FORMATION OF VESSEL-LIKE CHANNEL USING ALGINATE FIBER AS A SACRIFICIAL STRUCTURE

Keigo Nishimura[1], Nobuhito Mori[1], Yuya Morimoto[1], and Shoji Takeuchi[1, 2]
[1]Institute of Industrial Science, the University of Tokyo, JAPAN
[2]Takeuchi Biohybrid Innovation Project, ERATO, JST, JAPAN

ABSTRACT

This research reports a method to fabricate a loop-shaped vessel-like channel for perfusion of culture medium in a cell-laden collagen gel. We used alginate fibers as sacrificial structures for construction of loop-shaped vessel-like channels. By integration of a loop-shaped alginate fiber with connection ports in a cell-laden collagen gel, we formed a tube-connectable loop-shaped channel. Finally, we seeded endothelial cells onto inner wall of the channel to fabricate vessel-like channel coated with endothelial cells. As a result, we achieved perfusion of culture medium via the loop-shaped vessel-like channel. We believe that this method will be useful for in vitro construction of three-dimensional (3D) tissues with complex-shaped vessel-like channels.

INTRODUCTION

Blood vessels are ubiquitous networks throughout human body, and work as a pathway of oxygen and nutrition for cells [1]. In the field of 3D tissue construction, the pathway of oxygen and nutrition is also necessary to fabricate a thick tissue because cell viability decreased exponentially from the surface of the tissue due to the diffusion limitation of oxygen and nutrition [2]. In order to fabricate the pathway of oxygen and nutrition, many studies have formed vessel-like channels in 3D tissues [3–6]. Recently, 3D printing technology has been used in this field to fabricate culture devices integrated with connection ports connected to the vessel-like channels in thick 3D tissues for perfusion of culture medium [7, 8]. Using the 3D printing technology, the design of the culture device was easily adjustable. In these studies, 3D tissues with a channel coated by endothelial cells was constructed in the culture device connected to external pump, and perfused with culture medium [9]. However, it remains difficult to fabricate complex-shaped channels other than a straight-shaped one because the channels in 3D tissues were formed by pulling out rigid wires embedded in the tissues; the complex-shaped channel is necessary for widespread supply of oxygen and nutrition to 3D tissues.

Here, we used alginate fibers as sacrificial structures to fabricate a complex-shaped channel because of their flexibility, degradability and biocompatibility [3]. Owing to the flexibility, various shaped alginate fibers were available (e.g., loop, zigzag and branched) (Figure 1). In this research, to form a loop-shaped channel as an example of complex-shaped channels, we firstly formed a loop-shaped alginate fiber and set the fiber between connection ports of a device. Then, we embedded the fiber in a cell-laden collagen gel and dissolved it with enzyme to form a loop-shaped channel. After seeding human umbilical vein endothelial cells (HUVECs) in the channel, we performed perfusion of culture medium by connecting the device to an external peristaltic pump (Figure 2).

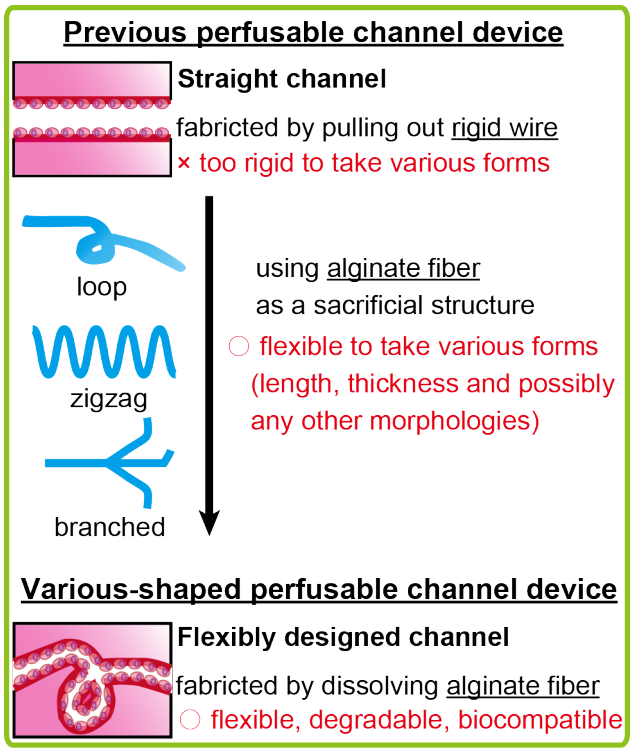

Figure 1: Conceptual illustration of perfusable channels fabricated by using alginate fibers as sacrificial structures. In conventional researches, shapes of the perfusable channels were straight because the channels were formed by pulling out rigid fibers. In this research, we formed a loop-shaped channel using a flexible alginate fiber.

