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Fabrication Factory for Tubular Vascular Tissue Mimics based on Automated Rolling Manipulation and Thermo-Responsive Polymers

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Abstract

This study presents a novel methodology for fabrication of tubular vascular tissue mimics by an automated factory using thermoresponsive polymers which have temperature-induced reversible phase transition characteristics; Poly(N-Isopropylacrylamide)-Grafted Gelatin (PNIPAM-gelatin), PNIPAM and gelatin. The custom-designed factory consists of a moving tray, a roller unit driven by rack-and-pinion mechanics, and a local temperature-control unit. PNIPAM-gelatin serves as the matrix, providing temperature-dependent adhesion and detachment of cell sheets. We describe two prototypes of vascular tissue mimics: a tubular medial tissue mimic composed of randomly oriented Smooth Muscle Cells (SMCs), and a hierarchically structured vascular tissue mimic (intimal/medical tissue mimics) composed of longitudinally oriented Endothelial Cells (ECs) on the luminal surface and circumferentially oriented multilayered SMCs in the outer layer. We observed these prototypes under electron microscopes and a Confocal Laser-Scanning Microscope (CLSM). An advanced model of our prototype factory would have the potential to produce high-quality on-demand vascular grafts without incorporating any foreign materials.

Keywords: Tubular vascular tissue; Automated roll tissue machine; Thermo-responsive material

Introduction

The use of living cells as key components of regenerative therapies in tissues and organs should provide opportunities for functional therapy beyond what can be achieved with artificial organs. Over the past two decades, tissue engineering studies have revealed that hybrid artificial organs, which are prepared by hand, such as hybrid vascular grafts and cartilage adopt the morphology and function of the corresponding natural tissues in the period following implantation; in other words, time-dependent remodeling occurs within the body [1,2]. The field awaits two types of advances: cell-based engineered tissues, which resemble function and morphology of native tissues and are suitable for therapeutic applications in the restoration, repair, and replacement of diseased tissues, and quality-controlled and rapid methodology for on-demand fabrication of engineered tissues. Recent studies have explored biofabrication technologies that use robotics-based cell handling techniques driven by extrusion, inkjet, dispensing, or laser-induced forward transfer processes [3-19]. Although these technologies promise deposition ('printing') of cells on planar surfaces that is highly resolved in terms of spot-to-spot separation and spacing, the Two-Dimensional (2D) tissue mimics prepared by the current region-specific precision cell deposition technology fall far short of real tissue architectures. Therefore, it is necessary to develop an assembly device focused on construction of morphologically complex 3D tissue mimics.

In this study, we designed and prototyped a custom-designed "factory" for automated fabrication of tubular vessel tissue using simple mechanical manipulation process and thermoresponsive polymers. The factory rolls up cell sheets automatically, combining mechanical manipulation of a sliding cell-bearing tray under control of a rack-and-pinion mechanism with a thermo-responsive polymer that allows temperature-dependent cell adhesion and detachment. The preparation of a rolled cell sheet is shown schematically in Figure 1. A cell sheet formed on a PNIPAM-gelatin-coated tray slides under control of a pushing rod driven by a linear motor (Figure 1B). The thermoresponsive property of Poly(*N*-Isopropylacrylamide)-Gafted Gelatin (PNIPAM-gelatin) coating allow cell sheets to be detached from the tray surface by locally lowering the temperature. Detached cell sheets are spontaneously transferred to a gelatin-coated roller that automatically rotates under control of a rack-and-pinion mechanism. The rolled cell sheet detaches at an elevated temperature (37°C) (Figure 1C). This factory, which is simple to operate, produced two types of tubular vascular tissue mimics: a medial tissue mimic with random cell orientation, and a vascular tissue mimic in which cells are hierarchically organized and highly oriented (an intimal/medial tissue mimic).

