FIBER-SHAPED ARTIFICIAL TISSUE WITH MICROVASCULAR NETWORKS FOR BOTTOM-UP TISSUE RECONSTRUCTION

Ryo Sato[1], and Hiroaki Onoe[1]
[1]Department of Mechanical Engineering, Faculty of Science and Technology,
Keio University, Kanagawa, JAPAN

ABSTRACT

This paper describes a fiber-shaped microscale tissue with blood vessel networks. We co-cultured Hep-G2 (Human hepatic epithelial cell line) and HUVEC (human umbilical endothelial cell) in a collagen/alginate core-shell hydrogel microfiber fabricated by using a microfluidic device. We observed difference in construction of blood vessel networks in the hepatic tissue by varying in the ratio of co-cultured cells and the diameter of the core. In addition, by arranging the fiber-shaped tissues to construct macroscale tissue assembly, we confirmed the connection of blood vessel networks between the assembled fiber-shaped tissues.

INTRODUCTION

Along with the recent development of regenerative medicine, attempts to reconstruct a tissue or organ are being conducted extensively in the field of tissue engineering [1]. In this field, a bottom-up assembly method has been expected as a powerful technique to construct three-dimensional macroscale tissue artificially *in vitro* [2]. By this method, macroscale heterogeneous tissues have been constructed hierarchically and spatially by assembling hundred-micrometer-scale cell-laden building blocks having various shapes [3]-[5].

In those building blocks, fiber-shaped artificial tissue [6]-[10] can be fabricated continuously and rapidly by using a microfluidic device. In addition, the fiber-shaped tissue suit for construction of a high-order macroscale tissue by using a weaving and bundling technique [7]. Furthermore, the fiber-shaped tissue is considered to be suitable for constructing blood vessels [8] and muscle fibers [9] and nerve tissues [10] having a similar shape. Especially among them, the vascular network plays a role of carrying nutrition and oxygen inside the tissue. Thus, artificially reconstruction of those vascular network is essential in the field of tissue engineering. However, integration of such fiber-shaped blood vessel building blocks with another macroscopic tissue has not been reported yet.

In this study, we propose a fiber-shaped building block that includes HUVEC networks into the fiber-shaped tissues (Hep-G2) (Figure 1). The both cells (Hep-G2 and HUVEC) suspended in the collagen gel are cultured in a fiber-shaped alginate calcium hydrogel (diameter: approximately 230 µm), and are connected with each other to form a fiber-shaped tissue. Since this fiber-shaped building block is prepared using a microfluidic device, it is easy to control diameter and length. Furthermore, by culturing these tissues side by side, we show a possibility of the fiber-shaped building block for assembling vascularized macroscopic tissues *in vitro*.

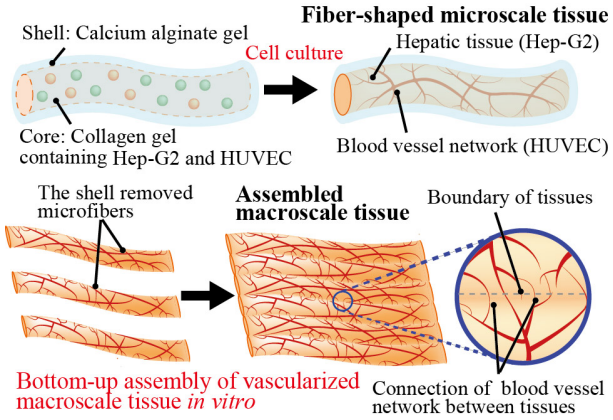

Figure 1. Concept of our fiber-shaped microscale tissue including blood vessel networks. The fiber-shaped tissue containing Hep-G2 and HUVEC was fabricated by using a microfluidic device. By assembling the fiber-shaped tissues including blood vessel networks, a vascularized macroscale tissue can be fabricated.

