Fabrication of *in vitro* three-dimensional multilayered blood vessel model using human endothelial and smooth muscle cells and high-strength PEG hydrogel

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Abstract

Here, we fabricated a three-dimensional multilayered blood vessel model using human cells and high-strength PEG hydrogel. The hydrogel tube was physically suitable for perfusion culture, and cells were cultured on the hydrogel surface by binding with fibronectin. Using the layer-by-layer cell multilayered technique, we successfully constructed an artificial blood vessel.

Main Text

Tissue engineering, which involves the creation of artificial tissues and organs, is an interdisciplinary field that aims at overcoming currently incurable chronic and degenerative diseases. The main strategy for fabricating such constructs is creating hybrid artificial tissues comprising tissue-specific cells and tissue-shaped molding scaffolds that support the attachment and proliferation of the cells (1). Using this technique, many types of bio-hybrid artificial tissues, such as skin, bone, and cartilage, have been constructed and used clinically (2-4).

Hybrid tissues with human cells are increasingly in demand for use as *in vitro* test samples for cosmetics and medicines (5) as well as for creating artificial disease foci, including those for chronic diseases. Disease foci typically consist of tissue-specific cells, immunocytes, and extracellular matrix (ECM); further, blood vessels are crucially involved in the development of chronic diseases in humans. To construct accurate *in vitro* samples for chronic and vascular diseases, artificial human blood vessel tissues are a primary requirement. This study therefore aimed to fabricate three-dimensional (3D) blood vessel tissues using multilayered human cells as a base, in order to eventually construct artificial disease foci.

Blood vessels are hierarchical multilayered tube structures composed of endothelial cells (ECs), smooth muscle cells (SMCs), pericytes, fibroblasts, and ECM. ECs are located at the inner surface of the vessels in a single layer in direct contact with flowing blood. ECs undergo shear stress from the blood flow and blood pressure. SMCs and pericytes surround the EC layer and

regulate the blood vessel diameter and physiological stability. They mainly undergo continuous stretching from the pulsatile blood flow. A bio-hybrid artificial blood vessel would need to mimic these layers and properties, and therefore needs 3D multilayered extendable and high-strength tube structures that allow culture in a medium or blood perfusion.

Previous studies have attempted to fabricate blood vessel-like structures *in vitro* using hydrogels, such as collagen and gelatin derivatives (6-8). Such hydrogels are considered a useful tool in biomedical research because of their elasticity, solute permeability, and cell compatibility (9,10). On the other hand, these hydrogels do not necessarily possess the high mechanical strength that is required for manipulation or perfusion culture. Thus, hydrogel materials with high mechanical strength and cell compatibility are required for successfully fabricating artificial blood vessels.

Recently, Sakai et al. reported that tetra-arm polyethylene glycol (PEG) hydrogel shows high mechanical strength with a homogeneous network structure (11). The tetra-arm hydrogel has a highly uniform diamond-like network structure and its maximum breaking stress is comparable to native articular cartilage (11,12). Therefore, we used the tetra-arm PEG hydrogel as a scaffold for constructing artificial blood vessels in this study.

The tetra-arm PEG hydrogel was constructed according to the method described by Sakai et al. (11), using commercially available tetra-amine-terminated PEG (TAPEG; SUNBRIGHT PTE-100PA) and tetra-NHS-terminated PEG (TNPEG; SUNBRIGHT PTE-100GS), which were purchased from NOF CORPORATION (Tokyo, Japan). We used an 18-gauge stainless needle (TERUMO, Tokyo, Japan) as the inner template and the polyethylene needle protector as the outer template of the tube shape. Then, 60 mg/mL of TAPEG and TNPEG were dissolved in phosphate buffer (pH 7.4) and mixed at room temperature. The mixed solution was immediately poured into the tube template and allowed to react for 12 h. The inner and outer diameters of the tube were 1.2 and 3.0 mm, respectively, and the length was 15 mm (Fig. 1A). After the PEG hydrogel tube

was removed from the outer template by desiccation, hydrogel tube swelling with PBS was induced again, and the inner needle was then removed. The tube's inner diameter was similar in size to *in vivo* small arteries.

