**Engineering of perfusable double-layered vascular structures using contraction of
spheroid-embedded hydrogel and electrochemical cell detachment**

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Abstract

Perfusable vasculatures are essential for engineering three-dimensional thick tissues and organs in the fields of tissue engineering and regenerative medicine. Here, we describe an approach for the fabrication of double-layered vascular-like structures (DVS) composed of a monolayer of human vascular endothelial cells (HUVEC) covered with a dense human smooth muscle cell (SMC) layer. HUVECs were attached to a gold needle via the oligopeptide self-assembled monolayer and grown to form a HUVEC monolayer that was subsequently embedded in a photo-crosslinkable gelatin hydrogel containing SMC spheroids in a culture chamber. During four days of culture, the hydrogel significantly contracted and formed a dense SMC layer around the needle. The binding between the HUVEC layer and the gold needle was cleaved by applying a negative potential to desorb the oligopeptide and the needle was extracted from the chamber, resulting in a perfusable DVS composed of HUVEC and SMC layers. The DVS was cultured under perfusion, and the cells in the DVS showed greater expressions of SMC-specific genes compared to those of spheroids. The DVS possessed a dynamic contraction ability in response to acetylcholine as observed in the *in vivo* SMC layer. This study proposes a promising approach for the fabrication of perfusable vasculatures for the engineering of fully vascularized tissues and organs.

1 **Introduction**

2 Organs such as the liver, pancreas, and kidney contain vascular networks for mass
3 transport, constantly delivering oxygen and nutrients throughout solid tissues and organs. Many
4 approaches have been reported to replicate vascularized tissues *in vitro*. A typical approach is
5 to encapsulate vascular endothelial cells in a hydrogel that can spontaneously form vascular
6 networks. Successful engraftment and curative effects have been demonstrated using
7 prevascularized tissue constructs prepared by encapsulating HUVECs with iPS-derived
8 hepatocytes in a hydrogel (1). The vasculatures in the constructs were connected to *in vivo*
9 vascular beds for blood to flow into the tissue constructs. However, because the diameter of
10 these spontaneously formed vasculatures is typically less than 50 μm , it is difficult to connect
11 an external pump to perfuse culture medium, limiting further the expansion of this approach to
12 larger dimensions and higher cell densities.

13 Approaches for perfusable vasculature fabrication have been proposed, including
14 microchannel preparation with sacrificial templates or needles in a hydrogel and subsequent
15 seeding of vascular endothelial cells inside microchannels (2-4). We also reported an approach
16 for perfusable vascular fabrication where a unique electrochemical reaction was used to detach
17 cells from a needle (5, 6). Vascular endothelial cells were first attached to a gold-coated needle
18 via an electro-active oligopeptide layer which could then be cleaved from the needle by the
19 application of a negative potential and transferred to a hydrogel, resulting in the fabrication of
20 a microchannel enveloped by vascular endothelial cells (7, 8). The advantage of this
21 electrochemical approach is that the cells and cell layers could be rapidly detached and
22 transferred to a hydrogel within 2-10 minutes (9-11). This rapid fabrication is significant when
23 organ cells such as hepatocytes and pancreatic cells are encapsulated at a high density in a
24 hydrogel to replicate these tissues, because calculations regarding the dissolved oxygen concentration
25 and oxygen consumption of hepatocytes suggest that oxygen in the medium is depleted within an hour even

1 when cells are encapsulated at one-tenth the density of the liver (7). In the conventional subsequent cell
2 seeding approach, perfusion of culture medium should be stopped for at least a few hours to let
3 endothelial cells attach onto the microchannels. In the proposed approach, the transferred
4 vascular endothelial cells migrated and sprouted into the surrounding hydrogels in following
5 perfusion culture to form branched and perfusable vascular networks (12, 13). However, the
6 drawback of this approach is that the vasculatures consist only of an endothelial cell layer, and
7 a rigid culture chamber is necessary to mechanically support it against hydraulic pressure
8 generated by perfusion of culture medium. This may be problematic when a fabricated tissue
9 is transplanted into the body.

