Microfluidic perfusion culture system for multilayer artery tissue models

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We described an assembly technique and perfusion culture system for constructing artery tissue models. This technique differed from previous studies in that it does not require a solid biodegradable scaffold; therefore, using sheet-like tissues, this technique allowed the facile fabrication of tubular tissues can be used as model. The fabricated artery tissue models had a multilayer structure. The assembly technique and perfusion culture system were applicable to many different sizes of fabricated arteries. The shape of the fabricated artery tissue models was maintained by the perfusion culture system; furthermore, the system reproduced the *in vivo* environment and allowed mechanical stimulation of the arteries. The multilayer structure of the artery tissue model was observed using fluorescent dyes. The equivalent Young's modulus was measured by applying internal pressure to the multilayer tubular tissues. The aim of this study was to determine whether fabricated artery tissue models maintained their mechanical properties with developing. We demonstrated both the rapid fabrication of multilayer tubular tissues that can be used as model arteries and the measurement of their equivalent Young's modulus in a suitable perfusion culture environment. © 2014 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4903210>]

I. INTRODUCTION

The primary function of arteries is to transport oxygen, nutrients, and waste. Arteries also indicate the health of each tissue, since poor circulation and vascular lesions result in circulatory system diseases.¹ Arteries are a multilayer structure comprising adventitia, media, and intima. Each layer has a specific role. The outer layer, adventitia, is composed of fibroblasts; media, the central layer, is made of smooth muscle and elastic fiber; and intima on the inside is made of endothelial cells and subendothelial tissue. The outer layer must be composed fibroblasts because the artery must withstand the pressure created by the heart (blood pressure). Smooth muscle in the central layer controls the blood pressure by its contractile and relaxant responses and supports the extracellular matrix (ECM). Endothelial cells prevent blood clotting and secrete various nutrients into the blood flow. Also, smooth muscle and endothelial cells respond to the blood flow and blood pressure. Various nutrients, wastes, and signals interact with each layer. Because each layer plays a complex role in the function of the artery,^{2,3} there is increasing interest in understanding the *in vivo* mechanisms by which arteries develop. The development of artificial arteries that mimic the structure of natural arteries is being driven by tissue engineering.^{4,5}

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The lack of techniques for fabricating macro-scale artery models from cells is hampering research on the mechanical properties of arteries.^{2,6,7} In particular, artificial arteries 2–6 mm in diameter are easily blocked, leading to coarctation and thus making these artificial arteries unsuitable for clinical use. Several recent reports have described the construction of artery models using cells in microfluidic chip, using a biodegradable scaffold and low density of cells.^{8–10}

The mechanical properties of arteries are important for understanding the characteristics of arteries *ex vivo*. Arteries actively respond to blood flow and blood pressure^{11,12} by changing their mechanical properties. To date, various mechanical properties of artificial arteries have been investigated.¹³ However, few studies have been published on the mechanisms by which arteries adapt and respond to mechanical stimuli like blood flow and blood pressure using a tubular tissue. There is therefore need for artery tissue models composed of a high density of cells that can be easily assembled into a multilayer tubular structure. It is also desirable to perfuse culture these artery tissue models, since determining their characteristics *ex vivo* requires observation and measurement of their mechanical properties in perfusion culture that mimics *in vivo* environments. Here, we propose both a new technique for assembling engineered multilayer tubular tissues as artery tissue models and a new perfusion culture system. A mechanical property of the model arteries, the equivalent Young's modulus, was measured by using this system. The assembly technique and perfusion system described here will help in understanding the mechanical properties of tubular tissues.

