

Molded hyaluronic-acid gel as a micro-template for blood capillaries

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ABSTRACT

Fabrication of blood capillaries in tissue-engineered tissue is necessary for creating thick three-dimensional (3D) tissue with a high cellular density. For inducing blood capillaries in the tissue *in vitro*, molded hyaluronic acid (HA) capillary-shape gel was made as a template for blood capillaries by photolithography and power free pumping techniques. Fabricated HA capillary-shape gel was sandwiched with two cell sheets consisting of neonatal normal human dermal fibroblast (NHDF), human umbilical vein endothelial cell (HUVEC), or co-cultured NHDFs and HUVECs, and eventually covered with the cells. Although a slight degradation of the HA gel was observed in the sandwiched tissue with HUVEC or NHDF cell sheets, significant degradation of the HA gel was observed in the sandwiched tissue with co-cultured cell sheets. Moreover, by continuing to culture the co-cultured tissue with HA gel, a tube formation was observed at HA gel-located site. Sandwiched HA capillary-shape gel with two cell sheets has a potential for creating blood capillaries *in vitro* and fabricating vascularized artificial organs.

Keywords:

Cell sheet

Tissue engineering

Blood capillary

Hyaluronic acid gel

1. Introduction

Tissue engineering has been widely investigated for providing regenerative tissues *in vitro*. Although the results of early clinical trials using regenerative tissues for the augmentation of urinary bladder are reported [1], biodegradable scaffold is unable to create thick tissue with a high cellular density [2]. For fabricating tissues with a high cellular density, our laboratory establishes cell sheet engineering [3], which utilizes a thermo-responsive surface allowing cells to proliferate to confluence at 37 °C and to become a cell monolayer. By reducing temperature to lower than 32 °C, the cells spontaneously detach themselves from the surface for around 30 min and become as a monolithic cell sheet. Since thermo-responsive polymer is covalently immobilized onto the surfaces of culture dishes, harvested confluent cells are found to detach themselves from the surfaces as a monolithic cell sheet by decreasing temperature with its healthy intact extracellular matrix (ECM) and transmembrane proteins. This technology creates various functional cell sheets for treating various diseases such as stenosis after esophageal cancer resection and heart failure [4,5]. Additionally, because of its intact ECM and transmembrane proteins, the cell sheet can be easily layered for creating multi-layered cell sheets, which can lead to organ-like three-dimensional (3D) tissue. However, the inner part of thick 3D layered cell sheets having more than four-layered cell sheets is necrosed because of poor oxygen and nutrients supplies [6]. Therefore, the fabrication of blood-capillary-mimicked lumens in prepared thick 3D tissue is important for maintaining its healthy condition and growth, because not only the tissue needs to be supplied oxygen and nutrients, but also various metabolic wastes should be removed from the tissue through blood capillaries.

Several studies succeeded in fabricating blood capillaries, which are prepared from endothelial cells by cell micropatterning processes [7-10]. Cell non-adhesive polymers such as polyacrylamide (PAAm) or poly (ethylene glycol) diacrylate (PEGDA) are immobilized on culture surfaces with designed patterns by a photolithography technique for fabricating micropatterned culture surfaces. On the micropatterned culture surface, endothelial cells are cultured, resulting in the formation of blood-capillary-like-luminal structure within networked endothelial cells. Although micropatterning of endothelial cells is able to fabricate a partial tube structure, a continuous tube structure along with networked endothelial cells is unable to be fabricated. Biocompatible and biodegradable material that works as a template for a rapid and stable tube structure formation with designed size has a potential for fabricating a continuous tube structure *in vitro*. From this point of view, biological extracellular matrix component is thought to have a possible potential for fabricating blood vessels. Among the constituent of extracellular matrix, HA is preferable as a template material for constructing a tube structure because HA gel can be degraded and inhibit cell migration [11], thus no cell invasion into the inner part of HA gel is expected.

In this study, crosslinked HA gel was fabricated to have a capillary-shape as a template for creating a vascular luminal structure *in vitro*. HA gel was surrounded and covered by cells, which would be expected to only proliferate on the surface of crosslinked HA gel without invading into the HA gel, and form a tube structure, which eventually became a capillary. However, dispersed and freely-movable cells can hardly cover the outer surface of HA capillary-shape gel because crosslinked HA gel is known to reduce cell adhesion [11, 12]. Therefore, cell sheet engineering was used for a tool for covering HA capillary-shape gel with cells in this study. HA gel was

sandwiched with fibroblasts, endothelial cells, or co-culture of fibroblasts and endothelial cells, and the histological analysis of sandwiched cell sheets with HA gel was performed. Sandwiched HA capillary-shape gel between two cell sheets was expected to form a tube structure where HA gel would be perfectly covered with cells derived from cell sheets.

