Facile fabrication processes for hydrogel-based microfluidic devices made of natural biopolymers

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We present facile strategies for the fabrication of two types of microfluidic devices made of hydrogels using the natural biopolymers, alginate, and gelatin as substrates. The processes presented include the molding-based preparation of hydrogel plates and their chemical bonding. To prepare calcium-alginate hydrogel microdevices, we suppressed the volume shrinkage of the alginate solution during gelation using propylene glycol alginate in the precursor solution along with sodium alginate. In addition, a chemical bonding method was developed using a polyelectrolyte membrane of poly-L-lysine as the electrostatic glue. To prepare gelatin-based microdevices, we used microbial transglutaminase to bond hydrogel plates chemically and to cross-link and stabilize the hydrogel matrix. As an application, mammalian cells (fibroblasts and vascular endothelial cells) were cultivated on the microchannel surface to form three-dimensional capillary-embedding tissue models for biological research and tissue engineering. © 2014 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4871936>]

I. INTRODUCTION

Cultivation of mammalian cells in a three-dimensional (3D) platform has recently drawn great attention in the field of tissue engineering, regenerative medicine, and biochemical research. Compared to conventional two-dimensional cell culture on a planar substrate, 3D cultures are more similar to the physiological conditions. 3D culture is especially advantageous in maintaining cellular functions and vitalities and thus lends itself to cell-based drug screening assays,^{1,2} construction of tissue models,^{3,4} differentiation control of stem cells,^{5,6} and physiological studies of cells, such as cancer cells^{7,8} and vascular endothelial cells.^{9,10} Among the most frequently used methods to achieve 3D culture is the formation of cell aggregates with non-adhesive wells, patterned surfaces, or microfluidic devices.^{11–14} Controlling the size of the wells/microchannels, inoculated cell numbers, or both allows accurate control of spheroid size. However, in such densely packed cell aggregates, cells at the center of spheroids are not viable because the supply of oxygen and nutrition is limited.^{12,15} This problem is especially severe in relatively large spheroids bigger than several hundred micrometers.

Hydrogel substrates equipped with microfluidic channels have recently been developed to provide 3D cell culture environments. The relatively soft characteristics of the hydrogels enable cell proliferation and migration within the hydrogel matrices. In addition, the microchannels embedded within the hydrogels act as vascular networks that efficiently deliver oxygen and nutrition to the cells. Until now, natural and synthetic polymers, such as alginate,^{16,17} collagen,^{18,19} agarose,^{20,21} and crosslinked PEG,^{22,23} have been used as matrices for hydrogel microchannels. Hydrogel-based microfluidic systems have been applied not only to the construction of tissue models^{19,24} but also to the investigation of the physiological characteristics of cells in *in vivo*-mimicking vascular microenvironments.²⁵ For example, the growth behaviors of vascular endothelial cells in the presence of cancer cells have been investigated using collagen-based hydrogel microchannels.²⁶ In addition, hydrogel microchannels have been used to form multi-layer tissue models via perfusion culture through the microchannels.²⁷

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Among the most standard processes for fabricating hydrogel microchannels is the molding-based preparation of a hydrogel plate with microgroove structures and subsequent bonding to a flat hydrogel plate.^{20,22} Compared with other strategies, including thin wire-based molding²⁸ and the removal of sacrificial structures,²⁹ this photolithography-based method yields complicated microchannel structures with arbitrary geometries. In addition, multilayer microchannel structures would be obtained by stacking hydrogel layers. However, facile fabrication of hydrogel microchannels, especially a bonding process indispensable for the preparation of enclosed microchannel structures, has not been fully realized. A previous study used jigs to pressurize hydrogel plates and ensure mechanical contact¹⁶ but presents several problems in terms of difficult manipulation and possible microchannel deformation. Bonding processes using partial hydrogel melting/dissolving have also been developed,^{17,20} but are applicable only to reversibly melting/dissolving hydrogels and pose difficulty for controlling the degree of microchannel deformation. As represented by the fabrication of polydimethylsiloxane (PDMS) microchannels through simple bonding via O₂-plasma treatment, a simple strategy for chemically bonding hydrogel plates would facilitate the wide use and application of hydrogel-based microfluidic devices.