Experiments

Materials

Poly(*N*-isopropylacrylamide), (PNIPAM), was prepared by conventional radical polymerization. PNIPAM-grafted Gelatin (PNIPAM-gelatin) was prepared by dithiocarbonation of lysine residues of gelatin, followed by "iniferter" living photo-polymerization under ultraviolet irradiation, according to the authors' previous method [20-23] (Figure 1B). Details of the preparation are described in our previous articles. The average number of graft chains per gelatin molecule (mol. wt. 9.5×10^4 per mol; total lysine residues, 36.8 per molecule) was 33.5, and the average molecular weight of graft-polymerized PNIPAM was approximately 5.0×10^4 g/mol. Lower Critical Solution Temperatures (LCST) of aqueous PNIPAM and PNIPAM-gelatin solutions were approximately 32°C and 34°C, respectively; below that temperature,

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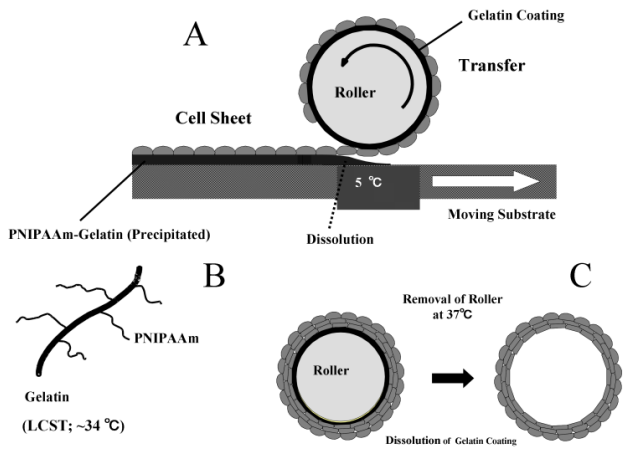

Figure 1: Schematics of the principle of rolled vessel fabrication (A): A cell sheet (continuous monolayer) formed on a thermoresponsive matrix of gelatin partially grafted with poly (N-isopropylacrylamide), PNIPAM-gelatin (B), was rolled on a gelatin-coated roller. Tissue was detached from the gelatin-coated metallic roller at temperatures below 30°C (C). LCST is Lower Critical Solution Temperature.

both polymers are soluble in water, but above that temperature, they both spontaneously precipitate.

Cell culture

An EGM-2 Bullet kit (Cambrex Bio Science, Walkersville, MD) was used as the culture medium for human umbilical vein Endothelial Cells (EC; Cambrex). ECs were trypsinized (trypsin-EDTA for 5 min at 37°C), plated in tissue culture dishes (Corning, Corning, NY) containing EGM-2 medium, and grown in an incubator at 37°C in 5% CO_2. Human aortic Smooth Muscle Cells (SMC; Cambrex) were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Grand Island, NY) containing 10% Fetal Bovine Serum (FBS; Hyclone, Logan, UT). At the time of use, cells were resuspended in appropriate culture medium at a density of 1×10^7 cells/ml. Living ECs and SMCs were stained using the lyophilic cell tracker agents DiO and DiI, respectively (Molecular Probes Inc., Eugene, OR). The poly(methyl methacrylate) cell culture tray (culture space: 6 cm×16 cm) was coated with 1.5 ml of 1% PNIPAM-gelatin aqueous solution and dried prior to cell seeding. The lyophilic cell trqackers used, both of which were purchased from Aldrich, were DiO, (2Z)-2-[(*E*)-3-(3,3-dimethyl-1-octadecylindol-1-ium-2-yl)prop-2-enylidene]-3,3-dimethyl-1-octadecylind,ole; perchlorate, and DiL, 3*H*-Indolium, 2-[3-(1,3-dihydro-3,3-di-methyl-1-octadecyl-2*H*-indol-2-ylidene)-1-propenyl]-3,3-dimethyl-1-octadecyl, perchlorate; 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate.

Patterned cell sheet

A commercially available polyimide sheet was ablated using a KrF excimer laser (wavelength; 248 nm) microprocessing apparatus (C4500, C4550, Hamamatsu Photonics Co., Shizuoka, Japan) equipped with a microscope, driven under programmatic control according to procedures we developed previously. This procedure yielded a micro-contract-stamp with lined banks (width, 200 μm; height, 30 μm; bank-to-bank space, 200 μm). This stamp was coated with 1% PNIPAM-gelatin solution, and then pressed onto the PNIPAM-coated culture space in the culture tray. The PNIPAM-gelatin–coated patterned culture space was seeded with ECs and SMCs at the appropriate locations.