II. MATERIALS AND METHODS

A. Design of multilayer tubular tissues

Figure 1(a) shows the concept for fabricating multilayer tubular tissues. First, sheet-like multilayered tissues are made using the Layer-by-Layer (LbL) technique.^{14,15} In this technique, fibronectin-gelatin (FN-G) nano films are deposited onto single cell surfaces to promote cell-cell interactions and cause the cells to adhere to each other, thus mimicking the ECM. Sheet-like multilayered tissues are obtained with defined thickness and high cell density. The sheet-like tissue is retrieved from the culture membrane and floated on the surface of the medium. Using water transfer printing,¹⁶ decorating technique for a curved body, the sheet-like tissue is transferred onto the surface of a supporting body and lifted from the medium. Sheet-like multilayered tissues were repeatedly floated and transferred other multilayered tissues repeatedly. Collagen gel is placed in the channel of the assistive jigs for preserving multilayer tubular tissues. Then, the fabricated multilayer tissue is put on the channel in the assistive jigs and clipped with assistive jigs and covered with collagen gel. After the supporting body is removed, multilayer tubular tissues can be fabricated from sheet-like tissues rapidly. The detailed fabrication process is shown in supplementary Figure S1 and supplementary Movies S1 and S2.²⁸

B. Cell culture

We used mouse smooth muscle cells (mSMCs; MOVAS7). MSMCs were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma Aldrich Corp., MO, USA) containing 10% fetal bovine serum (FBS; Thermo Scientific, MA, USA) and 1% penicillin-streptomycin. MSMCs were trypsinized (trypsin-EDTA for 5 min at 37 °C) from the culture dishes, plated at a density of 1.0×10^7 cells/well in 6 well cell culture inserts (Corning, Inc., NY, USA), and grown in an incubator for 4 days at 37 °C in 5% CO₂. After 3 days culture, a sheet-like multilayered tissue was fabricated on the membrane of the culture insert. Specifically, 1.7×10^7 mSMCs were used to fabricate sheet-like multilayered tissues on the membrane.

The sheet-like tissues had to be retrieved from the membrane prior to being assembled into tubular tissues from sheet-like tissues. However, the sheet-like tissues shrank after retrieval from the culture membrane due to cellular tensile forces. The relationship between the size of the initial flat seeded area to target diameter of the tubular tissue is important for successful

FIG. 1. (a) Concept behind the fabrication of multilayer tubular tissues. A sheet-like tissue is fabricated using the LbL technique and transferred onto a supporting body using the assembly technique. The transfer step is repeated to assemble several sheet-like multilayered tissues. Assistive jigs are utilized to lift the sheet-like tissues vertically from the water, and then the supporting body is removed. This approach allows the rapid fabrication of multilayer tubular tissues. (b) Schematic of the perfusion culture system. This culture system provides mechanical and chemical stimuli to the tubular tissues and allows the measuring of the equivalent Young's modulus with culturing.

fabrication of tubular tissues. The relationship between the seeded area S (mm²) and the target diameter d (mm) of tubular tissue is given by

$$S > d \times \pi \times l/a \quad (a: \text{retrieval rate}). \quad (1)$$

In Eq. (1), a is the retrieval rate, that is, the rate of retrieving the sheet-like area from the culture membrane compared to the initial seeded area on the culture membrane and l (mm) indicates the target length. In this proposed technique, we assembled tubular tissue from sheet-like tissue. Therefore, the required seeded area was calculated from [(the circumference of the target artery) $\times$ (the length of the target artery)]. Our previous research showed that the retrieval rate of sheet-like tissue when the cells were seeded in 6-well plates (23.1 mm diameter well) was 0.64 ($n=10$). In this study, we targeted tissue models of the carotid artery. The target diameter d was 3 mm and the target length l was 10 mm. Therefore, the necessary required seeded area S was $S > 3 \times \pi \times 10/0.64 = 13 \times 13$. Due to tissue shrinkage, sheet-like tissue squares 13 mm $\times$ 13 mm were required to construct tubular tissues, 3 mm in diameter and 10 mm in length.

C. Design of supporting body and assistive jigs

A double glass capillary was used as a stiff supporting body. The sheet-like tissues were repeatedly assembled on the tubular structure with connecting the two outer glass capillaries from the air-liquid interface. An inner glass capillary was passed through the inside of two outer glass capillaries. In Figure 1(a), the blue tube indicates the inner glass capillary and the light blue tubes indicate the outer glass capillaries. The tubular tissues were fabricated by removing the inner glass capillary from the outer glass capillaries. The inner glass capillary is 2.0 mm in diameter (Narishige, Inc., Tokyo, Japan) and the outer glass capillaries (Narishige, Inc., Tokyo, Japan) have an outside diameter of 3.0 mm and an inside diameter of 2.2 mm. We can fabricate tubular tissues with various diameters by just converting the diameters of the glass capillary, for example, 2–6 mm in diameter.