2. Materials and Methods

2.1. Synthesis of methacrylated HA

Methacrylated HA (MeHA) was synthesized by a method described previously [13]. Briefly, 0.5 g sodium HA (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 100 mL distilled water. For degradation of HA, 1000 U hyaluronidase (3.5 mg) (BBI Enzymes, South Wales, UK) was added into the solution, which was incubation for 16 h. The solution was boiled for 5 min for deactivating hyaluronidase, which was removed through 0.22 μm filter. After being precipitated in cold acetone, HA powder was obtained.

To 100 mL of 2 w/v% degraded HA, 20-fold excess of methacrylic anhydride (14.78 mL) (Sigma-Aldrich) was added, and the solution pH was adjusted to be 8 with 5N NaOH. Then, the solution was incubated at 4 °C for 24 h. After precipitating with the progress of time, MeHA was obtained from the solution and washed with ethanol for removing unreacted methacrylic acid anhydride. The methacrylate substitution ratio of MeHA was determined by $^1\text{H-NMR}$ spectroscopy.

2.2. MeHA labeling

MeHA was labeled with biotin for histological analysis. TFPA-PEG₃-biotin (Thermo Fisher Scientific, Rockford, IL, USA) was used to insert biotin in the C-H bonds of MeHA by UV irradiation at 300 – 370 nm. Approximately 12-fold of biotinylation reagent (26.6 μL) was added to 1 mL of MeHA solution (10 mg/mL). After being exposed by a UV lamp (250 W) (UVE-251S+EL-200) (San-Ei Electric, Osaka, Japan) for 5 min at room temperature, the solution was centrifuged in a spin

column for removing unreacted biotinylation reagent.

2.3. *Polydimethylsiloxane (PDMS) mold fabrication*

Micro-mold for casting capillary-shape HA gel was made from polydimethylsiloxane (PDMS) (Dow Corning Japan, Tokyo, Japan) by a photolithography technique [14]. Briefly, PDMS elastomer and curing agent were mixed in a 10:1 ratio. The mixture was poured onto silicon masters (Fig. 1A), which had been microfabricated with photoresist (SU-8 3050) (Nippon Kayaku, Tokyo, Japan) and had a 50 μm width lane, and then the mixture was cured at 45 $^{\circ}\text{C}$ for 30 h (Fig. 1B). The cured PDMS mold was removed from the silicon master and cut prior to use (Fig. 1C).

2.4. *Fabrication of molded HA capillary-shape gel*

HA capillary-shape gel were prepared by a previously reported photolithography and power-free pumping technique [15]. Briefly, PDMS molds were plasma-treated at 20 W for 30 s for obtaining hydrophilic surface. PDMS mold with the engraved side down was put on a sheet of cover glass (24 x 50 μm , 0.17 – 0.25 in thickness) (Matsunami, Osaka, Japan) treated with octadecyltrichlorosilane (Shin-Etsu Chemical, Tokyo, Japan), and then the PDMS mold on the cover glass was vacuumed for 20 min in a container. After the mold was taken from the vacuum container, a drop (approximately 50 μL) of 4 w/v% MeHA solution, containing 0.5% Irgacure 2959 (Ciba, Tokyo, Japan) in phosphate buffered saline (PBS) was put on the side wall of the mold attached with cover glass, and within 5 min, MeHA solution automatically permeated into the engraved part of mold and filled the empty engraved lane. MeHA solution

was then photocrosslinked by UV irradiation at 345 nm at a dose of 50 mW/cm² for 5 min (Fig. 1E). The PDMS mold was removed to fabricate molded HA capillary-shape gel on the cover glass (Fig. 1F). HA capillary-shape gel on the cover glass was spin-coated with 10 – 30% polyvinyl alcohol (PVA) (Kanto Chemical, Tokyo, Japan) at 7,000 rpm for 30 s for easy handling of HA gel (Fig. 1G). After PVA layer dried, PVA coated HA gel was carefully removed from the cover glass with acetone (Fig. 1H), and put on a piece of silicone rubber (15 x 15 μm, 0.5 μm in thickness) (As One, Osaka, Japan) for drying, and sterilized prior to being sandwiched with two cell sheets. For confirming the possible effect of PVA solution on the appearance of molded HA capillary-shape gel, the immiscibility between PVA and MeHA solutions was investigated. MeHA solution (4%) was mixed with various concentrations of PVA (10 – 30%) individually, and mixed solutions were carefully inspected by naked eyes and photographed.