This study explored the facile fabrication processes of hydrogel-based microchannels using two types of hydrogel materials, alginate, and gelatin. Alginate is a biocompatible polysaccharide obtained from algae and is gelled in the presence of multivalent cations, such as Ca²⁺, Mg²⁺, and Ba²⁺.^{30,31} To bond alginate plates, we proposed a simple but efficient strategy; one of two plates was coated with a polyelectrolyte layer of poly-L-lysine (PLL), and the coated and non-coated alginate plates were brought just enough into contact for electrostatic bonding. In addition, to prevent the severe shrinkage of the alginate hydrogel during the gelation process, we used propylene glycol alginate (PGA), a non-gelling derivative of alginate, to suppress the volume shrinkage of the precursor solution of sodium alginate (NaA). To fabricate gelatin-based hydrogel microchannels, we proposed an enzymatic reaction-based bonding process using microbial transglutaminase (MTG), which was also used to cross-link the gelatin hydrogel matrix so that it would not melt at 37 °C. Our experiments examined these two processes and investigated optimal fabrication conditions, including reaction period and chemical concentrations. In addition, we performed perfusion culture of mammalian cells within the hydrogel microchannels to determine their applicability to biological experiments.

II. EXPERIMENTAL SECTION

A. Materials

Sodium alginate (NaA; viscosity of 100~300 mPa s at 2% solution at 25 °C), Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA solution, penicillin-streptomycin solution, and PLL with various molecular weights (15 000–30 000, 150 000–300 000, and > 300 000) were obtained from Sigma-Aldrich Corp. (MO, USA). PGA (viscosity higher than 150 mPa s at 1% solution at 20 °C) and gelatin (from bovine bone) were obtained from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). Large particulates were removed from the PGA powder via filtration through a 100- μ m mesh. MTG (from *Streptomyces mobaraensis*) was kindly provided by Ajinomoto Corp. (Tokyo, Japan). GRGDSP peptide-conjugated alginate polymer (NOVATACH MVG GRGDSP) was obtained from FMC Health and Nutrition Corp. (PA, USA). EBM-2 endothelial cell Basal Medium-2 and EGM-2 Endothelial Cell Growth Media Kit were obtained from Lonza (Basel, Switzerland). Fetal bovine serum (FBS), Alexa Fluor 488-conjugated phalloidin, DMEM powder, and Hoechst 33342 were obtained from Life Technologies (CA, USA). SU-8 3050 was obtained from Nippon Kayaku Corp. (Tokyo, Japan). Silicon wafers were obtained from Wakatech Co., Ltd. (Yokohama, Japan). All other chemicals were of analytical grade.

B. Fabrication of alginate hydrogel microchannels

The fabrication process of Ca-alginate hydrogel microchannels is shown in Fig. 1(a). First, silicon molds were fabricated using standard soft lithography.³² A negative photoresist, SU-8 3050, was spin-coated on a silicon wafer and exposed to UV light through a photomask.

FIG. 1. Processes for preparing hydrogel microchannels. (a) Ca-alginate hydrogel microchannels made from an aqueous solution of sodium alginate (NaA) and PGA and bonded via PLL coating. (b) Gelatin hydrogel microchannels prepared by bonding two gelatin hydrogel plates using MTG.

After curing, the non-crosslinked SU-8 regions were removed using ethyl lactate. Then, an aqueous precursor solution of NaA and PGA was poured onto the mold. Small bubbles in the precursor solution were removed via aspiration. After the flat surface of the precursor solution was confirmed, an aqueous solution of a gelation agent (0.1 M CaCl₂ aq.) was gently poured onto the precursor solution. After incubation overnight at room temperature, a completely gelled Ca-alginate hydrogel plate was peeled off from the mold. A flat alginate hydrogel plate was also prepared using a flat silicon wafer as the mold.

Next, the flat plate was dipped in an aqueous solution of 0.05% (w/v) PLL for 3 min, followed by washing with DI water twice. Inlet/outlet holes ($\Phi = 1.0$ mm) were punched through the non-coated hydrogel plate with the microchannel structures. Aliquots of DI water on the surface of the hydrogel plates were removed with gentle wiping using a low-lint-release wiper (Bemcot Super QT-8, Asahi Kasei Fibers Corp., Tokyo, Japan). The flat PLL-coated plate and the non-coated plate were brought into contact and incubated at room temperature. Finally, silicone tubes with an outer diameter of 1.0 mm were attached to the inlet and outlet holes.