METHODS

Microfluidic device

To fabricate the fiber-shaped microscale tissue, we used a double core-shell type microfluidic device based on the previous work [7]. This device was composed of glass capillaries and connectors made of resin. A glass capillary tube (outer diameter: 1 mm, inner diameter: 0.6 mm, Narishige, G-1) was sharpened by a tip-puller (Narishige, P-10). Then, the tip of these glass capillaries was cut by a micro forge (Narishige, EG-44) and the tip diameter was adjusted to approximately 200 µm. A square glass tube (outer diameter: 1.4 mm, inner diameter: 1 mm, VITROCOM, 8100-100) was used to fix the inner glass capillary tube and guide the outer flow. A connector was fabricated by a 3D printer (Keyence, AGILISTA). These glass capillary and connectors were assembled on the slide glass (MATSUNAMI, S2124). All inlets were connected to syringes via Teflon tubes (VICI, 1/16”×0.5 ETFE) and all syringes were set to syringe pumps.

Cell suspension preparation

To prepare a collagen suspension of Hep-G2 and HUVEC for encapsulating them into the hydrogel microfiber, cells were operated as follows: (0) Hep-G2 and HUVEC cells were prepared in DMEM (SIGMA, D5796) and EGM-2 (Lonza, CC-3156), respectively. (1) When the cells reached to ~80% confluence, all of the medium was removed and 5 mL of Dulbecco's Phosphate-Buffered Saline (DPBS) was added to rinse the cells. (2) After removal of DPBS, 2 mL of 0.25% trypsin-EDTA (GIBCO, 25200-072) was added to detach the cells. Subsequently, 8 mL of the medium added and the medium containing cells was collected in the 50 mL centrifuge tube. (3) After centrifugation of the tube at 500 G for 5 minutes, the

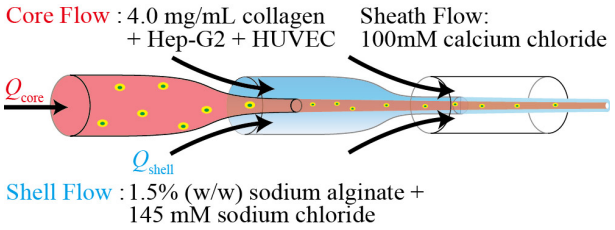

Figure 2. Schematic of the microfluidic device. Since each slow is a laminar flow in the microfluidic device, double coaxial flow can be formed without mixing with each other.

medium was aspirated, 10 mL of the medium was added and the cells were suspended. (4) Using the cell counter, the cells of the suspension were counted and the number of each cell was adjusted to 1.0×10^7 cells. (For the fiber-shaped tissue with Hep-G2:HUVEC=1:1) (5) The each suspension was mixed in centrifuge tube and the tube was centrifuged at 500 G for 5 minutes. (6) After centrifugation, the medium was removed and pre-gel collagen solution (4 mg/mL, derived from bovine dermis) (KOKEN, IAC-50) was added at 4°C. Finally, the cells were suspended in collagen without any bubbles.

Fiber Fabrication

For the shell flow, we prepared a 3% (w/w) sodium alginate solution (NaAlg, Wako, 194-13321) sterilized with a 0.22 µm filter and mixed at 1:1 ratio with a 290 mM sodium chloride (Wako, 191-01665) solution sterilized with an autoclave. In order to polymerize the shell flow, we prepared a solution containing 100 mM calcium chloride (Wako, 090-00475) and 3% (w/w) sucrose (Wako, 196-00015), and was sterilized with an autoclave.

The operation for fiber fabrication was performed at 4°C to prevent the gelation of the collagen solution. For sterilization, the microfluidic device was filled with 70% (v/v) ethanol for 20 minutes. Subsequently, we rinsed the devise with a saline. After that, we performed the following operations: (1) Each syringes are filled with cell suspension for the core flow, with the solution mixture containing 1.5% w/w NaAlg and 145 mM NaCl for the shell flow, and with the solution containing 100 mM $CaCl_2$ and 3% sucrose for the sheath flow (2) The syringe pumps were started to infuse the core flow, the shell flow (flow rate Q_{shell} = 2.5 mL/min) and the sheath flow. Since laminar flows were formed in the microfluidic device (Figure 2), each flow did not mix with each other. A microfiber containing the cells was formed in the microfluidic device and collected in a centrifuge tube filled with a saline. (3) Once the desired length of fiber was fabricated, the core flow, the shell flow and the sheath flow were stopped. (4) The microfiber containing the cells in the centrifuge tube was transffered to the 100 mm dish with the saline. The saline in the dish was replaced with the mixed medium (DMEM:EGM-2=1:1). (5) The dish with fiber-shaped tissue was incubated at 37°C and the collagen gel of the core gelated. Subsequently, the microfiber containing the cells was cultured at 37ºC in water saturated 5% CO_2 environment.