2.5. Cell culture and cell sheet fabrication

Neonatal normal human dermal fibroblast (NHDF) (Lonza, Walkersville, MD, USA), up to 10 passages, and human umbilical vein endothelial cell (HUVEC) (Lonza), up to 8 passages, were used in this study. NHDFs and HUVECs were seeded on poly (*N*-isopropylacrylamide) (PIPAAm) grafted dishes (CellSeed, Tokyo, Japan) at a density of 5.2×10^4 cells/cm² and cultured with fibroblast growth medium (FGM-2) (Lonza) and endothelial cell growth medium (EGM-2) (Lonza) at 37 °C in a humidified atmosphere with 5% CO₂, respectively. NHDF cell sheets were fabricated with FGM-2. For fabricating HUVEC cell sheets, cells were cultured with EGM-2 supplemented with 20% fetal bovine serum (FBS) (Japan Bio Serum, Hiroshima, Japan).

For preparing co-cultured cell sheets, HUVECs and NHDFs were seeded at a density of 2.6×10^4 cells/cm² individually, and cultured with medium for HUVECs with 20% FBS. All cells were cultured on temperature responsive dishes for 2 – 3 days, which allowed cells to detach themselves spontaneously as a monolithic cell sheet by reducing culture temperature to 20 °C.

2.6. *Sandwiching HA gel between two cell sheets*

Molded HA capillary-shape gel was sandwiched by two cell sheets (Fig. 1K). For this layering process, a cell sheet manipulator, which can prevent the self-shrinkage of cell sheet detached from a thermo-responsive dish, was used [16]. Briefly, 7.5% gelatin (Sigma-Aldrich) hydrogel dissolved with Hank's Balanced Salt Solution (Sigma-Aldrich) was applied to the plunger surface of cell sheet manipulator for holding a detached cell sheet. The cell sheet manipulator with gelatin was gently placed over confluent cultured HUVECs, NHDFs, or co-culture of HUVECs and NHDFs on PIPAAm-grafted surfaces. The manipulator and dish were incubated for around 20 min at 20 °C, which allowed the cell sheet under the manipulator to be detached from the surface of culture dish. The manipulator holding the cell sheet was moved from the surface and put on another cell sheet holding molded HA capillary-shape gel (Fig. 1K), and the manipulator and the dish were incubated again for 30 min at 20 °C for ensuring the newly covered cell sheet to attach to the HA gel holding cell sheet. The gelatin hydrogel attached to the manipulator were removed by incubating for 30 min at 37 °C, resulting in a sandwiched cell sheet. The sandwiched cell sheets were cultured with EGM-2.

2.7. *Immunohistochemical analysis*

The sandwiched cell sheet was washed with PBS and fixed with 4% paraformaldehyde for 20 min. After being permeabilized with 0.2% TritonX-100 (Sigma-Aldrich) for 5 min, the sandwiched tissue was treated with Alexa Fluor conjugated streptavidin (Invitrogen, Carlsbad, CA, USA) for staining biotinylated HA gel. For immunostaining HUVECs and NHDFs, the sandwiched tissue was treated with Alexa Fluor conjugated phalloidin. For co-cultured cell sheets, HUVECs were immunostained with anti-mouse CD31 (DAKO, Glostrup, Denmark), followed by Alexa conjugated secondary antibody (Invitrogen). Cell nuclei were stained with Hoechst 33342 (Invitrogen). Cell images were observed by a confocal fluorescence laser-scanning microscope (Carl Zeiss, Hallbergmoss, Germany) and processed with Zeiss LSM Image Browser software (ZEN2009).

2.8. *Measurement of residual HA gel*

HA gel found in the cross section of specimen was quantified from the ten images of sandwiched cell sheets. ImageJ software was used to quantify the area of HA gel in the cross sectional images. All data were expressed as mean \pm standard deviation (S.D.).

3. Results

3.1. Fabrication of molded MeHA capillary-shape gel

MeHA was synthesized for obtaining photo-crosslinked HA gel. The degree of substitution of methacrylate moieties to HA was approximately 22.7%, which was determined by HA methyl group (at 1.8 ppm) and multiplet methacrylic acid peaks (at 6.0 and 5.6 ppm) on $^1\text{H-NMR}$ spectrum (Fig. 2).