First, we tested the mechanical response of the PEG hydrogel tube by allowing water to flow within the tube. The constant water flow was induced using a syringe pump. The flow rate of the water was intermittently increased from 0 to 0.4 mL/s, and the inner diameter of the tube was observed by microscopy. The inner diameter of the tube increased according to the flow rate (Fig. 1C). The rate of increase of the inner diameter of the tube was approximately 6% at 0.4 mL/s. Our results demonstrated that the PEG hydrogel tube could be expanded repeatedly and behaved similar to an elastic body. The rate of increase in the diameter of *in vivo* human arteries in response to blood pressure fluctuations varies according to the location of the artery in the body, being approximately ~10% (13). Thus, we considered that our fabricated hydrogel tube was possibly similar to *in vivo* blood vessels.

On the basis of the tube diameter and flow rate, the shear stress applied on the inner surface was calculated by Poiseuille's law (Fig. 1D):

$$\tau = \frac{4\mu Q}{\pi a^3}$$

where τ is the shear stress, a is the inner radius of the tube, μ is the viscosity of water at room temperature (0.00098 Pa·s), and Q is the flow rate. The increase in the shear stress declined with tube expansion (Fig. 1D). The inner surface of the fabricated hydrogel tube received up to 19 dyn/cm^2 of pressure within the range until the rate of 0.4 mL/s of water flow. *In vivo*, the degree of shear stress of blood flow depends on the size and location of blood vessels; for human arteries, the shear stress is approximately 2–16 dyn/cm^2 (14). Thus, we were able to fabricate a high-strength hydrogel tube using the tetra-arm PEG hydrogel.

Next, we estimated the molecular permeability of the tetra-arm PEG hydrogel according to the molecular diffusion in the hydrogel. We examined the diffusion coefficients (D) of the

fluorescence molecules, Alexa Fluor 488 Alkyne (Alexa-alkyne; MW, 774 Da) and Alexa Fluor 488 labeled dextran (Alexa-dextran; MW, 10 kDa) (Life Technologies, Tokyo, Japan) by fluorescence correlation spectroscopy (FCS) (FV-1000D; Olympus, Tokyo, Japan). The FCS measurement was performed at 23 °C. The acquired data were analyzed by the software supplied by Olympus with a fitting program. The ratios of the D values between those in water (D_0) and those in the hydrogel (D_g) of these molecules are shown in Fig. 1E. The molecules can diffuse in the hydrogel at approximately 0.7-fold diffusion coefficients compared to diffusion in water. Thus, our used tetra-arm PEG hydrogel has molecular permeability properties.

Then, we investigated the possibility of using the PEG hydrogel as a cell culture substrate. PEG contains a high volume of water and generally does not interact with biomolecules. To overcome this, we modified the NHS group of TNPEG using gelatin (1 mg/mL in PBS) or fibronectin (200 μg/mL in PBS) placed over the surface of the PEG hydrogel. For cell culture, we used the human fetal lung fibroblast TIG-1 strain (15), obtained from the Health Science Research Resources Bank (Osaka, Japan), along with human aortic smooth muscle cells (HASMCs), human aortic endothelial cells (HAECs), and human umbilical vein endothelial cells (HUVECs), purchased from Kurabo (Osaka, Japan). HASMCs, HAECs, and HUVECs from cell passages 4–6 and TIG-1 cells from the 35^{th} to 40^{th} population-doubling level were used. TIG-1 cells did not adhere to the normal hydrogel surface or to the gelatin-modified hydrogel surface (Fig. 2a,b). However, the TIG-1 cells successfully attached to and grew on the hydrogel surface modified with fibronectin (Fig. 2c). When the NHS group of TNPEG was blocked by glycine ahead of fibronectin modification, TIG-1 cells could not adhere to the surface (Fig. 2d). HASMCs and HAECs also adhered to and grew on the fibronectin-modified hydrogel surface (Fig. 2e,f). Thus, we successfully performed cell culture on the PEG hydrogel surface by modifying it using fibronectin. The cell adhesive activity of fibronectin is stronger than that of gelatin (16), while the density of the free NHS group of the hydrogel surface is limited. Therefore, gelatin bound on the

hydrogel surface would be insufficient to adhere the cells.

