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Citation: AIP Advances **8**, 015006 (2018); doi: 10.1063/1.5015947
View online: https://doi.org/10.1063/1.5015947
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Fabrication of triple-layered bifurcated vascular scaffold with a certain degree of three-dimensional structure

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(Received 15 November 2017; accepted 28 December 2017; published online 9 January 2018)

Constructing vascular scaffolds is important in tissue engineering. However, scaffolds with characteristics such as multiple layers and a certain degree of spatial morphology still cannot be readily constructed by current vascular scaffolds fabrication techniques. This paper presents a three-layered bifurcated vascular scaffold with a curved structure. The technique combines 3D printed molds and casting hydrogel and fugitive ink to create vessel-mimicking constructs with customizable structural parameters. Compared with other fabrication methods, the technique can create more native-like 3D geometries. The diameter and wall thickness of the fabricated constructs can be independently controlled, providing a feasible approach for vascular scaffold construction. Enzymatically-crosslinked gelatin was used as the scaffold material. The morphology and mechanical properties were evaluated. Human umbilical cord derived endothelial cells (HUVECs) were seeded on the scaffolds and cultured for 72 h. Cell viability and morphology were assessed. The results showed that the proposed process had good application potentials, and will hopefully provide a feasible approach for constructing vascular scaffolds. © *2018 Author(s). All article content, except where otherwise noted, is licensed under a Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).* https://doi.org/10.1063/1.5015947

I. INTRODUCTION

Vascular diseases are among the leading causes of death throughout the world.[1,2] There is an immense need for tissue engineered artificial blood vessels because of lack of suitable autologous vessels.[3,4] Synthetic materials such as Dacron and polytetrafluoroethylene (PTFE) have been successfully applied to the high flow rate cases, but are not suited for lower flow rate conditions.[5–7] Small-diameter (inner diameter<6 mm) synthetic vascular grafts have shown poor patency rates, thus increasing the risks of thrombosis, which could further cause early graft occlusion, thrombosis and aneurysm.[8] Tissue-engineered vascular grafts showed a great potential for clinical applications.[9]

The walls of the native elastic arteries, muscular arteries and veins have three distinct layers. The innermost layer (intima) is a monolayer of endothelial cells (ECs). The middle layer (tunica media) is comprised of smooth muscle cells (SMCs). The outermost layer (tunica adventitia) is made of fibroblasts.[10,11] Recently, some researchers have constructed vascular scaffolds with triple-layered structure.[12] Liu et al. created a three-layered composite vascular scaffold. The innermost and

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outermost layers of the scaffold were formed with electrospun polycaprolactone (PCL). The middle layer was composed of physically crosslinked polyvinyl alcohol (PVA) and sodium alginate (SA), which was formed via freeze-thaw cycling method.[13] Hasan et al. described a promising system for forming a triple-layered perfusable vascular-like structure. This system is based on a concentric layer-by-layer organization of vascular cells. GelMA was selected as the perfusion material, which was crosslinked by UV light each time after a single layer was formed.[14] However, the utility of these methods was limited by their incompetency of forming bifurcated structure, with which the scaffolds could better mimic the functions of original blood vessels.[6]

Many researchers have been focusing on developing in vitro models with 2D or even 3D features.[15,16] These vessel-like models help to understand vascular disease pathophysiology, thus assisting clinical diagnosis. However, the over-simplification of some of the vascular models might limit their predictive capability and the conclusion from such models are usually unconvincing. These in vitro models suffer from not being able to construct physiological properties of blood vessels such as the cell-matrix interactions, the layered architecture and the complex blood flow in 3D vessel-like conditions. Also, some common pathological characteristics such as aneurysms and stenoses usually cannot be accurately reproduced in these models, thus contributing little to clinical diagnosis.

Some Aortic surgeries such as arch replacement surgery and coronary artery surgery often require grafts with a certain degree of three-dimensional structure. And these treatment surgeries should be individualized for each patient.[17] However, methods of forming tissue-engineered vascular grafts are still rare because of the limitation of equipment and process. 3-dimensional (3D) printing is a novel additive manufacturing (AM) technology to manufacture 3D structures by accumulating the printable materials layer-by-layer.[18–20] It gained its wide application in the field mold fabrication.[21] Utilizing 3D printing technology, a novel additive and subtractive hybrid manufacturing technique combining 3D printed molds, casting hydrogel and fugitive ink was proposed.