For transferring molded HA gel onto confluent cells, PVA coating was used to keep and protect its structure during the transferring procedure. When MeHA solution was mixed with 10% PVA solution, no phase separation was observed, resulting in one-phase solution (Fig. 3A). However, PVA solution over 15% gave a phase separation between PVA and MeHA solutions (Fig. 3B-E), indicating that PVA coating over 15% PVA solution would keep the structure of HA gel. However, molded HA capillary-shape gel coated with 10 and 15% PVA solution resulted in the deformation of HA gel structure (Fig. 3F, G). On the other hand, HA gel coated with 20 – 30% PVA solution resulted in the orderly structure of HA gel (Fig. 3H-J).

The lane width of PDMS mold for preparing molded HA capillary-shape gel was selected to be 50 μm , which was corresponding to the size of blood capillary. SEM micrograph of PDMS mold revealed that the depths of channel of the mold were approximately 22 μm , which was also appropriate for a blood capillary mold. MeHA solution automatically permeated into the channel of PDMS mold by a power-free pumping technique, and then MeHA solution was photo-crosslinked, resulting in the formation of molded HA capillary-shape gel on the coverslip (Fig. 4A). Molded HA capillary-shape gel coated with over 20% PVA solution was transferred onto confluent

cultured cells. Since 20% PVA was easily melt by medium, the remaining HA gel was successfully sandwiched with two cell sheets (Fig. 4B).

3.2. Immunohistochemical analysis of molded HA capillary-shape gel sandwiched tissue by two cell sheets

Fig. 5A, B, and C show the confocal microscope images of cross sections of specimens where molded HA capillary-shape gel was sandwiched by two NHDF cell sheets. Although NHDF cell sheets hardly adhered each other at the vicinity of HA gel and thus HA gel was unable to be covered by NHDF cell sheets after 1 h incubation (Fig. 5A), two NHDF cell sheets adhered and produced a double-layered cell sheet by sandwiching HA gel after 24 h incubation (Fig. 5B). After 48 h, HA gel was found to be still covered with NHDF cell sheets. Partial HA-gel degradation was found to be observed at 48 h incubation, but no complete HA gel degradation was observed (Fig. 5C and 6D).

Cross sections of confocal microscope images of HA gel sandwiched with two HUVEC cell sheets were shown in Fig. 5D, E, and F. Although HA gel was unable to adhere HUVEC cell sheets after 1 h incubation (Fig. 5D), HUVEC cell sheets covered HA gels after 24 h incubation (Fig. 5E). After 48 h incubation, HA gel was found to be slightly degraded (Fig. 5E and 6D). Interestingly, HA gel was partially covered with HUVEC cell sheets, and single-layered structure was observed.

For obtaining a possible double-layered tissue with a capillary architecture, co-cultured cell sheets containing NHDFs and HUVECs were prepared for sandwiching HA gel. Top views of the confocal microscope images of molded HA capillary-shape gel sandwiched with co-cultured cell sheets after 1, 24, and 48 h incubation were shown

in Fig. 6A, B, and C, respectively. Unlike the sandwiched tissue with NHDF or HUVEC cell sheets, the initial degradation of HA gel was observed after 24 h incubation (Fig. 6B). HA gel, shown in green, significantly decreased within 24 h. Residual HA gel was quantified by the images, and it showed a drastic degradation of HA gel when HA gel was sandwiched by co-cultured cell sheets (Fig. 6D). Cross sections of confocal microscope images of HA gel sandwiched between two co-cultured cell sheets after 1, 24, and 48 h incubation were shown in Fig. 6E, F, and G, respectively. HUVECs were stained with anti-CD31 antibody for distinguishing them from NHDFs. After 24 h incubation, HA gel was covered with HUVECs in the co-cultured cell sheets. In addition, the sandwiched cell sheets formed a double-layered structure in a way similar with the sandwiched tissue with NHDF cell sheets (Fig. 6F). After 48 h incubation, HA gel was found to be further degraded, and HA gel-located area became empty hollow, which was speculated to form a tube-like structure surrounded by HUVECs (Fig. 6G).