Assistive jigs were used to help maintain the tubular structure. After the several times assembly to fabricate multilayer tubular tissues, tubular tissues could be embedded in and covered with collagen gel by clipping with the assistive jigs. The tubular tissues deform when placed in medium and cultured if the tissue is not covered with collagen gel. Therefore, we covered the tubular tissues with collagen gel to maintain the tubular structure in medium. Porcine tendon acid-soluble Type I collagen (Cellmatrix Type I-A; Nitta Gelatin, Inc., Osaka, Japan)^{17,18} was used to form a collagen gel 50 μm thick. Gel was formed by making a sol and maintaining it at 37 °C for at least 30 min. Following assembly of the tubular tissue, both surfaces of the tissue were covered with the collagen gel, and then the tissue was incubated at 37 °C in 5% CO_2 for 1 h. This solidified the collagen gel, thus maintaining the tubular structure of the artery model. Two types of assistive jigs were used: to maintain the tubular structure, and to connect, position, and move the tubular tissues. The jigs had channels 4 mm in diameter and 15 mm long filled with collagen gel to cover and maintain the tubular structures of the fabricated tubular tissue (Fig. S1(7)–(9)).

D. Observation of multilayer tubular tissues

A confocal laser microscope system (A1RSI-N; NIKON Corp., Tokyo, Japan) was used to observe cross-sections of the fabricated tubular tissues. The sheet-like tissues were stained after 4 days culture and then assembled into the tubular structure. The tissue model was excited with laser light under the fluorescence microscope (objective lens: 4 $\times$). PKH 67 (Sigma Aldrich Corp., MO, USA) and Hoechst 33 342 solution (Dojindo, Inc., Kumamoto, Japan) were used to observe the multilayer structure of the tubular tissues. In general, PKH 67 stains the cell membrane, while Hoechst 33 342 stains nucleic acids. The excitation and emission wavelengths of PKH 67 are 490 nm and 504 nm, respectively, and those of Hoechst 33 342 are 352 nm and 461 nm, respectively. These two stains were used to observe the multilayer structure. One sheet-like tissue was stained with PKH 67 and the other sheet-like tissue was stained with Hoechst 33 342. Multilayer tubular tissue was then constructed from one of each stained sheet-like tissues.

A confocal microscope system (MVX10; Olympus Corp., Tokyo, Japan) was used to observe flow in the fabricated tubular tissues. As above, the sheet-like tissues were stained after 4 days culture, assembled into tubular structures, and then exposed to laser excitation light under the microscope (objective lens: 1 $\times$).

E. Perfusion culture system and monitoring system

A perfusion culture system was used to culture, monitor, and evaluate the tubular tissues during exposure to various stimuli. The system was composed of a centrifugal pump, a culture chamber, a pressure sensor, a flow meter (OF10ZAWP; Aichi Tokei Denki Co., Ltd., Aichi, Japan), and a medium reservoir. The components were connected to each other with silicone tubes to form a closed circuit and the system was filled with medium. Following fabrication of the tubular tissues using the LbL technique and water transfer printing, the tissue was inserted in the culture chamber connected to the outside pump system and monitored with perfusion

culture. The culture conditions can be varied by applying mechanical stimuli,^{19–21} for example, internal pressure by the pump system, or/and chemical stimuli such as O₂, NO, or different growth factors (Fig. 1(b)). The tubular tissues could be observed and evaluated using sensors and microscope images of the culture chamber during perfusion mimicking various *in vivo* environments. Image processing was used to observe deformation caused by pulsatile flow perfusion,^{22,23} and the edges of the tubular tissues were observed from the microscope images.