In the present study, three-layered bifurcated vascular scaffold with curved structure was constructed. The outermost layer was formed by physically cross-linking polyvinyl alcohol (PVA) and sodium alginate (SA), which were prepared via the freeze-thaw cycling method followed by a calcium ionic interaction between alginate and $CaCl_2$ solution. The purpose of adopting physically cross-linking method is to avoid residual substances from chemical crosslinking.[22] The middle layer and the innermost layer were formed with enzymatically crosslinked gelatin. Gelatin gels crosslinked by mTG showed to be biocompatible to human cells.[23] Compared with traditional fabrication methods, this additive and subtractive hybrid manufacturing technique realized the fabrication of triple-layered bifurcated vascular scaffold with a certain degree of three-dimensional structure. Its geometry originates from the designed CAD models, which means some pathological features can also be easily added to the vascular scaffold if need be, showing its promising application in the field of constructing in vitro blood vessel models and assisting clinical diagnosis.

II. EXPERIMENTAL

A. Materials

Polyvinyl alcohol (PVA-124, degree of polymerization: 2400, degree of alcoholysis: 98-99.8%) and sodium alginate (SA) were purchased from Sinopham Chemical Reagent Co. Ltd. (China). Gelatin was purchased from Tianjin Bodi Chemical Co. Ltd. (China). Microbial transglutaminase (mTG) was purchased from Ajinomoto Inc. (Japan), its activity was approximately 100U/g. Pluronic F127 was purchased from Sigma Co. Ltd. (America).

For the outermost layer of the scaffold, PVA was dissolved in deionized water at a 14% (wt) concentration, the solution was stirred in a 95 °C water bath for 1h, then cooled to room temperature. SA was dissolved in deionized water at a 3% (wt) concentration. The PVA and SA solutions were uniformly mixed at a 1:1 ratio. The mixed solution was kept at room temperature until the bubbles disappeared. For the middle and the innermost layer, gelatin was dissolved in deionized water and was stirred in an 80 °C water bath for 30 min. When the temperature of the gelatin solution dropped to 37 °C, mTG was added and thoroughly blended with gelatin solution. The gelatin/mTG solution contained 14% (wt) gelatin and 1.4% (wt) mTG. The fugitive ink was composed of 40% (wt) Pluronic

F127 in deionized water. The ink was homogenized using a mixer until the powder was fully dissolved, and then centrifuged to remove any air bubbles. The fugitive ink was subsequently stored at 0 °C.

B. Fabrication of the mold system

The method employed for fabrication of vascular scaffold was realized via a mold system. The mold system contains five molds. They were printed directly (UP Plus 2, TierTime, China) from computer-aided design (CAD) files (SolidWorks 2016, Dassault Systèmes, France). The printing material is Acrylonitrile Butadiene Styrene (ABS). The front views and side views of the five molds are shown in Fig. 1. The cross section of the track on each mold's curved surface has a semicircular shape. The center trajectories of the tracks on each mold's curved surface are the same. The diameters of the five tracks are 5 mm, 3 mm, 1.19 mm, 3 mm and 5 mm, respectively. The shape of the fitting surfaces of the five molds are also the same, which corresponds to the three-dimensional structure of the vascular scaffold.