10 In relatively large vasculatures *in vivo*, vasculatures contain a SMC layer that provides
11 mechanical tolerance to blood pressure and regulates the diameter of vasculatures by
12 modulating its contraction and relaxation. The SMC layer is also closely involved in various
13 vascular diseases, such as arteriosclerosis. Thus, the ability to replicate such DVS *in vitro* is
14 beneficial for engineering fully-vascularized three-dimensional thick tissues for transplantation
15 and for understanding the mechanisms of vascular diseases. Although there are several reports
16 for fabricating DVS using 3D printing (14), aggregation of microgels and spheroids (15), and
17 polymer scaffolds (16), a vascular endothelial cell layer was typically added after the SMC
18 layers were fabricated. Considering that vasculatures are inserted into high-cell dense
19 constructs for engineering vascularized thick tissues, this endothelialization approach may
20 cause a shortage of oxygen because the perfusion of culture medium should be stopped during
21 seeding of vascular endothelial cells onto a SMC layer for at least few hours.

22 In the present study, we took an approach of direct fabrication of DVS constructs by
23 first culturing a tubular HUVEC layer followed by encapsulation with SMC spheroids. The
24 rapid detachment and formation of the HUVEC tubular layer allowed maintaining oxygen
25 supply during the process and the use of SMC spheroids provided high cell seeding density

1 (Fig. 1). We believe that this could be a practical approach for the fabrication of perfusable and
2 robust vasculatures with thick SMC layers for engineering three-dimensional tissues with
3 vasculature networks.

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Materials and methods

Cell culture

GFP-labeled human umbilical vein endothelial cells (GFP-HUVECs; CC-2517, Cambrex Bio Science, Walkersville, MD, USA) were grown in EGM-2 culture medium (Lonza, Basel, Switzerland), exchanged every 2 to 3 days. The cells were passaged using 0.25% trypsin-EDTA (Thermo Fisher, Japan) before 80% confluence. GFP-HUVECs at passages 6 to 9 were used for experiments. Normal human aorta smooth muscle cells (SMCs; CRL-1999, ATCC, Manassas, VA, USA) were grown in Ham's F-12 culture medium (Sigma-Aldrich, Japan) supplemented with endothelial cell growth supplement (ECGS, Sigma-Aldrich), 10% fetal bovine serum (Life Technologies, USA), and 1% penicillin-streptomycin (Life Technologies, USA). Culture medium was replaced every 2 to 3 days. Cells were passaged using 0.25% trypsin-EDTA before cells reached 90% confluence. SMCs from passages 2 to 5 were used for the experiment. EGM-2 culture medium was used for co-culturing two types of cells.

Fabrication of microwell array culture plate and SMC spheroid formation

A microwell array plate (Fig. 1B) was fabricated using a two-step molding process as previously described (17). Briefly, hemispherical concave microwells (2,000 wells in 20 × 20 mm region) were drilled on an olefin plate (ZEONOR 1430 R, Zeon Co., Japan) with a ball-end mill ($\phi 500\ \mu\text{m}$) using a milling machine (MDX-540S, Roland Co., Japan). The microwell configurations were then transferred to an epoxy resin by pouring its solution (epoxy: curing agent, 2:1; Shinetu, Japan) and curing at room temperature for 48 hours. Using this protruding mold, a microwell array culture plate was fabricated by casting a polydimethylsiloxane (PDMS; Shinetu, Niigata, Japan) prepolymer solution (silicon elastomer: curing agent, 10:1) and baking at 80°C for 20 minutes. The PDMS microwell array plate was then immersed in pure water and autoclaved (120°C for 1 hour) to remove potentially harmful chemicals

including uncured PDMS and catalyst, to sterilize for subsequent cell culture. To make the surface of microwells cell-repulsive, a sterilized Pluronic F-127 solution (5 ml of 4% aqueous solution; Sigma-Aldrich) was poured in the microwells and incubated for 8 hours at room temperature. Prior to cell seeding, the plate was washed three times with PBS (Thermo Fisher Scientific Inc., Japan) to remove excess Pluronic F-127. SMCs were seeded on the plate at a density of 4×10^6 cells/2,000 wells/4 ml (2000 cells/well). After 1, 2, and 3 days of culture, SMC spheroids were collected from the microwells by gentle pipetting.