F. Evaluation of the mechanical properties of multilayer tubular tissues

Tubular tissues can be monitored and evaluated using the proposed system. For example, if we assume that the tubular tissues thickness are uniform, that the collagen film thickness is uniform, and that the model arteries do not leak, we can evaluate the equivalent Young's modulus of the tubular tissues. The thin cylinder model shown in Figure 2 was used to model the extension of the fabricated tubular tissues. The cross-section of the fabricated tubular tissue was approximated by a circle. Figures 2(a) and 2(b) show the initial state and the extended state, respectively. We measured the equivalent Young's modulus using formula (2) based on the thin cylinder model²⁴

$$E = \frac{(2 - \nu)pr^2}{2ut}, \quad (2)$$

where u is the stretch of the artery tissue (mm), ν is the Poisson's ratio of the artery tissue, p is the inner pressure of the artery tissue (Pa), r is the first diameter of the artery tissue (mm), t is the thickness of the artery tissue (mm), and E is the Young's modulus of the artery tissue (Pa).

No medium was flowed through the tubular tissue during measurements of the equivalent Young's modulus. The stretches were measured from the difference in the diameter between the initial and extension state. Therefore, we could apply formula (2) to the tubular tissues because the vessel wall was impervious to the flow volume.

We prepared a tubular model of poly-(lactide-co-caprolactone) (PLCL) in order to confirm whether this thin cylinder model can be used to estimate the mechanical properties of the tubular tissues. Based on a previous study,^{19,25} a 5 wt. % solution of PLCL (molar ratio: 50:50, 5 g of PLCL and 95 g of chloroform, molecular weight: 4.05×10^5 , BMG, Inc., Kyoto, Japan) in chloroform (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was prepared. Then, NaCl microparticles (diameter: 90–106 μm) were added to the polymer solution to make a porous scaffold. The porosity of the prepared PLCL tubes was 12%.

FIG. 2. Schematic showing the simulated expansion of a fabricated tubular tissue model. The model is based on the thin cylinder model. (a) The tubular tissue remains static during steady flow perfusion culture. (b) During pulsatile flow perfusion culture, the tubular tissue expands from the initial state due to the applied pressure. The mechanical properties of the tissue are measured from the relationship between the applied pressures and the stretch.

III. RESULTS

A. Fabrication of multilayer tubular tissues

Sheet-like multilayered tissues $13\text{ mm} \times 13\text{ mm}$ (Fig. 3(a)) were fabricated using the LbL technique. After 3 days culture, 91% of the cells in the sheet-like tissues were viable. The sheet-like tissue was lifted vertically from the air-liquid interface at 25 mm/s using an auto stage (SGSP20–85(Z); SIGMAKOKI Co., Ltd., Saitama, Japan). The sheet-like tissue was transferred onto the surface of the supporting body and covered with collagen gel, and then the inner glass capillary was removed. The tubular tissue was 2.2 mm in diameter and 5 mm long and was connected at both ends to the outer glass capillaries by 3 mm in diameter (Fig. 3(b)). Figure 3(c) shows fluorescence microimages of the fabricated tubular tissue. Green indicates the first layer and blue indicates the second layer. These images show that the tubular tissue was successfully fabricated and has a ring-like cross-section and a multilayer structure, similar to a native artery. The figure shows that the tissue is $50\text{ }\mu\text{m}$ thick and each layer is $25\text{ }\mu\text{m}$ thick.

Medium was introduced into the fabricated tubular tissue to determine if liquid can be perfused through the artery. The experimental setup is shown in Figure 4. The tubular tissue was 2.2 mm in diameter, 3 mm in length, and $50\text{ }\mu\text{m}$ in thickness, and the flow volume through

FIG. 3. (a) Photo of a sheet-like multilayered tissue. (b) Photo of a tubular tissue attached to the perfusion double glass capillary. (c) Fluorescence microimages of a tubular tissue attached to the perfusion double glass capillary. The green tube is the first layer of cells stained with PKH 67 and the blue tube is the second layer stained with Hoechst 33342.