C. Fabrication of triple-layered bifurcated vascular scaffold with a certain degree of three-dimensional structure

A schematic representation of the step by step process for fabrication of triple-layered bifurcated vascular scaffold with a certain degree of three-dimensional structure is presented in Fig. 2. At first, mold1 and mold2 were fitted together, with their center trajectories coincident. Then inserted a plastic tube into the hole located on one side of mold1 as the inlet, and inserted another plastic tube into the hole located on the other side of mold1 as the outlet. Next, freshly prepared gelatin/mTG solution was housed in a syringe barrel and pipetted into the inner channel formed by mold1 and mold2. The solution delivery rate was maintained at 6 mL/min by a micro-pump attached to the syringe barrel. After 15 min at room temperature and then 30 min in 4 °C to induce gelation, mold2 was gently removed, leaving the hydrogel structure in the channel of mold1. In the same manner, fit mold3 together with mold1 and the gelatin/mTG solution was pipetted into the inner channel formed by mold1 and mold3 at the rate of 6 mL/min. The system was placed at room temperature for 15 min and then cooled to 4 °C for 30 min for gelation. Then, the fugitive ink Pluronic F127 was loaded in a syringe and was printed into the groove of the hydrogel left on mold1. The ink was delivered using a 16-gauge needle (inner diameter=1.19 mm). The printing rate was set to be 534 μL/min. The printing process was executed by a custom-built three-axle linkage platform. After the printing process, mold4 was fitted together with mold1 and the gelatin/mTG solution was pipetted into the inner channel. After crosslinked in the same manner mentioned above, mold4 was removed,

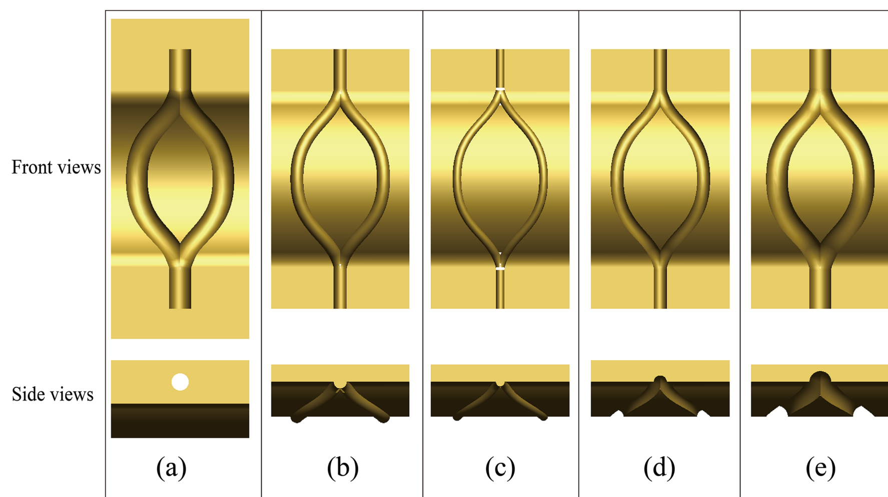

FIG. 1. The front views and side views of the five molds: (a) mold 1; (b) mold2; (c) mold3; (d) mold4; (e) mold5.