Preparation of GelMA

Photo-crosslinkable gelatin methacrylate (GelMA) was prepared as described previously (18, 19). A solution containing either 5% or 7% GelMA and 0.1% photoinitiator (Irgacure 2959, Wako chemical) was sterilized using a 0.22 μ m-pore filter (TPP, Switzerland).

Characterization of GelMA by encapsulating SMC spheroids

To characterize GelMA in terms of migration of SMCs, SMC spheroids prepared by culturing for 1 and 3 days in the microwell plate were encapsulated at a density of 50 spheroids/200 μ l in 5% GelMA solutions. The hydrogels were then crosslinked by UV exposure at 3 different intensities (5.0 mW/cm²-50 seconds, LOW; 7.0 mW/cm²-40 seconds, MID; and 7.0 mW/cm²-50 seconds, HIGH) with a UV lamp (USHIO, Japan). The spheroids were cultured for 3 days and the number and length of migrated cells from the surface of spheroids were quantified by taking phase-contrast microscopic images and analyzed using IMARIS software.

Oligopeptide design and gold needle preparation

The oligopeptide sequence CGGGKEKEKEKGRGDSP (Scrum, Japan) was designed for electrochemical cell detachment (Fig. 1E) (20). The oligopeptide is composed of three

functional domains, C, KEKEKEK, and GRGDSP (21). The cysteine (C) domain contains a thiol group, through which the oligopeptide is anchored to a gold surface with a gold–thiolate (Au-S) bond. The alternating lysine (K) and glutamic acid (E) domains induce a close packing of the oligopeptide layer on a gold surface by electrostatic intermolecular interactions, leading to a high-density self-assembled oligopeptide layer (22). The GRGDSP domain promotes integrin-mediated cell adhesion. The stainless needles were sputter-coated with a gold layer and modified with the oligopeptide by immersing in a 50 μ M oligopeptide aqueous solution at 4°C for 12 hours. After sterilizing with 70% ethanol solution for 5 minutes and washed three times with PBS, the needles were fixed in a culture chamber (see below). HUVECs were seeded on the needle with EGM-2 culture medium and cultured until cells covered the whole surface of the needle.

Preparation of culture chamber

A culture chamber was designed for the fabrication of a single DVS and subsequent perfusion culture (Fig. 1A). The chamber was made of PDMS and the volume was 80 μ l (2 mm in height, 20 mm in width, 2 mm in depth). Two glass pipes (650 μ m in inner diameter) were embedded into two walls of the chamber to guide the insertion and extraction of the needles and to connect silicone tubes (1.0 mm in inner diameter) from a syringe pump to a waste bottle. The PDMS chamber was fabricated using an acrylic plate mold cut using a computer-aided laser (Laser PRO C180; GCC, Taiwan) and bonded with an acrylic binder. After baking to cure PDMS in an 80°C oven for 30 minutes, the acrylic mold was removed and the glass pipes (diameter, 500 μ m; length, 8 mm each; Hirschmann Laborgeräte Eberstadt, Germany) were inserted into two holes in the opposite chamber walls and sterilized by autoclaving. Subsequently, the entire inner surface of the chamber was treated with 4% Pluronic F-127 solution for 8 hours to prevent cell adhesion. The chamber was rinsed three

times with PBS to remove the extra Pluronic F-127 solution.