FIG. 4. Schematics and photos of the perfusion experiment using tubular tissues. The orange section indicates the fabricated tubular tissue and the two blue sections indicate the outer glass capillaries in the left schematics. Red arrows indicate the diameter of the tubular tissue. Dashed lines indicate the edges of the tubular tissue. The center white section is the fabricated tubular tissue and the two glass sections are the outer glass capillaries in the right photos. Medium was pumped through the tubular tissue using a centrifugal pump (flow volume: 10 ml/min).

the tissue was 10 ml/min. The center orange section indicates the fabricated tubular tissue and the two blue sections on either side indicate the outer glass capillaries (Figure 4 left). Red arrows indicate the tubular tissue diameter. Dashed lines indicate the edges of the tubular tissue. In the right hand photo, the central white section is the fabricated tubular tissue and the glass on both sides is the two outer glass capillaries. We confirmed that liquid flows down the fabricated tubular tissue without leakage at the connection between the tubular tissue and the outer glass capillaries, thus demonstrating that we can fabricate multilayer tubular tissue using the proposed assembly technique. The tubular structure had sufficient high stiffness and cell density to flow the medium.

B. Perfusion culture of multilayer tubular tissues

We confirmed that the microfluidic perfusion culture system and observation system could be used to measure the mechanical properties of the tubular tissues. Initially, a PLCL tube (thickness: $380\text{ }\mu\text{m}$) was connected and perfused. During perfusion, the tube stretched due to the applied pressure: at 58 kPa applied pressure, the tube stretched by $300\text{ }\mu\text{m}$. The stretch caused by the applied pressure was measured at 5 points along the PLCL tube and averaged. The stretch along the length of the tube appeared to be uniform because the measured stretch at each of the 5 points was $300 \pm 15\text{ }\mu\text{m}$. Using the thin cylinder model shown in Figure 2, the relationship between stretch and applied pressure allowed evaluation of the equivalent Young's modulus of the PLCL tubes ($n=3$). Tensile tests of the PLCL tubes provided an equivalent Young's modulus of $800 \pm 10\text{ kPa}$, while calculations provided an equivalent Young's modulus of $860 \pm 30\text{ kPa}$. The agreement between the two sets of results showed that our proposed evaluation model is appropriate for determining the equivalent Young's modulus of the tubular tissues.

Three tubular tissues were prepared and each model was perfused using a centrifugal pump at the same time. Flow was monitored by a flow meter. The tubular tissues had been cultured for 4 days and were monitored continuously. During culture, the tubular tissue was perfused with the medium and stretched by the pulsatile flow. Figure 5 shows the perfusion conditions used with the fabricated tubular tissues. As shown in Figure 5(a), the pulsatile flow period was 0.5 Hz, with a flow volume of 60 ml/min for 1 s and then 40 ml/min for 1 s; as a result, a 300 Pa applied pressure stretched the tubular tissues by $125\text{ }\mu\text{m}$.

The stretch caused by the applied pressure was measured at 5 points along the tubular tissues and averaged. The stretch along the length of the tubular tissue appeared to be uniform

FIG. 5. (a) Schematic of the pump rotation rate generated by the centrifugal pump. The pump rotation rate is related to the flow volume. The frequency of pulsatile flow was 0.5 Hz; the flow volume in the tubular tissue was 60 mL/min at the max pump rotation rate and 40 mL/min at the minimum rotation rate. (b) Graph of pressure applied to the tubular tissue during pulsatile flow perfusion (approx. 300 Pa).

because the measured stretch at each of the 5 points was $125 \pm 12 \mu\text{m}$. Using the same approach as described above for the PLCL tubes, the equivalent Young's modulus of the fabricated tubular tissues (thickness: $60 \mu\text{m}$) was determined to be $130 \pm 20 \text{ kPa}$ ($n = 3$). The equivalent Young's modulus was determined to be $110 \pm 23 \text{ kPa}$ when the tubular tissues were stretched by $84 \pm 15 \mu\text{m}$ ($n = 6$). A collagen-only gel tube (thickness: $60 \mu\text{m}$) had an equivalent Young's modulus of 90 kPa. Therefore, the equivalent Young's modulus of the fabricated tubular tissues was measured using the proposed system.