4. Discussion

Microfabrication of HA gel has been studied for applying it to tissue engineering and regenerative medicine. HA gel was microfabricated by polymerizing MeHA solution photo-chemically under physiological condition. Photo-crosslinking of HA gel was beneficial, because not only the spatial control of microfabricated hydrogel was able to be achieved but also crosslinking rate was controllable [17]. Furthermore, as a power-free pumping technique, capillary force was utilized for permeating MeHA solution to the engraved part of PDMS mold, which also contributes the spatial control of microfabricated HA gel. Micromolding of PDMS is a common method for fabricating the microstructure of HA gel. However, micromolding tends to create HA gel thin film between the non-engraved part of PDMS mold and cover glass [18]. This problem was able to be solved by using a capillary force, which was unable to allow MeHA solution to permeate into the contacted areas between the non-engraved part of PDMS mold and the cover glass.

PVA coating on HA gel was useful for the easy handling of HA gel. However, a mixture of PVA and MeHA solutions (HA: 4%, PVA: 10%) gave a complete one-phase solution, indicating that they are miscible each other. Since miscible concentration of PVA solution might affect the shape of HA gel, the immiscible concentration of PVA to 4% MeHA solution was examined. HA gel coated with over 20% PVA solution gave the orderly shape of HA gel (Fig. 3H-J). Among 20 – 30% PVA concentrations, 20% was selected, because the dissolution of PVA is known to be dependent on its concentration, and the rapid dissolution of PVA after transferring HA gel onto confluent cultured cells was preferable.

For preparing a cell sheet architecture composed of double-layered cell sheets with a tube structure, degradable HA gel was used as a template and sandwiched between two cell sheets. After HA gel was degraded, a tube structure was expected to leave between two cell sheets. In this study, HA gel was sandwiched by two NHDF cell sheets, which gave a double-layered tissue with NHDF cell sheets. Partial HA-gel degradation was observed after the 48-h incubation of sandwiched tissue with two NHDF cell sheets. **HA gel could be slowly hydrolyzed, which resulted in the improved cell adhesion and further degradation was occurred.** HA is known to bind to a specific cell-surface receptor, CD44, which takes a critical role in HA turnover [19, 20]. High molecular weight HA is proposed to bind to cell surface by the combined effort of CD44 and extracellular hyaluronidase. Hyaluronidase cleaves the high molecular weight HA, and CD44 mediates the internalization and delivering of the cleaved HA to lysosome. Since fibroblasts express CD44 [21], HA degradation in this study was expected to follow the CD44-mediated pathway. Next, HUVEC cell sheets were used for sandwiching molded HA capillary-shape gel. HUVEC is a component of blood capillary structure, and endothelial cells can form capillary lumens. Since HUVECs also express CD44 [22], HA gel was expected to degrade in a similar way with NHDFs, after being sandwiched with two HUVEC cell sheets. Moreover, since the degradation products of HA are known to promote the proliferation and differentiation of endothelial cells for an angiogenesis [23], a possible vascularization was also expected to appear in HUVEC cell sheets with HA gel. **However, significant HA gel degradation was not detected when HA gel was sandwiched between two HUVEC cell sheets (Fig. 6D) due to the partial covering of HA gel with HUVECs.** Therefore, the HUVEC unattached part of HA gel was unable to be degraded. On the

other hand, HA gel, which was sandwiched between co-cultured cell sheets containing NHDFs and HUVECs, was significantly degraded within 24 h incubation (Fig. 6D). A possible reason for the significant degradation of HA-gel in NHDFs and HUVECs co-culture might be due to cell-cell interactions. Co-culture of fibroblasts and endothelial cells are known to show an increased responsiveness of fibroblasts on IL-1, which up-regulates the expression of CD44 [24]. These results supported two evidences that (1) the co-culture of NHDF and HUVECs had a synergetic effect on the degradation of HA gel, and (2) NHDFs were necessary for constructing double-layered structure. Additionally, co-cultured cell sheets gave a thicker tissue structure due to NHDFs, which may help HUVECs stay covering HA gel. After HA gel degraded, a tubular structure covered by HUVECs was observed (Fig. 6G). Since 3D tissue, fabricated by layering the multiple numbers of co-cultured cell sheets containing NHDFs and HUVECs, expected to mimic a physiological condition, this co-cultured system with HA gel might be suitable for forming a tubular structure. This result was found to agree with the previous study, which shows the implantation of HA based hydrogel into cardiac muscle induces an artery like lumen *in vivo*. The implanted HA gel is speculated to be automatically covered with endothelial cells, because cardiac muscle is rich in endothelial cells, and then the hydrogel gradually degraded to leave a hollow tube structure covered with endothelial cells, which could become an artery [25]. Our study revealed that the diameter of tubular structure covered with HUVECs was smaller than that of original HA gel (Fig. 6G). **The diameter of tubular structure was about a few dozen μm , which was appropriate for blood capillaries and larger than previous method for fabricate lumen [8-10], indicating the fabricated tubular structure was robust and not easily occupied by cells. For fabricating continuous and stable tube**