Immunofluorescent staining

We visualized a construction of HUVEC networks in

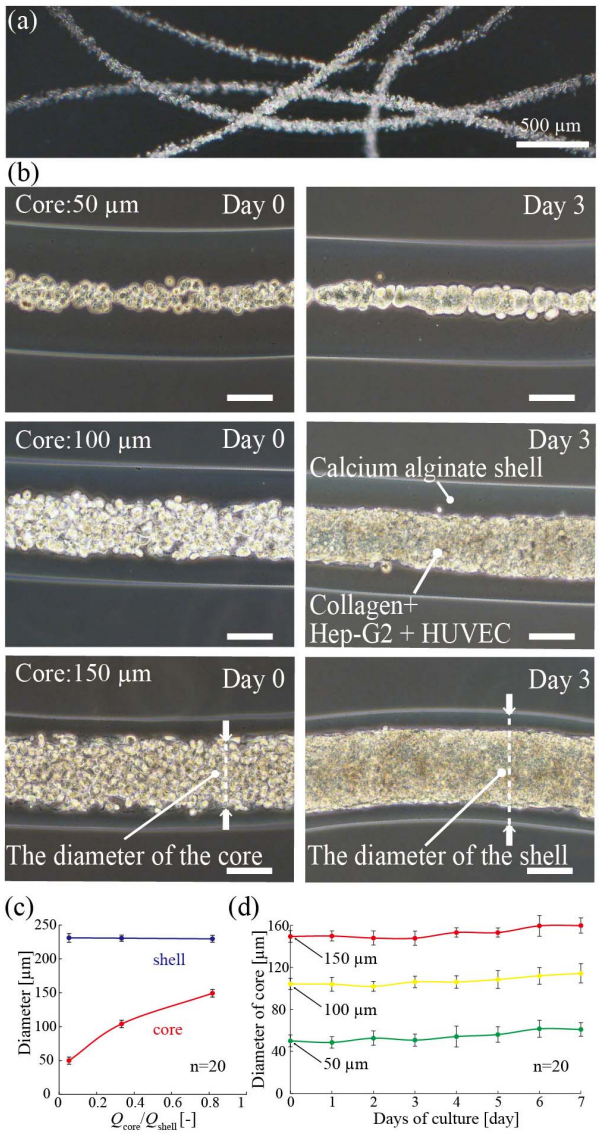

Figure 3: Fabricated fiber-shaped microscale tissue (a) A cell-laden core-shell hydrogel microfiber was fabricated. (b) Tissue formation in the microfibers with different diameter. Scale bars: 100 µm (c) By changing the ratio of core and shell flow rate, the core diameter can be adjusted. (d) The change of the diameter of the fiber-shaped tissues during culture.

the fiber-shaped hepatic tissue by immunofluorescent staining. The fiber-shaped tissue cultured in the medium was fixed with 4% paraformaldehyde phosphate buffer solution (Wako, 163-20145). After 15 minutes fixation, the fiber-shaped tissue was permeabilized with 0.1% Triton-X100 (Alfa Aesar, A16046) in DPBS for 10 minutes and soaked in 1% Bovine Serum Albumins (BSA) in DPBS to block non-specific bindings. Subsequently, the fiber-shaped tissue was incubated with Purified Mouse Anti-Human CD31 (BD Biosciences 555444) in DPBS overnight. After that, the fiber-shaped tissue was rinsed with DPBS and incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody (Invitrogen, A-11001) in DPBS and DAPI (Invitrogen, D1306) for nucleus staining. After rinsing with DPBS, the fiber-shaped tissue was arranged on 35 mm glass base dish (IWAKI, 3961-035) and sealed with mounting agent.