Fabrication of DVS

SMC spheroids collected at day 1 (S1) or day 3 (S3) of the culture were suspended in 80 μ l of 5% GelMA solution at a density of 50,000 spheroids/ml. Further, the gold needle with HUVECs was set into the PDMS chamber and the GelMA solution with SMC spheroids was poured into the chamber. The GelMA was crosslinked with UV irradiation at 7.0 mW/m² for 50 seconds. The shrinkage of the hydrogel was assessed for 4 days of culture. The Ag/AgCl reference electrode was inserted into the chamber and a potential of -1.0 V was applied for 5 minutes to detach and transfer HUVECs from the needle to the surrounding spheroid-embedder hydrogel. The gold needle was then extracted through the glass guide pipe carefully to prevent damage of the inner HUVEC layer. For the perfusion culture, the glass pipe was connected to the micro-syringe pump and the EGM-2 culture medium was perfused at 20 μ L/min. The structure was observed with a phase-contrast, fluorescent microscope (IX-71, Olympus, Japan), and a confocal laser scanning microscope (LSM700, Carl Zeiss, Germany).

Contraction of SMC spheroids and DVS in response to drug stimulation

Fabricated spheroids and DVS were exposed to 500 nM acetylcholine chloride (A6625; Sigma-Aldrich, Japan) for 30 minutes and the changes in the diameters were recorded every 30 seconds by taking phase-contrast microscopic images. The images were analyzed with Image J.

Immunofluorescence staining

Cultures were fixed with 4% paraformaldehyde (Sigma-Aldrich, Japan) in PBS for 12 hours, treated with 0.2% Triton X100 for 6 hours, and then stained with rhodamine phalloidin

(Cytoskeleton, Inc., Denver, CO, USA) and 4',6-diamidino-2-phenylindole (DAPI) for 12 hours at 4°C. After washing three times with PBS, fluorescent images were taken with a fluorescence microscope and a confocal laser scanning microscope. For the SMC layers, the cultures were fixed with 4% paraformaldehyde and then incubated with mouse anti- α -actin antibody (1:100) (Thermo Fisher Scientific Inc., Japan) for 24 hours at 4°C. After washing three times with PBS, cells were incubated with a secondary antibody, Alexa Fluor 488 anti-mouse IgG antibody (Thermo Fisher Scientific Inc., Japan) for 2 hours. During the last 30 minutes, DAPI was added to the culture.

Elastin staining

Elastin staining was performed with an elastin stain kit (Modified Verhoff's; ScyTek LABORATORIES INC. USA) by following the manufacturer's instructions. Frozen sections were hydrated with distilled water, to which working solution was added. These were then incubated for 15 minutes. After washing with tap water, 10-15 drops of 2% ferric chloride differentiating solution were continuously added and then washed with tap water followed by 2 dips in distilled water. 4-5 drops of 5% sodium thiosulfate solution were applied and incubated for 1 minute. After washing with tap water and following 2 dips in distilled water, 4-5 drops of Van Gieson's solution were added and incubated for 5 minutes. The section was rinsed with 95% ethanol and observed under a light microscope.

Gene expression analysis

Reverse transcription polymerase chain reaction (RT-PCR) was performed to determine the relative changes in the expression of SMC-specific genes, including α -smooth muscle actin (α SMA), calponin, and smooth muscle myosin heavy chain (SM-MYH). These genes encode the smooth muscle isoforms of contractile apparatus proteins. Total RNA was

1 extracted from samples using an RNeasy mini kit (Qiagen, Netherlands). In the extraction of
2 mRNA from SMC spheroids, a disposable homogenizer (Nippi, Japan) was used to physically
3 grind cells. cDNA was synthesized via reverse-transcription using a ReverTraAce RT-qPCR
4 kit (Toyobo, Japan). Real-time RT-PCR was performed with Step One (Applied Biosystem,
5 Singapore) and detected using SYBR Green (Qiagen, Netherlands) according to the
6 manufacturer's instructions. The sequences of forward and reverse primers were 5'-
7 agccacatcgctcagacac-3' and 5'-gcccaatacgaccaaacc-3' for glyceraldehyde-3-phosphate
8 dehydrogenase (*GAPDH*), 5'-ctgtccagccatcctcat-3' and 5'-tcatgatgctgttagtggt-3' for α *SMA*,
9 5'-ccaaccatacacaggtgcag-3' and 5'-tcacctgtttccttctt-3' for *calponin*, 5'-
10 aactcgtgtccaacctggaa-3' and 5'-ttctcctcggttaacaactga-3' for *SM-MYH*. All primers were
11 purchased from TAKARA BIO Inc. (Japan). The results were analyzed by using Step One
12 Software v.2.2 (Applied Biosystem, Singapore). PCR measurements were repeated in
13 triplicates from 3 experiments.