Factory

We designed and assembled an experimental factory for fabrication of tubular vascular tissue mimics. Our factory consists of a temperature-control unit, a plastic-covered incubator, inside which the cell-sheet–bearing tray and roller unit (diameter of roller; 0.6 cm) are installed, a pushing rod connected to a linear motor unit (Oriental Motor Co., Ltd, Osaka, Japan) and a cold-water infusion pump. This custom-designed factory was assembled by Four Leaves Co., Ltd. (Osaka, Japan). The temperature inside the incubator is maintained at 37°C and just beneath the roller unit is maintained at 4-5°C which is due to cold water infused into a pipe embedded in the base matrix of the incubator.

Instruments

A phase-contrast microscopic image was obtained using an OPTIPHOT2-POL instrument (Nikon, Tokyo, Japan). A scanning electron microscope (SEM; S3400-NX, Hitachi High-Technologies Co., Tokyo, Japan) was used to observe the luminal surface of rolled tissue mimics at an acceleration voltage of 10 or 20 kV. Samples were fixed with phosphate buffer solution containing 2.0% (vol/vol) glutaraldehyde for 2 h and subsequently treated with a series of dehydration steps using graded aqueous solutions of 50, 60, 70, 80, 90, 99, and 100% ethanol (10 min per step). Samples, treated twice with 2-methyl-2-propanol were frozen at -20°C for 1 h, freeze-dried using an ES-2030 freeze dryer (Hitachi High-Technologies Co.), and sputter-coated with osmium (VIII) oxide. For Transmission Electron Microscopic (TEM) observation, specimens excised from the mid-portions of the rolled tissue mimics were post-fixed with 1% osmium tetroxide in cacodylate buffer for 1 h, washed with buffer five times, stained en bloc with 2% uranyl acetate for 1 h, dehydrated with a graded ethanol series, and embedded in Spurr's low-viscosity resin. Ultrathin sections (85 nm thick) were mounted on 300×75 μm mesh copper grids. Sections were double-stained with 3% uranyl acetate in 30% ethanol and Reynold's lead citrate, and examined using TEM (H-600, Hitachi,Tokyo, Japan) at 75 kV. Cell distribution in hierarchically organized tissues, using DiO-labeled ECs and DiI-labeled SMCs, was evaluated using Confocal Laser Scanning Microscopy (CLSM; LSM510, Carl Zeiss MicroImaging, Inc., Thornwood, NY).

Results

Principle and prototype factory

Our factory, shown in overview in Figure 2A, relies on the function of two key elements. The first of these elements is an automated, synchronized mechanism for sliding of the culture tray and rolling of the cell sheet. The culture tray, which is placed on bearing belts positioned on both sides of tray, is pushed at a controlled speed by a rod driven by a linear motor. A series of photographs taken during movement of the tray is shown in Figure 2B. The roller is rotated by engagement of pinions attached to the roller on both sides of the roller machinery and racks installed on both sides of the tray (Figure 2B). Thereby, the sliding movement of the tray is transformed into a rolling movement of the roller (Detailed schematic drawing is shown in Figure 3). The second key element is the use of thermoresponsive polymers. Both PNIPAM and PNIPAM-gelatin precipitate in aqueous solution at 37°C. PNIPAM is non–cell-adhesive, whereas PNIPAM-gelatin is cell-adhesive. Co-use of these polymers with an aide of micro-contact stamp technique creates regionally patterned cell sheets [24]. Gelatin (cell-

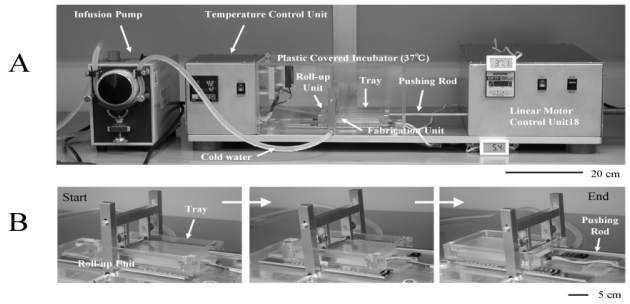

Figure 2: Overview of factory for fabricating rolled cell sheets (A): Fabrication unit consists of a polyacrylate container with temperature-control unit to maintain a temperature of 37°C, and a cold-water infusion pump enabling regionally specific reduction of the temperature of the moving tray. A linear motor with control unit enables the tray to be pushed at a constant speed. Serial photos depicting rolling of the cell sheet (B): A rod driven by a linear motor pushes the cell-sheet–bearing tray, which moves from the right to the left in the image shown.