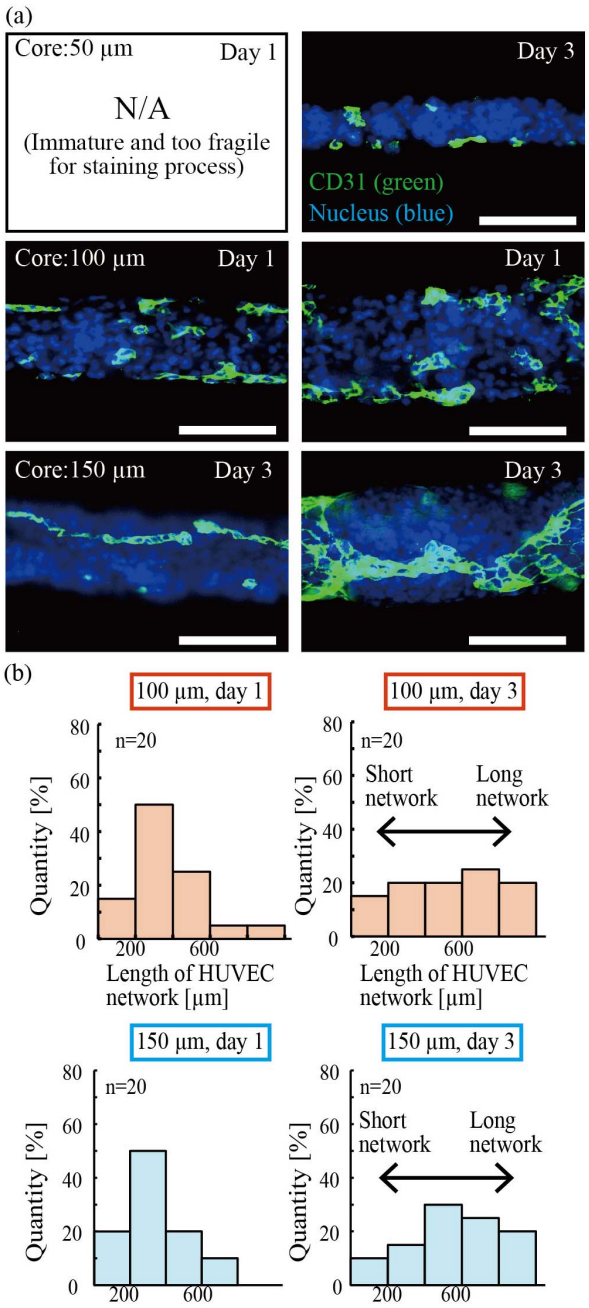

Figure 4: Fluorescent analyses on the fiber-shaped tissue. (a) The blood vessel network formation in the tissues. Scale bars 100 µm (b) Distribution of the length of HUVECs connection.

Fiber assembly

We confirmed whether microvascular networks are connected properly when we assemble the fiber-shaped tissue into the macroscopic constructs. Firstly, we prepared the 2 days cultured fiber-shaped tissue (diameter=100 µm, Hep-G2:HUVEC=3:1) and arranged them in parallel on a 35 mm glass base dish to assemble to a macroscale tissue after removing the calcium alginate shell of the fiber-shaped tissues by alginate lyase. To fix them and prepare an environment suitable for cell growth, we covered them with a collagen gel. Then the assembled tissue was observed how the HUVEC networks connected to each other over the assembled fiber-shaped tissues after 2 days of culture.

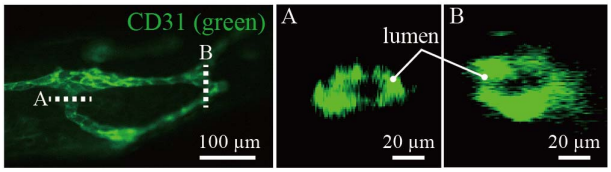

Figure 5:Cross-sectional images of the microvascular networks of the fiber-shaped tissue. These images show the presence of the lumens