Results and Discussion

Characteristics of SMC spheroids

Schematics for engineering DVS are shown in Fig. 1. Following this scheme, we initially prepared SMC spheroids with the PDMS microwell array plate to later encapsulate them at a high density in GelMA. In the PDMS plate, the cells aggregated in each microwell after 1 day of culturing and formed condensed spheroids after 3 days (Fig. 2A). A large number of spheroids (300 spheroids/culture plate) with a relatively uniform diameter were prepared with this PDMS plate, because almost the same number of cells enters the microwells simultaneously. PDMS has typically been used in the fields of lab-on-a-chip and BioMEMS mainly owing to its cytocompatibility, transparency, micro workability, and oxygen permeability (23). Because muscle cells generally have a high demand of oxygen and oxygen shortage can be readily caused in the core of spheroids when cells are aggregated, the PDMS microwell array plate was designed to supply oxygen not only from the top of the culture medium but also from the bottom through the PDMS (24). The diameter of spheroids decreased from ~150 μm to ~92 μm during the 3 days of culture (Fig. 2B), which is equivalent to a 67% compaction in volume. This significant change could affect the SMC phenotype and SMC-induced hydrogel shrinkage.

Changes in gene expression of smooth muscle markers were analyzed using RT-PCR. The analysis revealed that the expression of *calponin* and *SM-MYH* reached its highest peaks on day 1, while the expression of *α SMA* decreased over time (Fig. 2C). These marker genes were expressed as a differentiated contractile phenotype (25, 26) and their drastic decrease is correlated to the undifferentiation to a proliferative phenotype (27). In a typical SMC culture, SMCs alter the phenotype from differentiated to undifferentiated phenotypes when they form aggregates such as spheroids (28). This is consistent with the results obtained in this study, except that we found peaks of *calponin* and *SM-MYH* expressions on day 1 of the culture. The

spheroids were then prepared in the plate for 1 and 3 days and examined on the level of contraction in response to a muscle concentration inducer, acetylcholine. Immediately after the stimulation, the spheroid diameter decreased up to 12% in 10 minutes (Fig. 2D). The spheroids after 3 days of culture (S3) contracted only 6% 15 minutes after stimulation (data not shown). These results suggest that SMC alter their characteristics after 3 days of spheroid culture.

Migration of SMC spheroids in GelMA

The culture period for SMC spheroid preparation and UV exposure for crosslinking GelMA might play a role in the SMC-driven shrinkage of the hydrogel and thus in the successful fabrication of a high cell dense SMC layer around the needle. We examined these influences using the spheroids cultured for 1 and 3 days (S1 and S3) then encapsulated in GelMA under 3 different crosslinking conditions (5.0 mW/cm² for 50 seconds, LOW; 7.0 mW/cm² for 40 seconds, MID; and 7.0 mW/cm² for 50 seconds, HIGH). During 3 days of culture after the encapsulation, SMCs in S1 were found to migrate at least several hundred micrometers in GelMA in all three crosslinking conditions. Interestingly, the results of comparisons among three crosslinking conditions were in opposition to our expectations. We had predicted that a softer gel would facilitate more cell extension and migration, but the migration number and length, in fact, increased by increasing the UV exposure intensity and time (Fig. 3B, C). Further extension of UV exposure time (70, 80, 90 seconds, 7.0 mW/cm²) was found to hinder cell extension and migration completely. SMCs in S3 did not migrate into the GelMA and the morphology of these spheroids remained in the original shape, independent of the crosslinking conditions. Based on these results, we decided to use S1 and 7.0 mW/cm² for 50 seconds in the following experiments.