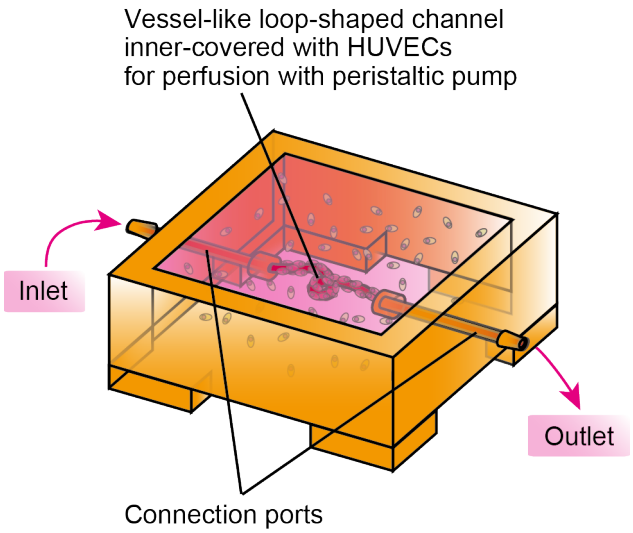

Figure 2: Conceptual illustration of a perfusable culture device with a vessel-like loop-shaped channel fabricated by dissolving a loop-shaped alginate fiber and seeding HUVECs.

EXPERIMENTAL METHODS

Materials

500 mL of Endothelial Cell Basal Medium (EBM) and growth factors for Endothelial Cell Growth Medium 2 (EGM2) (10 mL of fetal calf serum (FCS-10), 0.25 µg/500 µL human Vascular Endothelial Growth Factor (hVEGF-0.25), 5.0 µg/500 µL human basal Fibroblast Growth Factor (hbFGF-5), 10 µg/500 µL of Long Arg3 Insulin-like Growth Factor-1 (R3 IGF-1), 2.5 µg/500 µL of human Endothelial Growth Factor (hEGF-2.5), 100 µg/500 µL hydrocortisone (Hydrocortisone-100), 11.25 mg/500 µL heparin (Heparin-11.25) and 500 µg/500 µL ascorbic acid (Ascorbic Acid-500)) were purchased from PromoCell GmbH. Vascular Endothelial Growth Factor, human, recombinant (rhVEGF-A_{165}) and Fibroblast Growth Factor (basic), human, recombinant (rhbFGF) were purchased from Wako Pure Chemical Industries, Ltd. Dulbecco's modified eagle's medium (DMEM), penicillin-streptomycin solution and 10× Hanks' Balanced Salt Solution were purchased from Sigma-Aldrich Corporation. Fetal Bovine Serum (FBS) for supplementing DMEM was purchased from Biosera, Ltd. Phosphate buffered saline (PBS) was purchased from Cell Science & Technology Institute, Inc. Type I collagen solution (IAC-50) was purchased from KOKEN Co., Ltd. Sodium alginate was purchased from Junsei Chemical Co., Ltd. Calcium Chloride was purchased from Kanto Chemical Co., Inc. Sodium hydrogen carbonate was purchased from NACALAI TESQUE, Inc. HEPES was purchased from DOJINDO LABORATORIES.

EGM2 was prepared by supplementing EBM with growth factors for EGM2. Other culture medium intending to induce sprouting of vessels (EGM-sp) was prepared by supplementing EBM with some parts of growth factors for EGM2 (FCS-10, R3 IGF-1, hFGF-2.5, Hydrocortisone-100, Heparine-11.25 and Ascorbic Acid-500), 10 µg rhVEGF-A_{165} and 5 µg rhbFGF in 100 µL PBS. DMEM was supplemented with 10% (v/v) FBS, 100 U/mL penicillin and 100 µg/mL streptomycin.