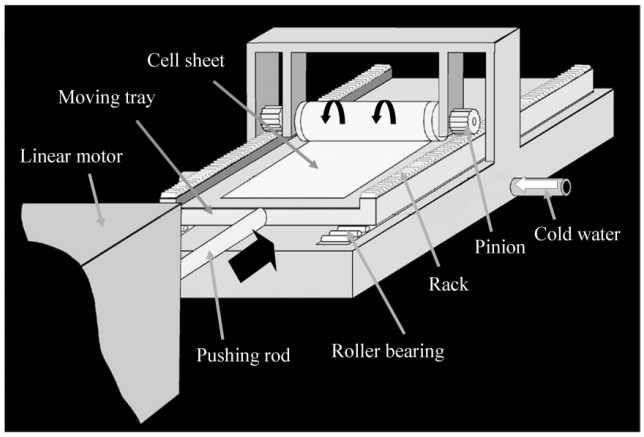

Figure 3: Detailed sketch of moving cell-sheet–bearing tray and roller device. The incubated cell-sheet–bearing plastic tray is first coated with PNIPAM-gelatin, followed by cell seeding and culture. Both upper sides of tray have racks that can be engaged with pinions attached to the gelatin-coated roller. The tray is sliding on the roller bearing installed at the base matrix of the fabrication unit. Cold water is infused in a pipe embedded in base matrix, which allows local reduction of temperature. Multiple rotation of roller produces tubular vascular tissue mimics.

adhesive), which gels in water at low temperature but dissolves in water at 37°C, is used as the cell-sheet-transfer matrix on the roller at low temperature, as well as for detachment of tubular tissue from the roller at 37°C (Figure 1A) (Figure 1C). In this study, we describe prototypes of two vascular tissue mimics: a medial tissue mimic composed of a randomly oriented SMC multilayer, and a hierarchically structured intimal/medial tissue mimic with cells that are highly aligned within their respective sub-tissues.

Model I: Medical tissue mimics: Cell-adhesive matrix, PNIPAM-gelatin, is coated in thin layers on the cell culture space within a tray, and DMEM containing a suspension of SMCs solution is carefully overlaid at 37°C. When a confluent cell sheet forms after the culture period, the tray is installed into a fabrication unit (Figures 3 and 4). The tray, supported by roller-bearing belts on both sides, slides gently and smoothly under control of a rod driven by a linear motor (2 mm/min). The temperature of the plastic-covered incubator is maintained at 37°C. In the tray just beneath the roller, cold water (4-5°C) is circulated in a pipe embedded in the base matrix of the device, causing the PNIPAM-gelatin to dissolve away. Lifted cell sheets are spontaneously attached to a cell-adhesive gelatin layer coated onto a roller (Figure 1A). The roller rotates under the control of a rack-and-pinion mechanism (Note that the roller was gently placed just on tray without enforcing placing). The continuous sliding of the tray at the lateral shift of 2 mm/min enables transfer of cell sheet to a rotating roller, thus forming a tubular multilayered tissue mimic, as demonstrated in Figure 5. No contact force was measured. In the culture space beyond the roller in the tray, no cell sheet is observed, indicating that cell sheet has completely transferred to the roller. After removal of the roller from the fabrication unit, it is immersed in DMEM solution at 37°C for one

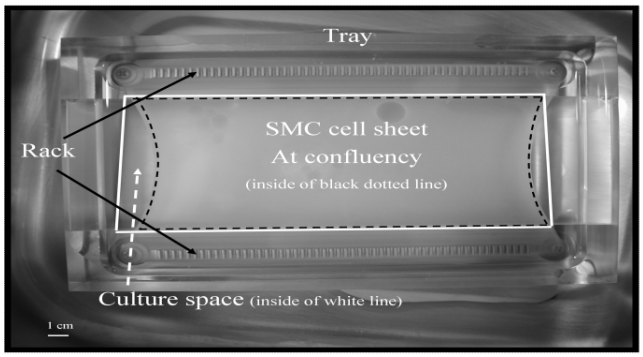

Figure 4: A representative photo of a tray bearing a cell sheet of SMCs. The cell sheet is shown inside the dotted black lines.