DVS fabrication and characterization

As shown in Fig. 1A, HUVECs were seeded on the needle coated with the oligopeptide self-assembled monolayer and grown to cover the surface. Subsequently, a GelMA solution containing SMC spheroids (S1) was poured into the chamber containing the needle. The thickness of the hydrogel was 1.9 mm after the crosslinking (Fig. 4A). We carried out the same experiments with S3. The shrinkage of the hydrogel containing S1 was rapid and significant compared to that of S3 (Fig. 4B). This was expected, as shown in Fig. 3, because the SMCs in S1, but not in S3, readily migrated into the hydrogel. The hydrogel with S1 shrunk by almost 50% during the 4 days of culture and the cell density became 6.0×10^8 cells/ml. This cell density is over 50 times greater than that of previous studies where single SMCs were encapsulated in a tubular hydrogel (29).

After 4 days of culturing in the chamber, an electrical potential of -1.0 V vs. Ag/AgCl was applied to the gold-coated needle for 5 minutes. The needle was then carefully extracted from the chamber (Fig. 4C), resulting in a DVS composed of a dense SMC layer enveloped in a monolayer of HUVECs (Figs. 4E-G). The length and inner and outer diameters were 18.78 ± 0.29 mm, 972.2 ± 26.7 μ m, and 488.0 ± 10.2 μ m, respectively. The standard deviations were calculated from the results of three independent experiments. We have previously fabricated a DVS by dip-coating and crosslinking GelMA on a needle covered with HUVEC, with the subsequent electrochemical detachment of the HUVEC layer (19). However, this approach did not result in a thick SMC layer. We repeated the GelMA coating process to increase the thickness of layer, but this only resulted in a low cell density layer. There are several studies that report on fabricating a SMC tube with a cell-dense thick layer. In one approach, multiple ring-like SMC aggregates were previously prepared on a microfabricated plate and then assembled with a support needle (30). In another approach, a SMC cell sheet was prepared and wrapped on a support needle (31). Both approaches were able to fabricate SMC tubes with a thick layer. However, when seeding vascular endothelial cells onto an internal surface of the

fabricated SMC tubes, the flow of culture medium should be stopped for a couple of hours so that cells can settle down and attach to the surface. However, this may cause a shortage of the oxygen supply and serious ischemia-reperfusion injuries once perfusion is restarted. The advantages of the approach used in the present study are: i) SMCs are encapsulated at a relatively high density but there are still pathways to diffuse oxygen and nutrients between the spheroids; ii) immediately after the transfer of an endothelial layer, perfusion of culture medium can be started; iii) in the perfusion culture, mechanical loads are applicable on the constructed DVS for maturation with an external pump. We have previously demonstrated that the electrochemical transfer of vascular endothelial cell layers is applicable not only to a single needle but also to spatially aligned multiple needles (12). We prepared a multi-needle consisting of 9 stainless steel needles (diameter, 500 μm ; pitch, 500 μm ; length, 10 mm, 3×3 arrangement), with which 9 endothelialized microchannels were simultaneously fabricated in a square hydrogel block of dimensions 3 mm $\times$ 3 mm $\times$ 10 mm. We believe that the same approach can be used to fabricate multiple DVSs by placing a spacer with lattice architecture between needles to compartmentalize each needle.

After 3 days of perfusion culture, the contractility of the DVS was examined by the addition of 500 μM acetylcholine in the culture medium. Changes in the appearance of DVS were recorded for the following 15 minutes. A dynamic contraction up to 7% was observed after 10 minutes (Fig. 4D), suggesting that the fabricated DVS possesses an adequate contractility similar to that of *in vivo* blood vessels (32). The elastin staining of the DVS cross-sections indicates that there are accumulated elastic fibers secreted by SMCs (Fig. 4H). The gene expression analysis of DVS after 3 days of perfusion culture revealed that *αSMA* , *calponin*, and *SM-MYH* were upregulated up to 3.1, 3.1, and 5.0 times compared to those of S1 before the encapsulation, respectively (Fig. 4I). Further studies may be needed to clarify the mechanisms responsible for the upregulation of these gene expressions. There could be

complex interactions through various cues, such as mechanical loading generated by perfusion, and signaling between HUVECs. The interactions between endothelial cells and smooth muscle cells or pericyte *in vivo* are well studied (33, 34). Signaling through soluble factors and direct contacts such as angiopoietin and notch signaling is essential for morphogenesis during development and maintenance of vascular functions in mature vessels.