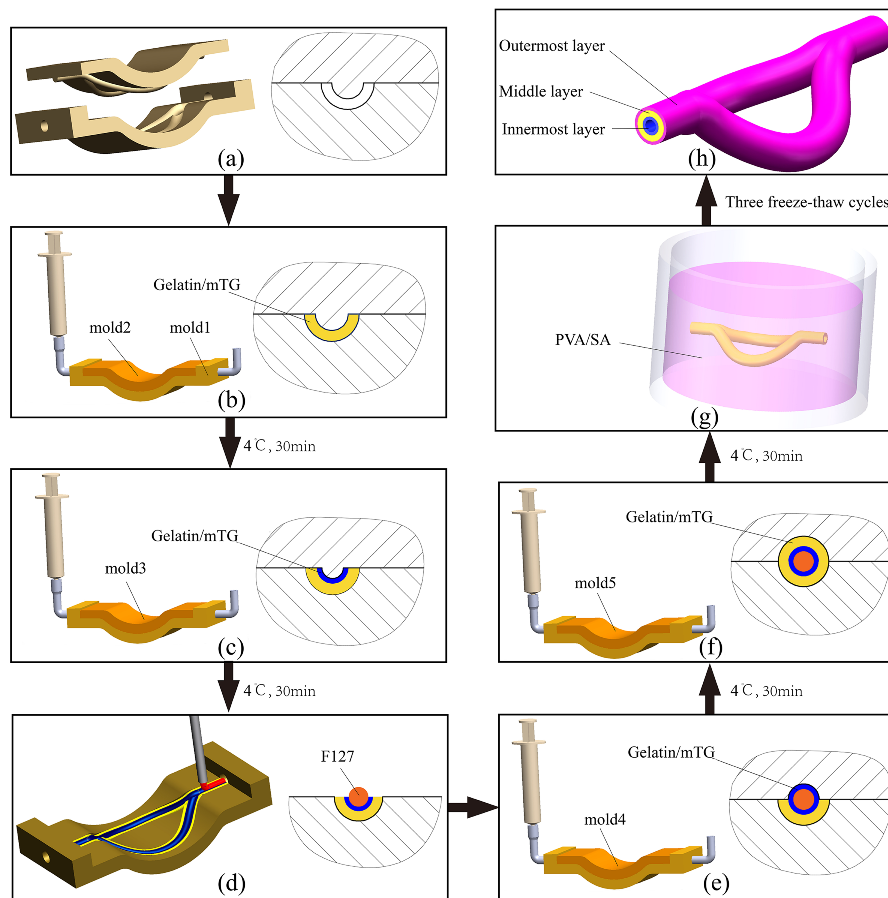

FIG. 2. Fabrication process of the vascular scaffold.

and mold5 was fitted together with mold1. In the same way, the gelatin/mTG solution was pipetted into the inner channel formed by mold1 and mold5. After crosslinked in room temperature for 15 min and then in 4 °C for 30 min, mold5 was gently removed, then carefully removed mold1, thus getting a double-layered scaffold, which corresponded to the innermost and middle layer of the vascular scaffold. The obtained scaffold was incubated at 37 °C for 5 h to get fully crosslinked. Then, the scaffold was immersed in 65 °C distilled water for 5 h to heat-inactivate the residual enzyme.

Then the double-layered scaffold was immersed in the PVA/SA solution for 10 s. When no droplets dripped after removal from the PVA/SA solution, the scaffold was physically crosslinked by three freeze-thaw cycles, which consisted of freezing at -30 °C for 12 h and thawing at 21 °C for 6 h, respectively. At the same time, in the process of freezing, fugitive ink Pluronic F127 liquefied and flowed away, thus forming the inner channel of the vascular scaffold. Finally, the scaffold was submerged in a 3% $CaCl_2$ solution for 15 min for the cross-linking process of alginate by calcium ionic.

D. Uniaxial compressive testing

The vascular scaffolds fabricated in this study possess high aspect ratio, determining its susceptibility to buckling when subjected to axial compressive loading.[8] Uniaxial compressive testing was operated on a universal materials testing machine (Z2.5, ZWICK, Germany). For the purpose of stability during the testing, the diameter of the cross-section was scaled up by three times its original diameter. The uniaxial compressive tests were performed at the rate of 1 mm/min at ambient temperature of 23 °C and humidity of 65%. To investigate the effect of the addition of mTG on the mechanical properties, scaffolds with different composition were fabricated to carry out the tests. Their respective

TABLE I. Specifications of the innermost and middle layer of different samples used in uniaxial compressive testing.

	Innermost layer		Middle layer	
Code Name	Gelatin (wt%)	mTG (wt%)	Gelatin (wt%)	mTG (wt%)
GmG	14	1.4	14	0
GGm	14	0	14	1.4
GG	14	0	14	0
GmGm	14	1.4	14	1.4

specifications and designated code names are listed in Table I. Six independent batches of scaffolds were fabricated and tested.

E. Cell seeding

Human umbilical cord derived endothelial cells (HUVECs) (ScienCell Research Laboratories, USA) were trypsinized, centrifuged and suspended in an endothelial cell culture medium (ScienCell Research Laboratories, USA) at a cell density of 5.0×10^7 cells/ml. Cells in the third passage were used for the experiments. For purpose of promoting cell adhesion, vascular scaffold with relatively small channel diameter (0.5 mm) was fabricated for the cell seeding experiment. The scaffold was soaked in 75% alcohol for 1 h and then washed 3 times with phosphate buffer solution (PBS) to remove the alcohol. After that, the sample was exposed to ultraviolet light for 30 min. The cell suspension was injected into the channel. After 4 h of cell attachment, the cellular construct was placed in fresh culture medium and cultured in a humidified incubator at 37 °C. It was statically cultured with medium changed each day. After 72 h of culture, the cellular morphology was observed with an inversed fluorescent microscope (Eclipse Ti-U, Nikon Instruments Inc., Japan).