Cell culture

HUVECs were purchased from PromoCell GmbH. We cultured these cells in EGM at 37°C in a CO_2 incubator (MCO-5AC, SANYO Electric Co., Ltd.) (5% CO_2 concentration). 2–3 days after thawing and seeding them, we subcultured 80–90% confluent cells once with 12 culture dishes (100 mm diameter). Another 2–3days after first subculture, we harvested 80–90% confluent cells from culture dishes by trypsin treatment and resuspended in EGM after centrifugation (4°C, 1200 rpm, 5 min). We used the cell suspensions for construction of HUVEC-laden collagen structures and fabrication of a HUVEC layer on inner walls of vessel-like channels.

Device design for perfusion of culture medium and insertion of alginate fibers

We designed a device using 3D CAD software (Autodesk Inventor 2016, Autodesk, Inc). Our device consisted of two connection ports for inserting an alginate fiber to fabricate a channel connectable with an external peristaltic pump (AC-2110II, ATTO Corporation) via

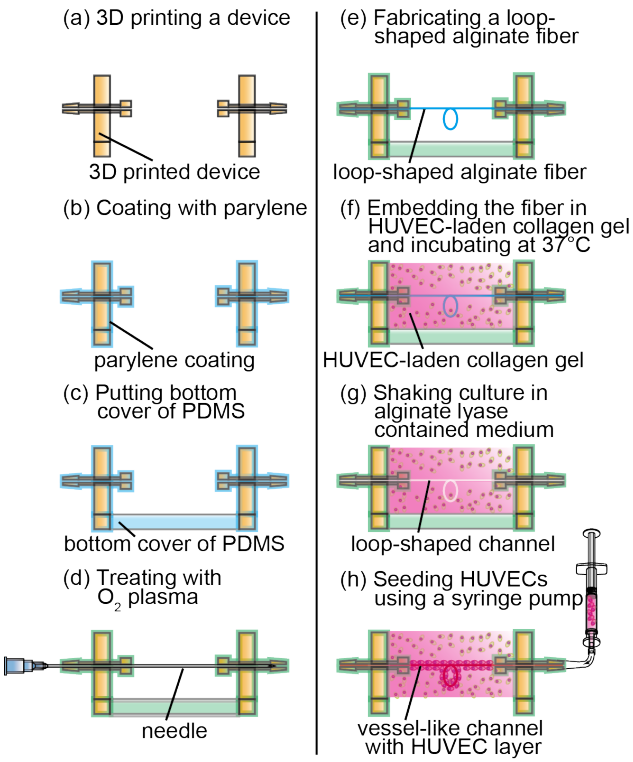

Figure 3: Fabrication process of a loop-shaped vessel-like channel in a device. (a) A device was 3D printed. (b) The device was coated with parylene. (c) Bottom of the device was sealed with PDMS cover. (d) O_2 plasma was treated on the surface of the device. (e) A loop-shaped alginate fiber was fabricated through two connection ports on the same straight line. (f) HUVEC-laden collagen solution was poured into the device to embed the fiber and the device was incubated at 37 °C for gelation. (g) The device was incubated by shaking culture in EGM2 containing alginate lyase for fabrication of a loop-shaped channel by dissolving the fiber. (h) Alginate lyase was washed out and HUVECs was seeded onto the channel by infusing EGM-sp with HUVECs using a syringe pump.

silicone tubes (1 mm in inner diameter and 3 mm in outer diameter, ATTO Corporation) and ethyltrifluoroethylene tubes (ETFE tube) (1/16" in outer diameter and 0.50 mm in inner diameter, VICI AG). The shape of the device was square prism (30 mm outer square, 24 mm inner square and 12.5 mm height) with three connecting ports (1 mm of inner diameter) for each side face and four legs (2 mm height) for each vertex of bottom face.

We fabricated the device by a 3D printer (AGILISTA-3100, KEYENCE Corporation) and coated with parylene in order to increase biocompatibility (Figure 3a, b). After sealing the bottom of the device with a polydimethylsiloxane (PDMS) cover, we treated the device with O_2 plasma to improve the adhesiveness with the collagen and cells (Figure 3c, d).