Finally, we fabricated a multilayered blood vessel model using HASMCs and HUVECs. For achieving the HASMCs and HUVECs multilayered culture, we used the layer-by-layer (LbL) cell multilayering technique (17,18). This technique forms nanometer-sized ECM films on the layered cell surface or single suspended cell surface to eventually construct 3D multilayered tissues (17,19). First, the inner surface of the PEG hydrogel tube was modified with fibronectin, and HASMCs and HUVECs were labeled by Celltracker Red (red fluorescence; Life Technologies) and Celltracker Green (green fluorescence), respectively. HASMCs and HUVECs were covered with a fibronectin-gelatin nanofilm using the LbL technique as described previously (19). Briefly, the trypsinized suspended cells were treated with 40 μg/mL of fibronectin for 1 min. The cells were collected by centrifugation and then treated with 40 μg/mL of gelatin for 1 min. After 7 repetitions of this LbL process, 150 μL of the HASMCs with fibronectin-gelatin nanofilm (1.0×10^5 cells/mL) was seeded on the inner surface of the PEG hydrogel tube. The PEG hydrogel tube was immersed in fresh HASMC medium, and the hydrogel was cultured at 37°C for 6 h. The hydrogel tube was turned upside down and more HASMCs in the fibronectin-gelatin nanofilm were again seeded on the inner surface of the tube and cultured for 12 h. Then, green fluorescence-labeled HUVECs (1.0×10^5 cells/mL) with the fibronectin-gelatin nanofilm were seeded onto the HASMCs layer of the hydrogel tube. After a further 24 h of culture in HUVEC medium, the hydrogel tube was observed using fluorescent microscopy (IX-71; Olympus). Red fluorescence-labeled HASMCs and green fluorescence-labeled HUVECs were observed within the inner channel of the hydrogel tube (Fig. 3a-c). They had adhered and spread on the inner surface of the hydrogel tube. An examination of a cross-section of the hydrogel tube revealed a clear layer of HUVECs and HASMCs, with HUVECs covering the layer of HASMCs (Fig. 3d,e). Thus, by using the LbL cell multilayered technique, we successfully constructed a multilayered 3D blood vessel model comprising HUVECs and HASMCs. In the meantime, the cell density of

the adhered inner surface of the hydrogel tube was inhomogeneous, probably due to our cell seeding method. Additionally, our constructed HASMCs layer was probably singular. To achieve homogeneous cell culturing and construction of the multilayered SMCs in the inner tube surface, we must develop a uniform and continuous cell-seeding method.

We then performed perfusion culture of the fabricated 3D multilayered blood vessel model. The flow rate was increased and maintained at 20 μL/s. The shear stress of this flow rate was approximately 0.81 dyn/cm^2 (the viscosity of water at 37 ºC is 0.000685 Pa·s). Under these conditions, the HUVECs and HASMCs did not show any morphological changes (data not shown). In future studies, we intend to apply a higher flow rate and maintain a long-term perfusion culture of the 3D multilayered blood vessel model fabricated in the present study.

In this study, we successfully fabricated a 3D multilayered blood vessel model using human cells and high-strength tetra-arm PEG hydrogel that was used to construct a flexible tube-shaped scaffolding for the vessel. We examined the physical properties of the tube-shaped PEG hydrogel, and we consider the hydrogel tube to be suitable for perfusion culture subjected to the mechanical stresses of blood flow. Cells could be cultured on the PEG hydrogel surface by covalent bonding with fibronectin on the hydrogel surface. Using the LbL cell multilayered technique, an artificial bio-hybrid multilayered blood vessel-like structure comprising HASMCs and HUVECs was successfully constructed, which allowed perfusion culture. At the present time, there are mainly 2 types of *in vitro* blood vessel models for biohybrid vascular research: one is a hydrogel model, while the other is a microfluidic channel. The materials used in the microfluidic channel, such as PDMS and glass, have high mechanical strength, while the microfabrication technique enables the construction of various sizes and shapes of microfluidic channels. Thus, the microfluidic channel with ECs is used to examine leukocyte inflammation reactions (20). On the other hand, the blood vessel models using hydrogel, including the present model, have some vessel properties including wall expansion and solute permeability. The *in vitro* studies for

vascular and inflammatory diseases need diverse types of *in vitro* blood vessel models. In particular, the *in vitro* models with physical properties and multicellular location similar to *in vivo* arteries contribute to the study of refractory chronic diseases. These models enable the inflammation reaction and subsequent tissue cell responses with fast blood flow in the vessels under arbitrary conditions. We believe that our constructed model will be a tool in the development of artificial foci for chronic diseases and in future research related to atherosclerosis and chronic inflammation.