III. RESULTS AND DISCUSSION

A. Morphology and structure

The fabricated triple-layered bifurcated vascular scaffold with a certain degree of three-dimensional structure is shown in Fig. 3. To verify the connectivity of the inner channel, yellow acrylic paint (Pre-tested Profession Oil, Grumbacher, USA) solution was perfused into the channel using a syringe pump (Fig. 3(b)). An inversed fluorescent microscope (Eclipse Ti-U, Nikon Instruments Inc., Japan) was used to determine the inner diameter (Fig. 3(c), (d)) of the scaffold. The side view (Fig. 3(a)) and top view (Fig. 3(b)) of the vascular construct confirmed its bifurcated structure in the three-dimensional space. The inner channel of the scaffold became visible after perfused with yellow acrylic paint solution (Fig. 3(b)), which demonstrated its connectivity. Microscopic morphology of the scaffold (Fig. 3(c), (d)) showed the diameter of the inner channel was approximately 1.2 mm, which was a little bit more than the designed dimension (1.19 mm). This might be because of the swelling of the fugitive ink in the extrusion process. Furthermore, the materials and thickness of each layer could be independently controlled.

Compared with previously seen vascular scaffolds, the proposed scaffold possesses a branched structure while keeping the three-layered feature. This is of particular importance because over-simplified in vitro vascular models have limited significance for simulating the actual in vivo environment.

B. Mechanical properties

Mechanical tests results are showed in Fig. 4. For uniaxial compressive testing, Fig. 4(a) shows the stress-strain curves of the 4 different samples listed in Table I. Their compressive modulus was shown in Fig. 4(b). It can be seen that the addition of mTG greatly enhanced the modulus of the scaffolds. The compressive modulus of GmGm is more than 6 times greater than that of GG. The main reason for this mechanical enhancement lies in the fact that the composition of gelatin includes glutamine

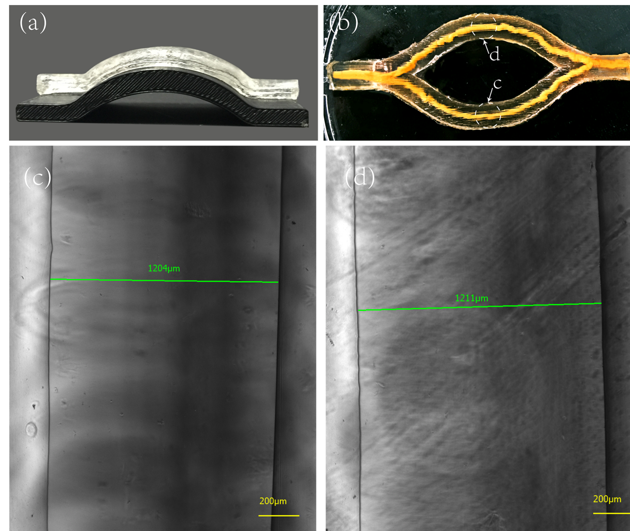

FIG. 3. Morphology of the vascular scaffold. (a) Side view of the scaffold. (b) Top view of the scaffold after perfused with yellow acrylic paint solution. (c-d) Optical images of the inner channel of vascular scaffold.

and lysine. The presence of these amino acids means there are more crosslinking sites in gelatin, thus promoting the chance of creating stiffer gels when provided with sufficient amounts of mTG under proper crosslinking conditions.

The mechanical properties of the enzymatically-crosslinked scaffolds are still relatively low compared to those of native blood vessels. Hence, the proposed vascular scaffolds are not suitable for clinical implantation during cardiovascular surgeries. However, the fabricated vascular scaffolds can be useful in in vitro studies of vascular diseases and development of in vitro blood vessel models.