Formation of loop-shaped vessel-like channel

We first formed an alginate fiber between two connection ports of the device. In the formation, the device was filled with 150 mM calcium chloride solution. A needle (0.6×32 mm, Terumo Corporation) connected with

1 mL syringe (Terumo Corporation) filled with 1.5%(w/w) sodium alginate solution was inserted from the left connection port to the right connection port (as shown in Figure 3d). The alginate fiber was formed by withdrawing the needle outside and injecting the sodium alginate at the same time. After that, we made the fiber a loop-shape manually using a tip of an ultrathin needle (0.40×19 mm, TERUMO Corporation) (Figure 3e). Next, we poured 3 mL of HUVEC-laden collagen solution into the device and incubated it at 37°C in the CO_2 incubator for collagen gelation (Figure 3f). The HUVEC-laden collagen solution was prepared by mixing 240 µL EGM-sp with 2.0×10^6 cells/mL HUVECs, 2.4 mL of 5% (w/w) type I collagen sol, 300 µL 10×HANKS, 30 µL of each 1.0×10^3 mM sodium bicarbonate and HEPES solution on ice. Subsequently, we added 0.04% (w/w) alginate lyase contained EGM and performed shaking incubation at 37°C in the CO_2 incubator to dissolve the loop-shaped alginate fiber (Figure 3 g).

After about 17 hours of shaking culture with the EGM containing alginate lyase, we exchanged culture medium to DMEM for removing the alginate lyase. Next, we connected a syringe pump (KDS210, KD Scientific Inc.) with the channel via a silicone tube, an ETFE tube and a connecting port of the device. Subsequently, we seeded HUVECs onto the inner wall of the channel by infusing EGM-sp with 2×10^6 cells/mL HUVECs at 0.02 mL/min flow speed for 10 min with the syringe pump (Figure 3h). After incubation of the cells seeded on the channel at 37°C in CO_2 incubator for 20 min, we inverted the device and incubated it for another 20 min in order to seed the cells uniformly onto whole inner wall of the channel. We then inverted the device again and incubated overnight to promote adhesion of the cells to the channel wall. Finally, we obtained a loop-shaped vessel-like channel with a HUVEC layer in the device.

RESULTS AND DISCUSSION

Properties of loop-shaped channel

To check a hollow structure of the loop-shaped channel, we dipped the device in red-colored DMEM after the dissolution of the alginate fiber. The diffusion of red-colored DMEM was observed at the loop-shaped channel in a HUVEC-laden collagen gel, indicating that the alginate fiber embedded in the collagen gel was dissolved with alginate lyase and culture medium permeated to the inside of the collagen gel owing to the hollow structure of the channel (Figure 4 (left)).

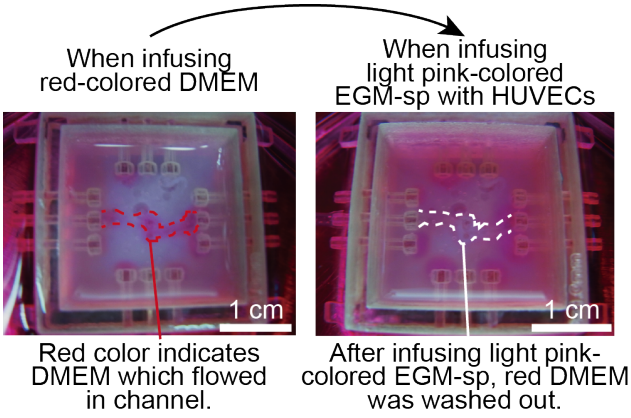

Figure 4: Images of a channel in the device (left) when infusing red-colored DMEM, (right) when infusing light pink-colored EGM-sp with HUVECs.