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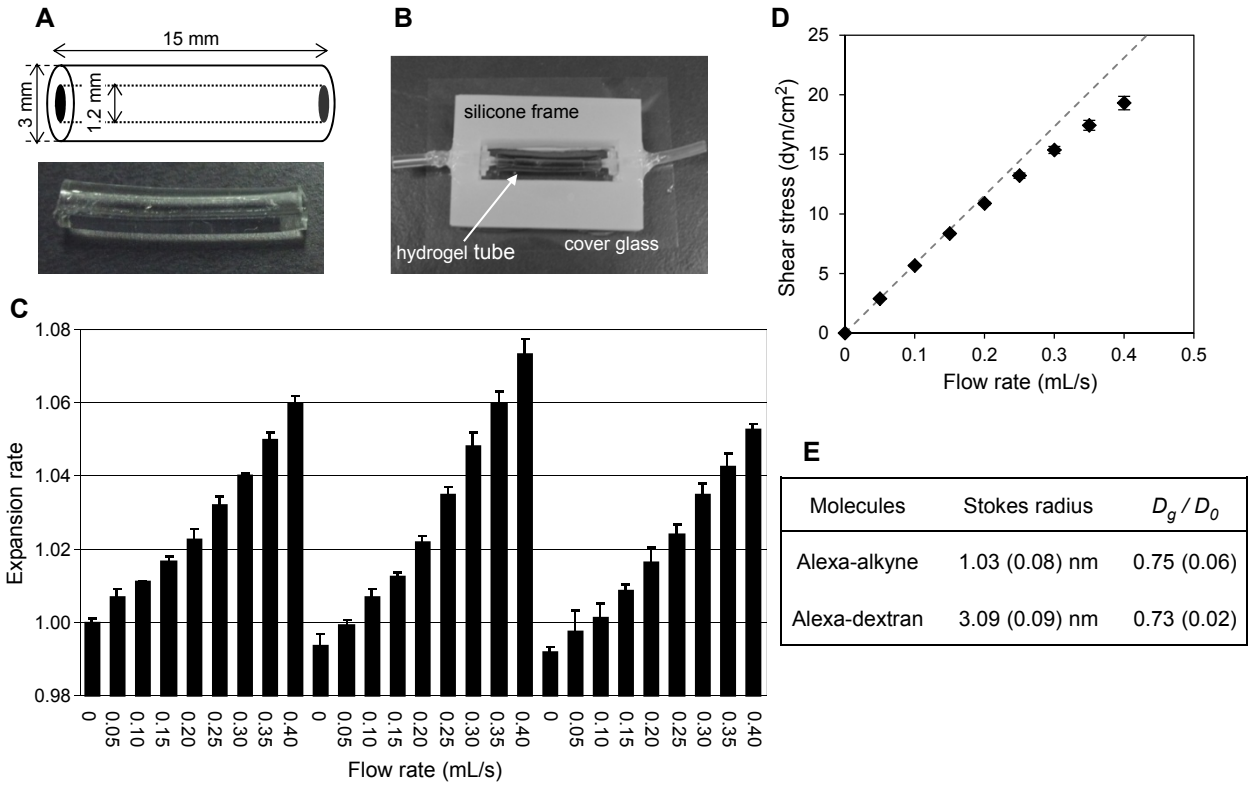
Figure legends

Fig. 1. Tube scaffold of tetra-arm PEG hydrogel. (A) Size and shape of the hydrogel tube. (B) The culture system using the hydrogel tube. (C) Mechanical features of the hydrogel tube with inner water flow. Each indicated flow rate was achieved beginning from 0 mL/s. The expansion in the tube diameter was observed by optical microscopy. Each expansion rate was calculated from 5 observations. (D) Applied shear stress for the inner channel surface for each flow rate. The broken line shows the shear stress of the tube without expansion. (E) The ratios of the diffusion coefficients of fluorescence molecules between those in water (D_0) and those in the tetra-arm PEG hydrogel (D_g). The Stokes radius (nm) and D_g/D_0 are shown.

Fig. 2. Phase-contrast micrographs of the cells cultured on the tetra-arm PEG hydrogel surface. TIG-1 cells cultured on the normal PEG hydrogel (a), gelatin-modified hydrogel (b), fibronectin-modified hydrogel (c), and glycine-blocked fibronectin-modified hydrogel (d). HAECs (e) and HASMCs (f) were cultured on the fibronectin-modified PEG hydrogel surface. All micrographs were obtained after 24 h culture.