structure, the ratio of NHDFs and HUVECs needs to be considered since migration of NHDFs to HUVEC-consisted tube structure stops the tube structure. Longer cultivation of the sandwiched tissue might induce further cell migration into the tubular structure and allow the tubular structure to be filled with cells. For maintaining the size of tubular structure and maturing the tubular structure to a functional blood capillary, the application of growth factors or a liquid flow into the lumen of the tubular structure is desirable in the future.

5. Conclusion

Molded HA capillary-shape gel was performed as a mold for vascular lumens. HA gel sandwiched between NHDF and HUVEC co-cultured cell sheets degraded rapidly, and a vascular luminal structure was observed after the degradation of the gel. This could be the first feasibility study to use gel as a template for size controlled blood capillaries. This method for creating blood capillaries *in vitro* has a potential for fabricating vascularized artificial organs.

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Figure Legends

Fig. 1. Schematic illustrations of the fabrication of hyaluronic acid (HA) capillary-shape gel and the sandwiching process of HA gel with two monolithic cell sheets. (A) Silicon masters for HA gel were fabricated by photolithography. (B) Polydimethylsiloxane (PDMS) elastomer and curing agent were poured onto the silicon masters. (C) Cured PDMS mold was cut out. (D) Photocrosslinkable HA was synthesized by methacrylating HA. (E) PDMS mold with the engraved side down was put on a sheet of octadecyltrichlorosilane treated cover glass (24 x 50 μm , 0.17 – 0.25 in thickness). Methacrylated HA (MeHA) solution was allowed to permeate into the engraved part of mold, and then the MeHA solution was photocrosslinked. (F) PDMS mold was removed, resulting in molded HA capillary-shape gel. (G) HA gel on the cover glass was spin-coated with polyvinyl alcohol (PVA). (H) PVA coated HA gel was removed from the cover glass. (I) Cells were cultured on temperature responsive dishes. (J) Cells detached themselves as a cell sheet by reducing temperature. (K) HA gels were sandwiched between two cell sheets.

Fig. 2. ^1H NMR of methacrylated hyaluronic acid (MeHA).