In conclusion, we have demonstrated a novel approach that is scalable for the three-dimensional alignments of DVS by assembling multiple needles as previously reported (12). Our next task is to examine whether such multiple DVSs can be used to induce perfusable and complex vascular networks *in vitro* and supply oxygen and nutrients to parenchymal cells encapsulated between the needles in a hydrogel. We believe this perfusable DVS fabrication approach could open up a new avenue for engineering vascularized tissues *in vitro* for regenerative medicine applications, such as liver and kidney tissues.

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Figure legends

Figure 1. Steps for fabrication of double-layered vascular-like structures (DVS). (A) Seed HUVECs on a gold needle coated with an oligopeptide self-assembled monolayer. (B) Prepare SMC spheroids with a PDMS microwell array plate. (C) Encapsulate SMC spheroids around the HUVEC-covered gold needle in GelMA in a culture chamber. (D) Incubate for four days. During this culture period, SMCs migrate into GelMA, which subsequently contract the hydrogel toward the needle via the attraction forces of SMCs. (E, F) Detach HUVECs from the gold needle along with electrochemical desorption of the oligopeptide layer. (G) Extract the needle and start perfusion of culture medium using a syringe pump.

Figure 2. Preparation of SMC spheroids. (A) Cell aggregation and subsequent compaction in the spheroid culture plate. (B) Quantification of the spheroid diameter in (A). Error bars represent the standard deviations calculated from total 10 spheroids in three independent experiments. Numerical variables were statistically evaluated by the Steel–Dwass test. $p < 0.05$ was considered significant. (C) Changes in gene expressions of SMCs. Error bars represent the standard deviations calculated from three independent experiments. (D) Contraction response against stimulation with acetylcholine chloride. The red and white circles in the image indicate the diameter of SMC spheroid before and 15 minutes after the stimulation, respectively. The graph indicates changes in the diameter. Error bars represent the standard deviations calculated from total 5 spheroids in three independent experiments.

Figure 3. Migration of SMCs in GelMA. (A) Comparisons of migration of SMCs from spheroids after 5 days of culture in GelMA. S1 and S3 indicate that spheroids were previously cultured in the microwell array plate for 1 and 3 days, respectively. The values on the top indicate UV irradiation intensity (mW/cm^2) / exposed time (sec). (B) The number and length

of migrated SMCs from the surface of spheroids. Error bars represent the standard deviations calculated from 10 spheroids in three independent experiments.

Figure 4. DVS. (A) Shrinkage of SMC spheroid-embedded GelMA layer around a needle during 4 days of culture. (B) Quantitative analysis of changes in the thickness of the hydrogel. Error bars represent the standard deviations calculated from three independent experiments. (C) DVS after extracting a gold needle. (D) Coarctation of the DVS in response to stimulation of 500 μ M acetylcholine chloride. Error bars represent the standard deviations calculated from three independent experiments. (E-G) Confocal microscopic images of the structure. Three-dimensional configuration of smooth muscle layer stained with Alexa F 488 conjugated anti α -actin antibody (E). Cross-section stained with DAPI and rhodamine phalloidin (F) and its magnified view additionally merged with an image of GFP-HUVEC layer in the same region (G). (H) Cross-section stained for elastin. (I) Gene expressions of SMCs at 1 day of culture in the microwell array plate (S1) and subsequent 5 days of perfusion culture as a DVS. Error bars represent the standard deviations calculated from three independent experiments.

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