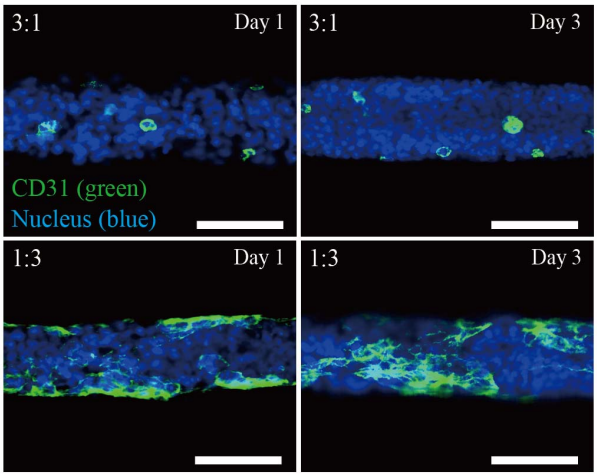

Figure 6: Fluorescent images of the fiber-shaped tissue with different in the ratio of the cells (HepG2:HUVEC=3:1, 1:3). Scale bars: 100µm

RESULTS

Fabricated fiber-shaped tissue

We fabricated a fiber-shaped tissue (shell diameter: about approximately 230 µm), which is composed of a shell part made of alginate calcium and a core part made of collagen gel and cells (Figure 3 (a))**.** To confirm the difference of the growth of blood vessel networks in cell culture environment having the different size, we fabricated three types of microfibers with different diameters of the core (50 µm, 100 µm, and 150 µm) (Figure 3 (b)) by changing the core and shell flow rates (Figure 3 (c)). At all the diameters, the cells were dispersed in the collagen gel immediately after fabrication, but the cells were connected each other and the fiber-shaped tissue is formed day 3. Figure 3 (d) shows the change of the diameter of the fiber-shaped tissues during culture. It shows that the diameter of the core slightly increased due to cell proliferation and progression.

Construction of a microvascular networks

Figure 4 (a) shows the confocal fluorescent images of immunostained HUVEC (green) and nucleus (blue) in the fiber-shaped tissues. Although HUVEC networks were not constructed in the microfiber with 50-µm core diameter, development of HUVEC networks was observed both in the microfibers with 100- and 150-µm core diameters. Analysis on the fluorescent images (Figure 4 (b)) indicates that HUVEC networks became longer by culturing for 3 days. The cross-sectional images of the HUVEC networks of the microfiber indicate the existence of lumen (Figure 5). This result indicates that the microvascular shape HUVEC tissues were formed in the fiber-shaped hepatic tissue. The cell ratio also affected on the HUVEC network

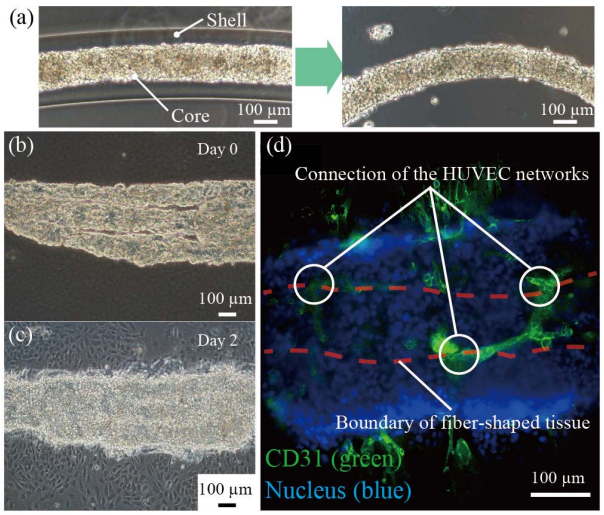

Figure 7. Macroscale assembly of the fiber-shaped tissues. (a) Removal of calcium alginate hydrogel by alginate lyase. (b) The fiber-shaped tissues were adjacently arranged in parallel. (c)The connection of the fiber-shaped tissue after 2 days cultivation. (d)Connection of the blood vessel networks between the microfibers.

formation (Figure 6). Connection of HUVEC was not observed in the 3:1 (Hep-G2:HUVEC) microfiber, but the connection of the HUVEC network was constructed in the 1:3 microfiber.

Observation of assembled fiber-shaped tissue

The calcium alginate shell of the fiber-shaped tissue (diameter: 100 μm, ratio of the cells 3:1) was removed by 15 minutes treatment with alginate lyase (Figure 7 (a)). The shell removed microfibers were arranged in parallel (Figure.7 (b)). After additional 2 day culture, arranged fiber-shaped tissues were connected to each other and larger macroscopic tissue was formed (Figure 7 (c)). The confocal fluorescent image of the immnostained assembled tissue obviously indicates that the connection of the HUVEC networks between the fiber-shaped tissues was constructed spontaneously (Figure 7 (d)). This result indicates a possibility of the fiber-shaped building block for assembling vascularized macroscopic tissues *in vitro*.