After that, to seed HUVECs onto inner channel wall, we infused EGM-sp with HUVECs into the channel via the connection port by a syringe pump. During this process, we observed that the flow of light pink-colored EGM-sp with HUVECs washing out red-colored DMEM in the channel, indicating that fabricated channel has appropriate hollow structure for perfusion of culture medium by the pump

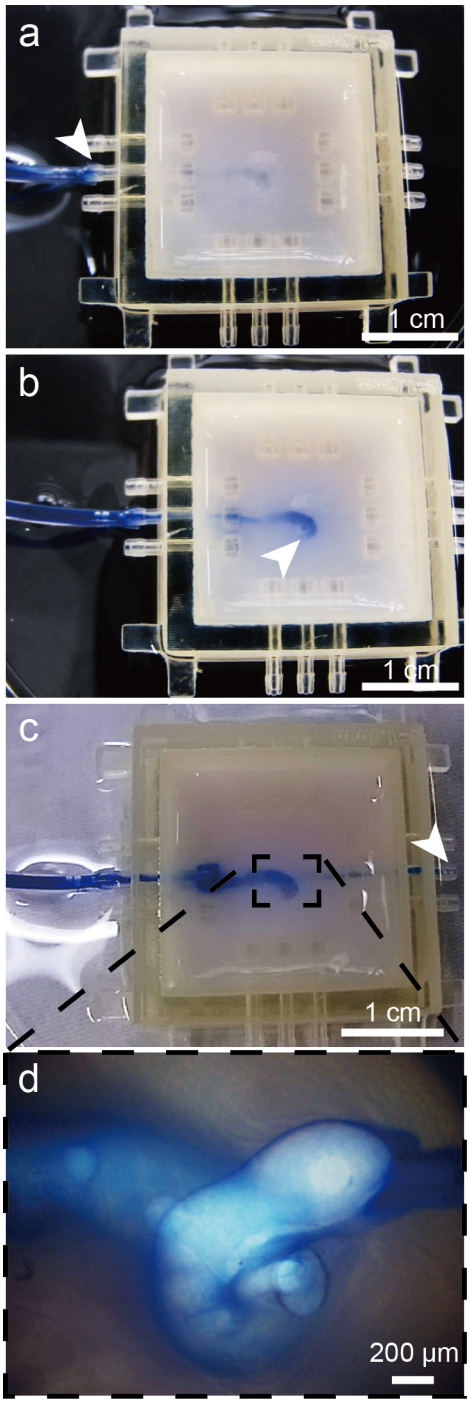

Figure 5: Images of blue ink infusion into a loop-shaped vessel-like channel. (a-c) Time lapse images of blue ink infusion at (a) 0 minute, (b) 2 minutes and (c) 10 minutes after starting the infusion. Arrowheads indicate arrival point of blue ink. (d) Microscopy of the loop-shaped channel.

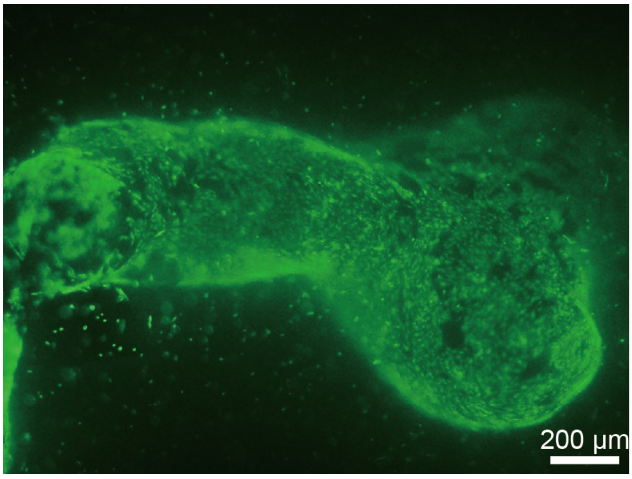

Figure 6: Fluorescence image of a loop-shaped vessel-like channel with stained HUVECs. Green shows HUVECs.

(Figure 4 (right)).

In addition, after seeding HUVECs and incubating the device overnight until adhesion of HUVECs onto the channel wall, we conducted live/dead assay. As a result, the viability of the cells in collagen gel was 77%. This result indicates that two processes, dissolution of alginate fiber and seeding of HUVECs, do not affect the viability of cells seriously.

