simulate blood flow that forms physiologically relevant WSS and flow velocity on the printed vessels. The flow simulation will aid in predicting the hemodynamics, calculating flow velocities in the vessels or in the aneurysm sac, and predicting the clotting responses after endovascular treatment.

The main difficulty in comparing PIV data against computational results is that the location, as in z-coordinate, of the PIV images is unknown. Choosing different slices along the z-coordinate of the 3D simulation may result in significant differences in the velocity magnitude. The flow velocities from the simulation model at slightly different z slices ($z = -0.65 \text{ mm}$, $-0.66 \text{ mm}$, $-0.67 \text{ mm}$) were analyzed at 300 µl min$^{-1}$ flow rate (supplementary information table S1 and figure S1). Although the three slices were only 10 µm apart, the velocity magnitudes were significantly different in each slice. The flow at the parent vessel showed smaller relative error at a slice closer to the center of the device ($z = 0.65 \text{ mm}$), whereas the errors for the rest of the device (left and right daughter vessels, aneurysm neck) decreased at a slice closer to the bottom wall ($z = -0.67 \text{ mm}$). This indicates that the in vitro vessels are not perfectly flat but are in a slight inclination which leads to discrepancies between the experimental and computational data. Identifying the angle of a slice with respect to the xy-plane remains as a challenge in comparing PIV and computational models. In addition, disturbances in the flow that were observed in some of the PIV images could have led to the higher errors.

Low packing density of aneurysms using embolization coils is an important contributing factor of aneurysm recurrence after endovascular therapy, which increases the recurrence rate especially when the percent packing volume is less than 25% [66, 67]. Clinical treatments using bare platinum coils showed mean packing density of 23% with a range of 8%–40% in randomized clinical trials [68–70]. The coil packing densities in our in vitro system were 32.5% and 36.5% which were well within the range of clinical trials and above the packing density of 25%. This indicates that our in vitro model can be used to calculate accurate packing density of aneurysms using embolization devices and serve as a realistic model for testing the efficiencies of endovascular devices in aneurysm clotting and healing.

The potential significance of the in vitro living aneurysm platform is the ability to observe the cellular responses or the healing process after embolization treatment. For future studies, cells can be cultured for longer periods of time after the embolization device deployment which may lead to the subsequent formation of endothelium at the neck of the aneurysm, which represents the ideal recovery outcome in aneurysm intervention treatments. It is currently highly challenging to observe or predict when and how the endothelium develops across the neck of the embolized aneurysms in vivo or in clinical studies [71]. However, the in vitro platform developed here can reveal the healing process near the occluded aneurysm in real-time, which can enable investigations directly upon the mechanism of vascular healing post-treatment. In addition, observing cellular behaviors under pulsatile flow, how the geometry of aneurysm neck affects the hemodynamics, and how the size of the aneurysm dome affects the treatment efficiency will be some possible future works that are of great interest.

5. Conclusion

Our 3D printed in vitro living aneurysm system is a powerful platform for the testing of novel endovascular interventions with the capability of enhancing the translation of experimental treatments from lab to clinical practice. PIV analysis demonstrated that the intra-aneurysmal circular flow is formed only at
a higher flow rate (20 ml min\(^{-1}\)) which was confirmed by computational flow modeling. Detachable coils were delivered through printed vessels using a microcathether without penetrating the aneurysm dome, with the deployed coils successfully packed tightly within the aneurysm sac while achieving a percent packing volume of 32.5% and 36.5%. After 30 min of filling the vessels with bovine plasma, a significant clot was formed only at the embolized aneurysm, with no clotting observed at any other locations in the vessels. This key demonstration of the ability to perform a medical intervention with living \textit{in vitro} constructs highlights the utility of this platform as a testbed to simulate treatment using embolization devices, analyze hemodynamics around the aneurysm sac, and observe the clotting properties of the deployed embolization devices. Furthermore, utilizing the \textit{in vitro} platform as an experimental validation of predictive models will be a first step towards developing computation-based personalized medicine.

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