CONCLUSION

We proposed the fiber-shaped artificial tissue with microvascular networks for bottom-up tissue reconstruction. By using the double coaxial type microfluidic device, we succeeded in encapsulating two types of cells (Hep-G2 and HUVEC) in the hydrogel microfiber, and confirmed the connection of the cells by cultivation. We fabricated the fiber-shaped tissues with different diameters and ratio of the cell. In the fiber-shaped tissues with different diameters, we showed that as the diameter becomes larger it is suitable for constructing a longer HUVEC networks. In the fiber-shaped tissues with different ratio of cells, the larger the proportion of Hep-G2 is, the harder it is to construct HUVEC networks. We demonstrated a possibility of the fiber-shaped building block including microvascular networks, by assembling the fiber-shaped tissues. We believe that our microfiber containing HUVEC network would be useful for creating macroscopic artificial tissues in the tissue engineering field.

ACKNOWLEDGEMENTS

This work was partly supported by Grant-in Aid for Scientific Research (B) (26289060), Japan Society for the Promotion of Science (JSPS), Japan

REFERENCES

[1] Langer, R. and Vacanti, J.P. “Tissue engineering”, *Science*, 260, 1993,pp 920–926

[2] D. L. Elbert. “Bottom-up tissue engineering”, *Current Opinion in Biotechnology*, 22, 2011, pp 674–680

[3] J. Yang, M. Yamato, C. Kohno, A. Nishimoto, H. Sekine, F. Fukai, and T. Okano “Cell sheet engineering: Recreating tissues without biodegradable scaffolds”, *Biomaterials,* 26, 2005, pp. 6415-6422.

[4] D. A. Bruzewicz, A. P. McGuigan, and G. M. Whitesides. ”Fabrication of a modular tissue construct in a microfluidic chip”, *Lab on a chip*, 8, 2008, pp 663-671.

[5] S. F. Wong, D. Y. No, Y. Y. Choi, D. S. Kim, B. G. Chung, and S. H. Lee. ” Concave microwell based size-controllable hepatosphere as a three-dimensional liver tissue model”, *Biomaterials*, 32, 2011, pp 8087-8096.

[6] Su-Jung Shin, Ji-Young Park, Jin-Young Lee, H. Park, Yong-Doo Park, Kyu-Back Lee, Chang-Mo Whang, Sang-Hoon Lee. “On the Fly Continuous Generation of Alginate Fibers Using a Microfluidic Device”, *Langmuir*, 23, 2007, pp 9104–9108

[7] H. Onoe, T. Okitsu, A. Itou, M Kato-Negishi, R. Gojo, D. Kiriya, K .Sato, S. Miura, S. Iwanaga, K. Kuribayashi-Shigetomi, Yukiko, T. Matsunaga, Y. Shimoyama, and S. Takeuchi, .” Metre-long cell-laden microfibres exhibit tissue morphologies and functions”, Nature Materials, 12, 2013, pp 584-590.

[8] K. H. Lee, S. J. Shin, Y. Park, and S-H. Lee .” Synthesis of Cell-Laden Alginate Hollow Fibers Using Microfluidic Chips and Microvascularized Tissue-Engineering Applications”, small, 5, 2009, pp 1264-1268.

[9] T. Neumann, S. D. Hauschka, J, E. Sanders, “Tissue Engineering of Skeletal Muscle Using Polymer Fiber Arrays”, Tissue Engineering, 5, 2003, pp 995–1003

[10] E. Kang, Y. Y. Choi, Su-Kyoung. Chae, Jin-Hee Moon, Joon-Young. Chang, Sang-Hoon. Lee .” Microfluidic spinning of flat alginate fibers with grooves for cell-aligning scaffolds”, *Advanced Materials*, 24, 2012, pp 4271-4277.

CONTACT

*R. Sato, tel: +81-45-566-1507;
E-mail: ryosato@keio.jp