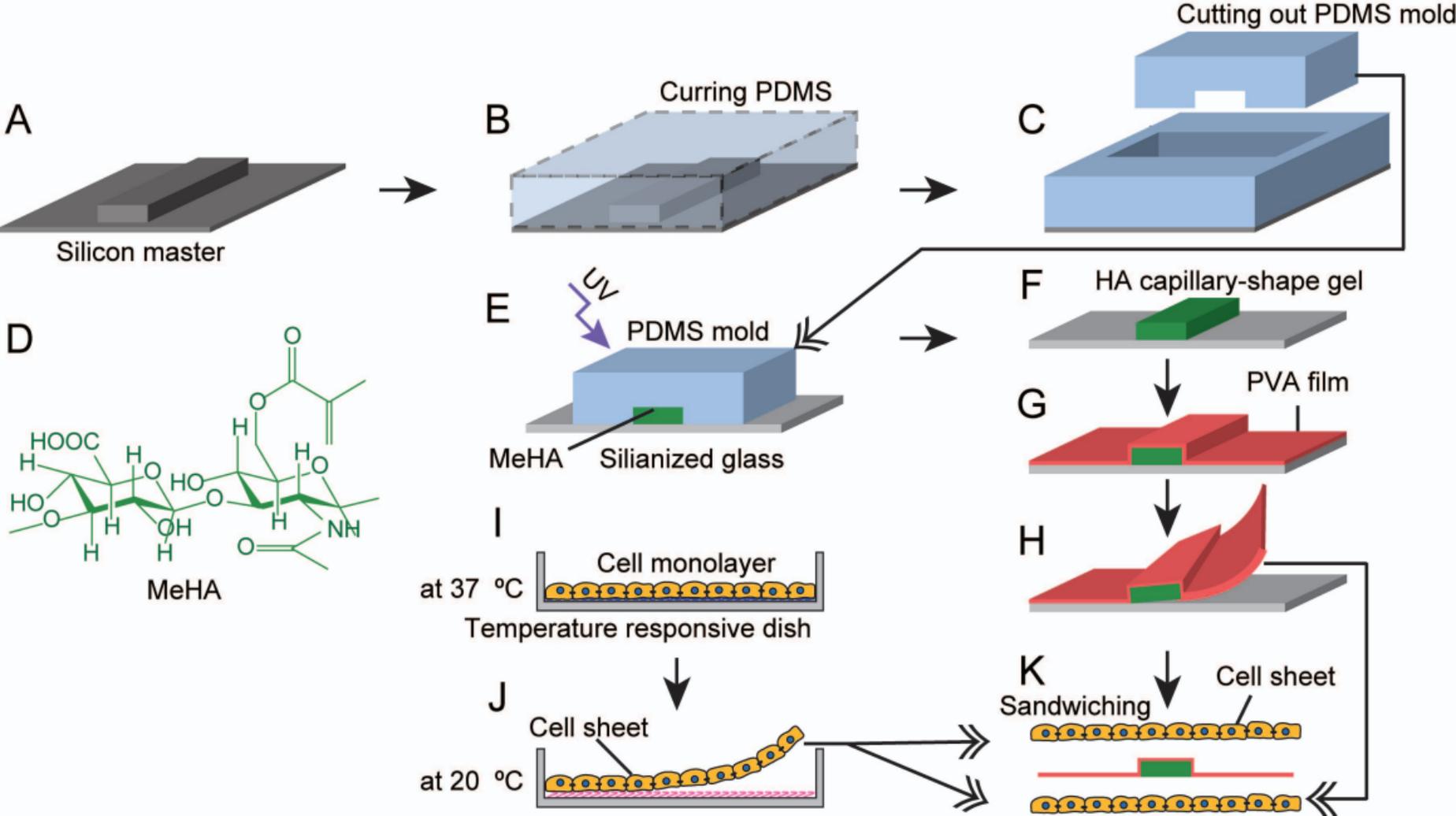
Fig. 3. Phase separation of 4% methacrylated hyaluronic acid (MeHA) and 10 – 30% polyvinyl alcohol (PVA) solutions (A-E) and the appearance of 4% HA capillary shape-gel after PVA spin-coating process (F-J). The white arrows in the upper photographs (B) – (E) show a phase separation. The red arrows in the lower photographs show 4% HA capillary-shape gel. Bar: 100 μm