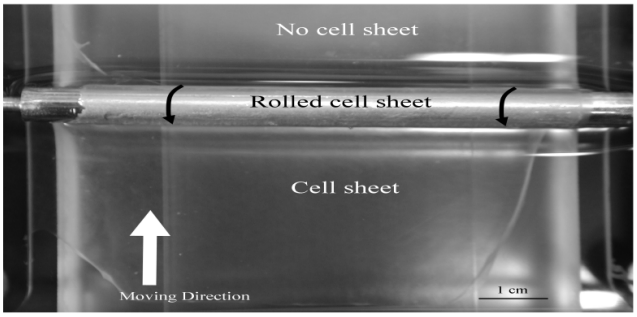

Figure 5: A representative photo of a rolled cell sheet. Note that the cell sheet (lower region) is rolled around the roller, and no cell sheet is observed the upper region of the tray.

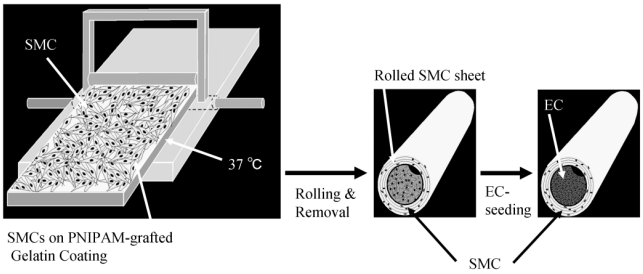

Figure 6: Schematics of Model I vascular graft composed of rolled smooth muscle cells. Upon cooling to room temperature, the gelatin coating the roller is dissolved away, leaving a rolled medial tissue mimic. When endothelial cells are seeded and cultured in a tubular tissue prepared in this manner, a prototype of vascular endothelial cell lining is formed.

hour. The very thin rolled tissue (thickness, ~200 μm) easily detaches from the roller upon dissolution of the gelatin coating (Figure 1C). The tubular tissues thus formed, "medial tissue mimics," are generally very thin and fragile, resulting in spontaneous collapse.

Model II: Vascular tissue mimics with highly aligned cells: Arteries of living tissues are composed of monolayers of ECs that are longitudinally aligned in the layer (intimal tissue) that contacts the blood, and multilayers of circumferentially aligned SMCs (medial tissue) in the outer part of tissue. To generate vascular tissue mimics with specific cellular orientation via one-step preparation, two patterned cell sheets are created in distinct regions of the cell culture space in the tray. Figure 7 shows schematics of such patterned cell sheets. ECs at the front region of the culture space are aligned parallel to the roller, and SMCs in the rear region are aligned in the direction of movement. Regional cell patterning, with different cell types aligned in different directions, was created by a micro-contact stamp method originally developed by Whitesides group [24]. In this study, a polyimide film is laser-ablated to create an array of banks. Figure 8 shows the serial steps of preparation of a rolled patterned tissue. First,

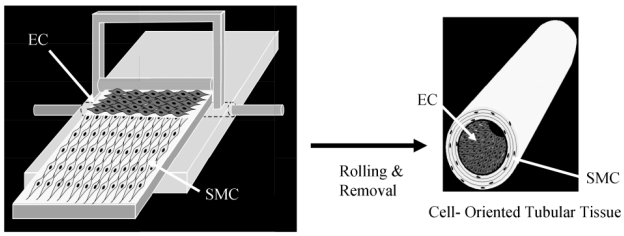

Figure 7: Schematics of Model II vascular tissue composed of endothelial cells (oriented longitudinally) and smooth muscle cells (oriented circumferentially). To generate this tissue mimic, the cell sheet on a tray contains two differently patterned cell sheets composed of two individual cell types in different regions of the tray. ECs are oriented parallel to the long axis of the roller, whereas SMCs in the rear of the tray are oriented in a perpendicular direction. After rolling of the cell sheet, the resulting tubular vessel should exhibit cellular alignments similar to those of a native vessel.