Fig. 3. Micrographs of the fabricated 3D multilayered blood vessel model at 2 days culture. Phase-contrast micrograph of the vessel model (a). Fluorescent micrographs of HASMCs (b) and HUVECs (c) on the inner surface of the model. Fluorescence micrographs of a cross-section of the constructed vessel model (d). The red cells are HASMCs, while the green cells are HUVECs. Highly magnified fluorescence micrograph of a part of the cross-section of the model observed by confocal laser scanning microscopy (FV-1000D) (e).



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Molecules	Stokes radius	D_g / D_0
Alexa-alkyne	1.03 (0.08) nm	0.75 (0.06)
Alexa-dextran	3.09 (0.09) nm	0.73 (0.02)

Fig. 1 Shinohara et al.

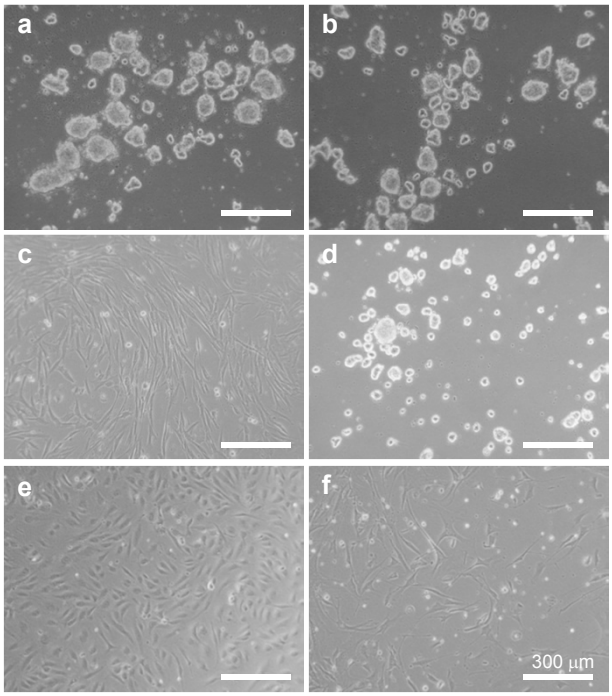

Fig. 2 Shinohara et al.

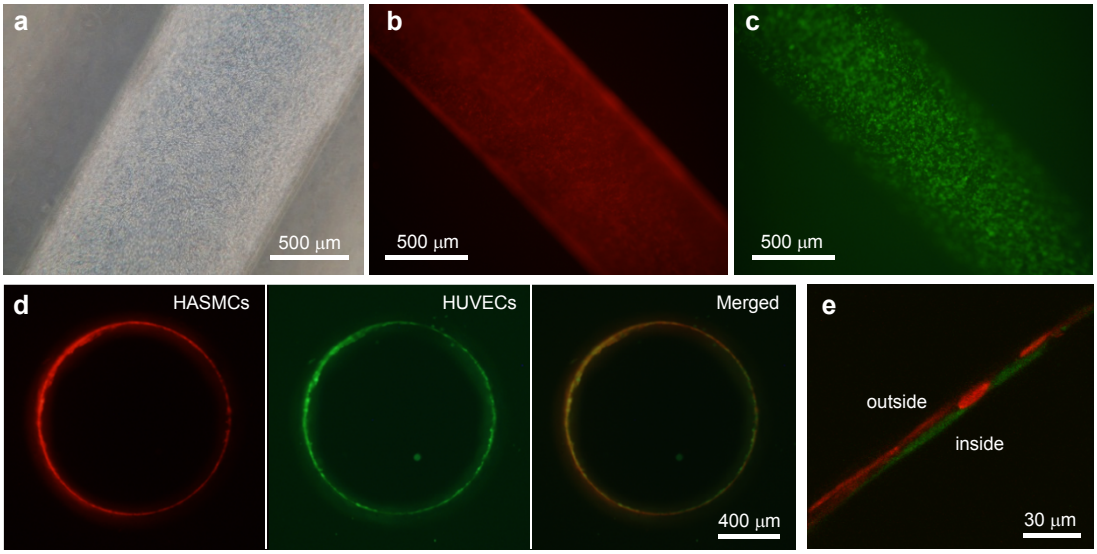

Fig. 3 Shinohara et al.