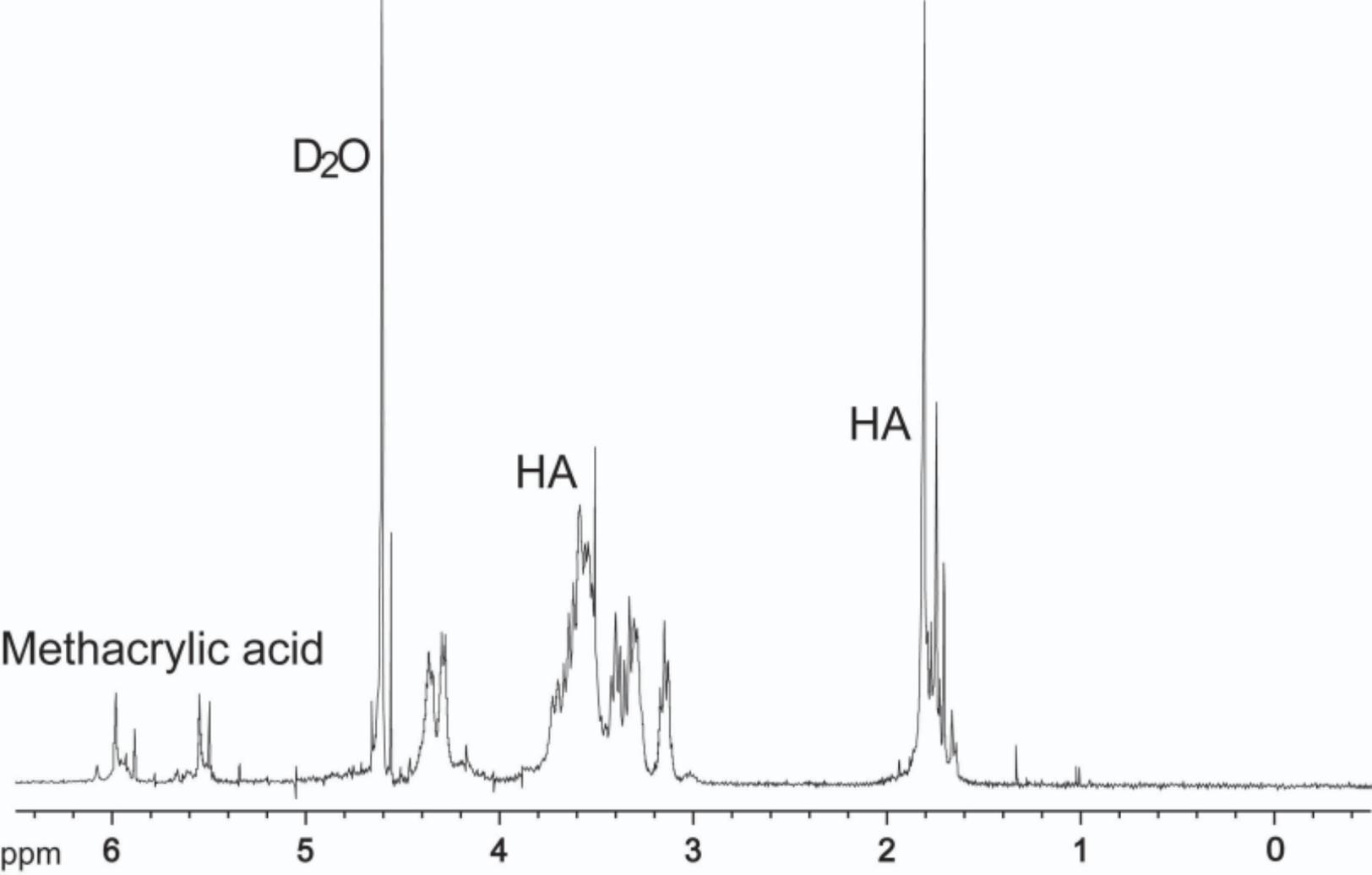
Fig. 4. Microscopic images of molded hyaluronic acid (HA) capillary-shape gel. (A) Light microscope image of HA gel (green) on a cover glass. (Top view) Bar: 20 μm (B) Confocal microscope image of HA gel sandwiched between two neonatal normal human dermal fibroblast (NHDF) cell sheets. The red, the blue, and the green fluorescence colors were NHDFs, nuclei, and HA gel, respectively. Schematic illustration shows the cross sectional image of sandwiched cell sheets with HA gel. The gray color indicates NHDFs; the blue, nuclei; the green, HA gel. Bar: 20 μm

Fig. 5. Immunohistochemistry of cross sectional images of sandwiched cell sheets with neonatal normal human dermal fibroblast (NHDF) (A-C) and human umbilical vein endothelial (HUVEC) (D-F) cell sheets. Hyaluronic acid (HA) gel sandwiched with NHDF cell sheets after 1 h (A), 24 h (B), and 48 h (C). HA gel sandwiched with HUVEC cell sheets after 1 h (D), 24 h (E), and 48 h (F). The red, the blue, and the green fluorescence colors were NHDFs (A-C) or HUVECs (D-F), nuclei, and HA gel, respectively. Bar: 20 μm . Schematic illustrations show the cross sectional images of sandwiched cell sheets with HA gel. The gray color indicates NHDFs; the blue, nuclei; the green, HA gel; the red, HUVECs.

Fig. 6. Immunohistochemistry of hyaluronic acid (HA) gel sandwiched with co-cultured cell sheets. Top views of sandwiched co-cultured cell sheets after 1 h (A), 24 h (B), and 48 h (C). Bar: 20 μm (D) Time-course of residual HA ratios between cell sheets. The open squares, triangles, and circles represent the residual HA ratios of neonatal normal human dermal fibroblast (NHDF), human umbilical vein endothelial

cell (HUVEC), and co-cultured cell sheets, respectively. Data show the mean of three determinations with standard deviations. ‡ $P < 0.01$; statistical analysis were by one-way ANOVA versus NHDF groups. ** $P < 0.01$; statistical analysis were by one-way ANOVA versus HUVEC groups. Cross sectional views of HA gel sandwiched with co-cultured cell sheets after 1 h (E), 24 h (F), and 48 h (G). The red, the blue, and the green fluorescence colors were HUVECs, nuclei, and HA gel, respectively. Bar: 20 μm . Schematic illustrations show the cross sectional images of sandwiched cell sheets with HA gel. The gray color indicates NHDFs; the blue, nuclei; the green, HA gel; the red, HUVECs.





PVA concentration (%)

10

15

20

25

30