IV. DISCUSSION

Figure 3(a) shows a sheet-like multilayered tissue made by the LbL technique and demonstrates the construction of thick artery tissues not achievable using most of the previous techniques. The tissue shrank approximately 35% during the early stages of culture; regardless, the fabricated tubular tissues were wide enough to allow formation of a tubular structure.

Figure 3(b) shows a fabricated tubular tissue. It has a multilayer tubular structure, similar to a native artery. Initially, it was difficult to maintain the structure of the artery and connect it to the artificial perfusion system, but this was ultimately achieved by using a double glass capillary and assistive jigs. If a simple glass capillary instead of a double glass capillary was used as the supporting body, the glass capillary could not be removed from the tubular tissue due to the low stiffness of the tissue. When a simple double glass capillary was used, the tubular tissues collapsed upon removal of the inner capillary. However, using the assistive jigs, the inner glass capillary could be easily removed from the tubular tissues to fabricate the tubular structure, and the tubular tissue could be easily connected to silicone tubes by using both ends of the outer glass capillaries. The supporting body after removing the inner glass capillary could not be positioned because it was composed of two outer glass capillaries. However, using the assistive jigs, the tubular tissue over the two outer glass capillaries could be positioned and inserted into the culture chamber.

Figure 3(c) shows fluorescent Z-stack confocal images of fabricated tubular tissue. This tubular tissue was uniformly transferred to the supporting body. The tubular tissue consisted of 2 layers with a total thickness of $50 \mu\text{m}$. Therefore, multilayer tubular tissues similar to native artery were obtained rapidly using water transfer printing. Figure 4 shows a perfusion experiment. A tubular tissue was inserted into the culture chamber and the tubular structure was maintained by the assistive jigs during perfusion. We confirmed that medium was transported in the fabricated tubular tissue without leakage. In future, endothelial cells will be applied to the inner surface of the fabricated tubular tissue, thus providing multilayer tubular tissues compatible with the capillary network.

By using a custom-designed perfusion culture system, fabricated tubular tissues were perfusion-cultured in a pulsatile flow mimicking the *in vivo* environment. Mechanical stimuli, such as internal pressure and shear stress inside the tubes, should be applied during long-term

culture; furthermore, perfusion culture promotes the development of elastic fibers in the tubular tissues.²⁶ Our proposed multilayer tubular tissues can be applicable to highly responsive bionic simulators of native artery, in contrast to artificial artery or artery tissues with biodegradable scaffolds. In addition, unlike previous studies, our proposed system allows direct, real-time monitoring of the effects of mechanical stimuli in the tissue models. In future, the mechanisms underlying arteriosclerosis and angiogenesis could be monitored *ex vivo* using this system; the addition of pressure and oxygen sensors could allow observation of the mechanical/biological properties of assembled tubular tissues. Furthermore, elastic fiber development and protein expression were observed during long-term culture using this system.²⁷ The system described here will be useful for research in tissue engineering and circulatory system diseases.

V. CONCLUSION

One goal of tissue engineering is the fabrication of tissue structure that mimics the physiological and mechanical properties of native arteries. Tubular tissue similar to native artery always develops responding to the surrounding environments such as chemical and mechanical stimuli. From these simulations, the physiological and mechanical properties of synthetic arteries can be evaluated *ex vivo*. We proposed a new assembly technique to fabricate multilayer tubular tissues and a new microfluidic perfusion culture system mimicking the *in vivo* environment. The multilayer tubular tissues fabricated by the new assembly technique could be studied using the microfluidic perfusion culture system and other evaluation systems. Moreover, this system would be useful for measuring the equivalent Young's modulus of arteries with perfusion culturing and allowing the real-time monitoring of the mechanical properties of tubular tissues. In future, the use of this system will help to simulate tissue development by real-time monitoring and evaluation *ex vivo*.

This fabricating technique was used to make customized, macro-scale, and three-dimensional tissue models. This technique is applicable to many different cell types, such as smooth muscle cells, fibroblast and endothelial cells, required to fabricate artery-like structures. More in-depth investigations using this perfusion culture system might be possible in order to expand our knowledge of complex cell-cell interactions and the three-dimensional organization of cells in well-defined tissue architectures.

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