C. Endothelialization of the inner channel

One of the most critical properties of vascular scaffolds is its biocompatibility.[24] And the formation of endothelialized layer is also critical for the proper function of a vascular scaffold. To investigate the biocompatibility of the scaffold, HUVECs were seeded and statically cultured in the inner channel. Fig. 5 shows the microscopic morphology of endothelial cells at the linear part (Fig. 5(a)) and bifurcated part (Fig. 5(b)) of the inner channel after 72 h of culturing. The cells in the inner channel were found to well spread on the wall and take normal cellular phenotype. The results indicated that

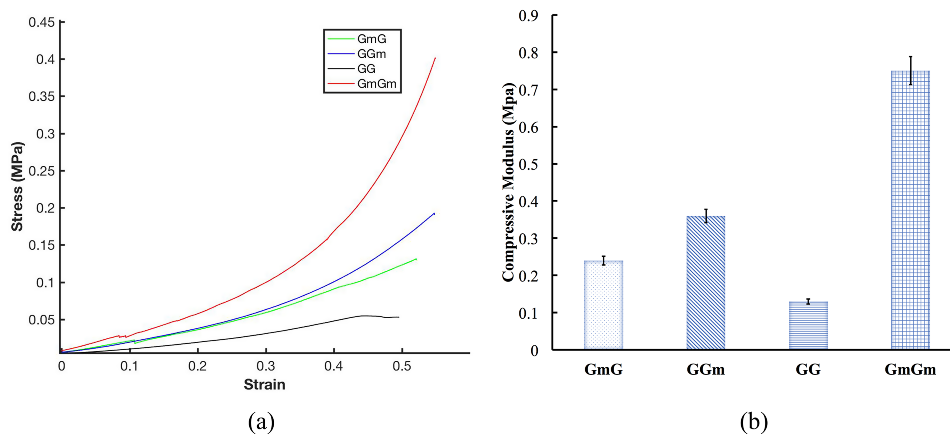

FIG. 4. Results of the uniaxial compressive testing. (a) Stress-strain curves from uniaxial compressive testing of the four samples. (b) Compressive modulus of the four samples.

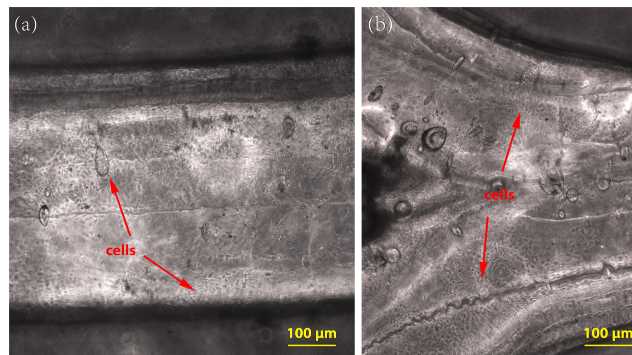

FIG. 5. Microscopic images of cell morphology at different locations of the inner channel after 72 h of culturing.

the vascular scaffold and the fabrication process were nontoxic for the growth of HUVECs and was suitable for cell attachment, spreading and endothelialization.

IV. CONCLUSIONS

In this study, a novel approach to fabricate triple-layered bifurcated vascular scaffold with a certain degree of three-dimensional structure was presented. The method used in this study combined 3D printed molds, casting hydrogel and fugitive ink, providing a facile technique to construct a range of vascular scaffolds whose morphologies are close to those of the original blood vessels. Compressive testing results showed that by crosslinking gelatin with microbial transglutaminase, the compressive strength of the scaffolds was greatly enhanced comparing with scaffolds without crosslinking. Also, enzymatically crosslinked scaffold had been shown to be favorable to the growth of HUVECs. To the best of our knowledge, there haven't been reports on the effect of infection or thromboembolism or other undesired effects when the materials used for constructing the scaffolds are in contact with living organisms. With the inherent advantages of 3D printing, it is envisioned that the proposed technique will play a significant role in the field of tissue engineering and in assisting researches of cardiovascular diseases.

ACKNOWLEDGMENTS

This work was funded by National Natural Science Foundation of China (NO.51475281, NO.51575333) and China National Funds for Distinguished Young Scientists (NO. 61625304).

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