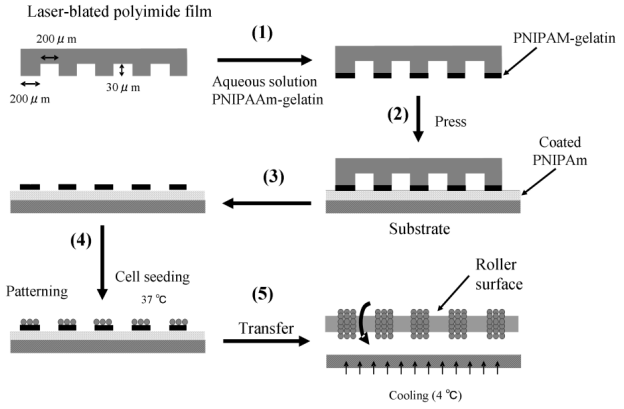

Figure 8: Fabrication procedure for generating co-patterned cell sheets using the micro-contact stamp technique developed by Whitesides et al. [24]. Laser-ablated polyimide film is used as the micro contact stamp, which is prepared in-house using a microscope-equipped excimer-laser apparatus, and coated with PNIPAM-gelatin on non-ablated banks (1). PNIPAM-gelatin film was pressed onto the PNIPAM-coated substrate (2). Thus, a PNIPAM-coated substrate with regionally specific deposits of PNIPAM-gelatin can be prepared (3). Next, SMCs and ECs are deposited in the appropriate patterned regions (4). After culture, the resulting patterned cell sheets are transferred to a roller (5).

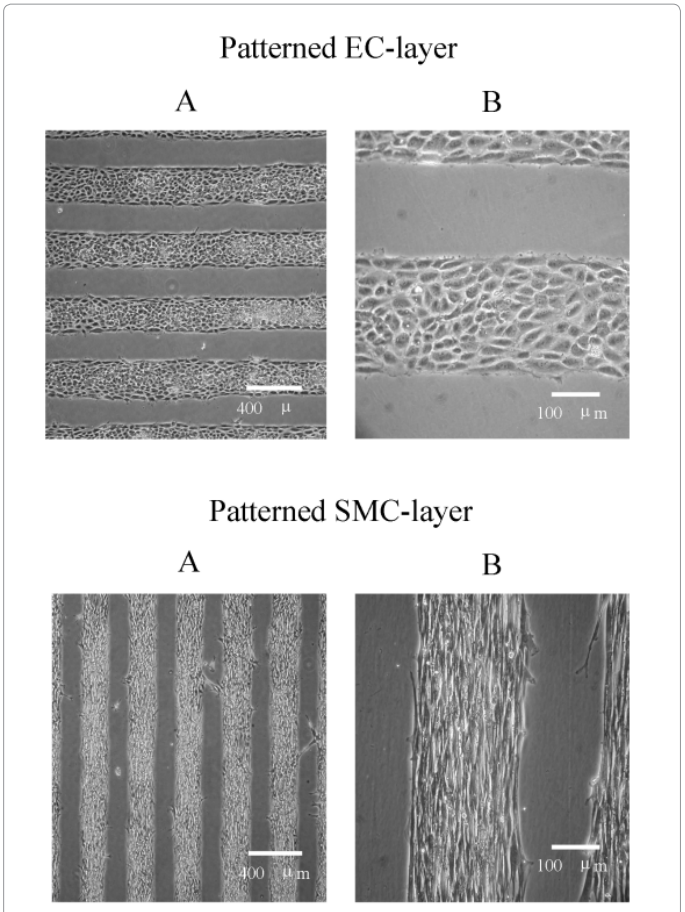

Figure 9: Representative photos of patterned cell sheets. The upper photo shows that confluent ECs are aligned to some extent parallel to the direction of the pattern. The lower photo shows that confluent SMCs are highly aligned. The right-hand photos (B) were taken at higher magnification than the left-hand photos (A).

the whole culture space is coated with aqueous PNIPAM solution and dried. Next, a PNIPAM-gelatin–coated micro-contact stamp is pressed onto the PNIPAM-coated surface in order to transfer PNIPAM-gelatin to the tray in a regionally specific manner.