Properties of loop-shaped vessel-like channel

To investigate the availability of perfusion, we infused PBS including 10% (v/v) blue ink into the channel at a flow rate of 0.02 mL/min. By time-lapse observation, we observed that blue ink flowed through the channel and flowed out from the outlet port in 10 min. This result indicates that the fabricated channel is usable for perfusion of culture medium (Figure 5a–c). In this experiment, we also observed the formation of loop-shaped vessel-like channel with microscope. This result shows that our method to use an alginate fiber as a sacrificial structure is useful for formation of complex-shaped channel (Figure 5d). We also observed that some ink was leaked out from the interstice between the connection ports and the channel when continuous injection of PBS including 10% blue ink. This result suggests that cell adherence at the interstice were not strong enough to bear a high hydraulic pressure. This leak problem will be solved by raising adhesiveness with surface treatment and design change of device.

Finally, to check whether HUVECs were seeded onto the inner wall of the channel, we stained HUVECs in the channel with live/dead assay kit (Figure 6). We observed that inner wall of the channel was covered with HUVECs. This result indicates that our method is useful to form a HUVEC layer on inner wall of the channel.

CONCLUSION

In this research, we fabricated a loop-shaped vessel-like channel for perfusion of culture medium in a cell-laden collagen gel using an alginate fiber as a sacrificial structure. We confirmed that the fabricated loop-shaped channel was perfusable and covered with HUVECs. We believe that this method will be applicable to construction of intricately widespread networks of blood vessels for reconstruction of 3D tissues and organs.

ACKNOWLEDGEMENTS

We thank Minghao Nie and Akane Ito for beneficial discussions and technical supports, respectively. This work was partially supported by Grant-in-Aid for Scientific Research (S) (Grant number: 16H06329).

REFERENCES

[1] K. Kinoshita, M. Iwase, M. Yamada, Y. Yajima and M. Seki, “Fabrication of multilayered vascular tissues using microfluidic agarose hydrogel platforms,” *Biotechnol. J.*, vol. 11, pp. 1-9, 2016.

[2] M. Radisic, J. Malda, E. Epping, W. Geng, R. Langer and G. Vunjak-Novakovic, “Oxygen Gradients Correlate with Cell Density and Cell Viability in Engineered Cardiac Tissue,” *Biotechnol. Bioeng.*, vol. 93, no. 2, 2006.

[3] M. I. Bogorad, J. DeStefano, J. Karlsson, A. D. Wong, S. Gerechtc and P. C. Searson, “Review: in vitro microvessel models,” *Lab Chip*, vol. 15, pp. 4242-4255, 2015.

[4] I. Vollert, M. Seiffert, J. Bachmair, M. Sander, A. Eder, L. Conradi, A. Vogelsang, T. Schulze, J. Uebeler, W. Holnthoner, H. Redl, H. Reichenspurner, A. Hansen and T. Eschenhagen, "In vitro perfusion of engineered heart tissue through endothelialized channels", *Tissue Eng. Part A*, vol. 20, pp. 854-863, 2014.

[5] D. Marino, J. Luginbühl, S. Scola, M. Meuli and E. Reichmann, “Bioengineering Dermo-Epidermal Skin Grafts with Blood and Lymphatic Capillaries,” *Science Translational Medicine*, vol. 6, issue 221, 221ra14, 2014.

[6] S. Kim H. Lee, M. Chunga and N. L. Jeon, “Engineering of functional, perfusable 3D microvascular networks on a chip,” *Lab Chip*, vol. 13, pp. 1489–1500, 2013.

[7] N. Mori, Y. Morimoto and S. Takeuchi, “Skin-equivalent integrated with perfusable channels on curved surface,” *Proceeding of the 28th IEEE International Conference on Micro Electro Mechanical Systems*, pp. 351–353, 2015.

[8] K. Sakaguchi, T. Shimizu, S. Horaguchi, H. Sekine, M. Yamato, M. Umezu and T. Okano, “In Vitro Engineering of Vascularized Tissue Surrogates,” *Sci. Rep.*, vol. 3, no. 1316, 2013.

[9] S. Sakai, S. Yamaguchi, T. Takei, and K. Kawakami, “Oxidized alginate-cross-linked alginate/gelatin hydrogel fibers for fabricating tubular constructs with layered smooth muscle cells and endothelial cells in collagen gels,” *Biomacromolecules*, vol. 9, pp. 2036–2041, 2008.

CONTACT

*K. Nishimura,
tel: +81-3-5452-6650; nishim@iis.u-tokyo.ac.jp