Two patterns of PNIPAM-gelatin coating are created in the culture space along the designed cell patterning for ECs and SMCs, according to those in Figure 7. Then, culture media containing suspensions of ECs and SMCs are gently added to the appropriate regions at 37°C. After culture for 2 h, non-adherent cells are gently washed away, and the attached cells are grown to confluence. Figure 9 shows phase-contrast microscopic images of patterned tissues of ECs and SMCs at confluence. Both types of cells adhered and formed patterned cell sheets only on PNIPAM-gelatin coated surfaces, whereas no cells adhered to PNIPAM-coated surfaces. ECs aligned to some extent, and SMCs aligned almost completely, in the directions of their respective patterns. Using the trays on which these aligned cellular sheets have formed, rolled tissue mimics are prepared, followed by removal of the mimics from the roll by incubation at 37°C (Figure 1C). At this point, we subjected samples to electron microscopy. An SEM photo reveals that ECs were highly oriented in the longitudinal direction of the tube, and a TEM photo reveals that at least ten circumferentially elongated SMC layers could be observed (Figure 10). Depth profiling by CLSM shows that DiO-stained ECs were localized at the upper layer, whereas

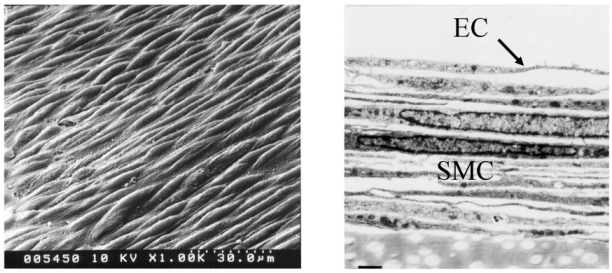

Figure 10: Scanning electron microscopic image (A) of the luminal surface of fabricated tissue mimics where ECs are highly oriented in the longitudinal direction, and transmission electron microscopic image (B) of circumferentially cut-specimen of rolled tissue mimics, showing longitudinally aligned, multilayered SMCs.

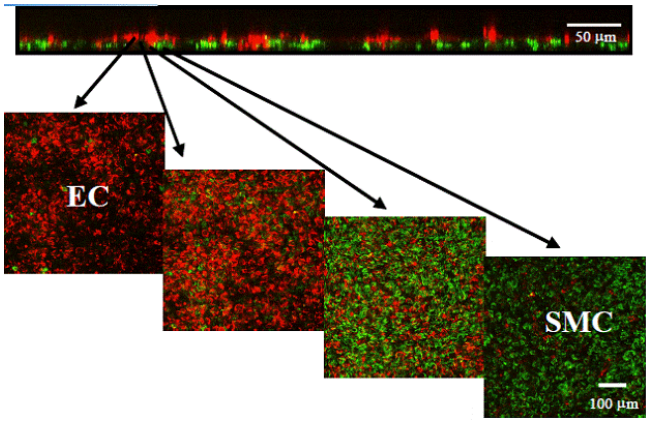

Figure 11: Representative photo of a cross-section view of rolled vascular mimics composed of two sub-tissues containing dye-stained ECs and SMCs. Sliced images were taken every four microns along the depth axis, indicating that highly populated ECs reside the outermost layer.

dense populations of DiI-stained SMCs resided in the deeper tissue layer (Figure 11).

Discussion

The development of rapid and precision fabrication technology, enabling manufacture of high-quality engineered tissue mimics for use in cell-based regenerative therapies, has been long-awaited. Once *ex vivo* engineered tissue mimics are routinely utilized in clinical settings, it will be required that a high quality-controlled and on-demand tissue fabrication technology that can be adapted to the dimensions and shapes of individual patients' diseased tissues. Recently described robotics-based bioprinting technologies, including extrusion, inkjetting, and laser-induced forward transfer processes, can precisely control regionally specific cell deposition ('printing') on planar surfaces; however, on-demand production of 3D tissue architecture incorporating live cells has not yet been achieved successfully [3-19]. As an extension of "cell sheet technology" using PNIPAM-grafted cell culture dishes, originally developed by Okano and his group, generation of multi-layered tissue mimics via serial deposition or stacking of monolayer sheet can produce functional 3D-planar tissue mimics such as cardiac tissue [25,26]. On the other hand, cell-free 3D scaffolds with complex shapes have been prepared on demand by computer-controlled techniques such as rapid prototyping of photopolymerizable polymers and direct 'writing' inkjet 'printing' of apatites to create artificial bone [27-29]. However, a one-step fabrication technology for producing 3D tissue mimics that incorporate live cells and resemble native tissue has not yet been developed.

In this study, we developed a prototype factory for fabrication of tubular vascular tissue mimics. The factory's operation relies on on-demand cell sheet adhesion and detachment using thermoresponsive polymers and an automated roller mechanism. PNIPAM-gelatin, which was originally developed as an *in situ* cell injectable extracellular matrix for applications involving cartilage and hemostasis, precipitates at physiological temperature to provide a cell-adhesive matrix [30,31]. Lowering the temperature below the LCST spontaneously induces detachment of adhered cells and cell sheet. The degree of cell-adhesiveness depends on the degree of graft derivatization and graft chain length: higher levels of derivatization and chain lengths of PNIPAM result in lower cell-adhesiveness and LCST [23]. These characteristics have been utilized for clonal cell harvesting from mixtures of different cell types by differential cell adhesiveness and harvesting of single target cell under a microscope equipped with a pinpoint cooling apparatus [32,33].

The combination of sliding a cell-sheet–bearing tray with automated rolling under control of a rack-and-pinion mechanism produced our Model I tissue mimic composed of SMCs, which was very thin (approximately 200 µm in thickness) and fragile. Once a thicker medial tissue tube can be obtained by multiple cycles of rolling, using many cell-sheet–bearing trays, such a rolled tissue mimic should exhibit mechanical integrity without spontaneous collapse. As schematically shown in Figure 6, infusion of concentrated EC suspension into a tubular tissue and further culture should produce vascular tissue mimics composed of two sub-tissues of EC monolayer at the inner side and an SMC multilayer at the outer side (intimal/medial tissue mimics).

In general, manipulation of cell orientation in distinct sub-tissues in a manner that mimics the hierarchical organization and function of the native tissue will likely prove essential for successful vascular tissue regeneration with high physiological demands. The alignment of both ECs and SMCs in a manner that resembles their arrangement in arterial tissues can be easily achieved by micro-contact stamping of two thermoresponsive polymers, producing a hierarchically organized vascular tissue mimic with a high degree of cell alignment in each sub-tissue (Model II: Figure 10).

This prototype factory, operated by a sliding rack-and-pinion mechanism in combination with thermoresponsive functional polymers and micro-contact stamping, was able to produce vascular tissue mimics. Multiple cycles of rolling the cell sheets and changes in the diameter and length of a roll could enable on-demand production of vascular tissue mimics. In this study, production design of fabrication of rolled tissue mimics using temperature-responsible polymers and roll-up mechanics has been focused, but structural analysis and histological appearance including tissue and cell specific markers and their cell function have not been studied. That is, at this stage, mechanical properties, function and structure of fabricated vascular tissue mimics, regardless of Model I or II, are far beyond those of natural vascular tissue. On-going study will be focused on generating tissue mimics that more closely resemble the function, morphology and mechanical integrity of native vascular tissue, which will be reported in near future.

Conclusion

We developed a custom-designed prototype factory for fabrication of tubular vascular tissue mimics. Sheets of vascular cells were rolled to form tubular tissue mimics. Manipulation was achieved using a combination of on-demand thermo responsive polymers enabling temperature-dependent cell adhesion and detachment with an automated roller which is operated by sliding of a cell-sheet–bearing tray under control of a rack-and-pinion mechanism. Using a micro-contact stamp technique, a hierarchically structured intimal/medical tissue mimics with a high degree of cellular orientation at each sub-tissue was prepared.

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