C. Fabrication of gelatin hydrogel microchannels

The fabrication process for the gelatin-based hydrogel microchannels is shown in Fig. 1(b). First, a similar molding process used in the fabrication of Ca-alginate hydrogel microchannels was used to prepare gelatin hydrogel plates. An aqueous solution of 20% gelatin and MTG (0.1–50 U/ml) was poured onto the silicon molds with and without the SU-8 structure, followed by incubation at 4°C for 30 min for gelation. The resulting gelatin hydrogel plates were peeled off from the molds. Then, two plates were brought into contact after dropping an aliquot of phosphate-buffered saline (PBS) with MTG (10 U/ml, $\sim 50 \mu\text{l}/\text{cm}^2$) onto the flat hydrogel plate. The plates were incubated up to 24 h until the cross-linking reaction was complete and the

stable bonds formed. Finally, silicone tubes were inserted into the inlet/outlet holes and glued by adding an aqueous solution of 20% gelatin with 2 U/ml MTG.

D. Cell culture within hydrogel microchannels

NIH-3T3 cells (from Riken BRC, Ibaraki, Japan) were cultivated in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Human umbilical vein endothelial cells (HUVECs, Lonza) were cultivated in EBM-2 with the EGM-2 kit. Cells were harvested from cell culture dishes with trypsin/EDTA treatment and suspended in saline after centrifugation. Cell suspension was filtered through a cell strainer (BD Biosciences, CA, USA) with a mesh size of 40 μm to remove large cell aggregates and then used for experiments.

The cell culture experiments in hydrogel microchannels used fabrication protocols slightly different from those described above. To prepare alginate hydrogel microchannels, we used a sterile, aqueous solution of 1.5% NaA, 3.0% PGA, 0.9% NaCl, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) as the hydrogel precursor solution. After the bonding process using PLL treatment, the fabricated microchannels were dipped in the cell-culture medium for at least 1 day to soak the medium into the hydrogel matrix. An aqueous solution of 0.05% PLL in saline was then introduced into the microchannels and incubated for ~ 3 min, followed by the introduction of 0.05% GRGDSP-alginate solution in saline and incubation for ~ 2 min to coat the entire microchannel surface with a PLL-GRGDSP-alginate layer. For the preparation of gelatin hydrogel microchannels, an aqueous solution of 15% gelatin with 2 U/ml transglutaminase and 1.35% DMEM powder was used. After dissolving the gelatin with heat, we added 0.37% sodium carbonate and poured the solution onto the mold. The prepared micro-devices were used after dipping in the cell culture medium with 10% FBS overnight.

NIH-3T3 cells were introduced into the Ca-alginate hydrogel microchannels and cultured for 4 days under static culture conditions. HUVECs were introduced into the gelatin microchannels and cultured under medium perfusion for 3 days. The medium was pumped into the microchannel using a syringe pump at a flow rate of 4 or 10 $\mu\text{l}/\text{min}$. After cultivation, cells within the hydrogel microchannels were fixed using 4% paraformaldehyde in PBS and then treated with 1% Triton X-100 in PBS followed by the staining of nuclei and cytoskeleton with Hoechst 33342 and Alexa Fluor 488-conjugated phalloidin, respectively.

III. RESULTS AND DISCUSSION

A. Preparation of alginate hydrogel microchannels

We first investigated the fabrication process of Ca-alginate hydrogel microchannels. Alginate-based hydrogels are potentially suitable materials for fabricating hydrogel microchannels because of their high mechanical strength and biocompatibility. In addition, the gelation condition is mild and requires no temperature control such as heating and cooling. Hence, alginate hydrogels facilitate the incorporation of cells into the hydrogel matrix and have been used as cell-encapsulating carriers and tissue engineering scaffolds.^{33,34} However, a critical problem arises in the preparation of alginate hydrogels; namely, they shrink drastically during the gelation process, making molding-based production of microchannels highly difficult. In previous reports, specific devices such as porous membranes have been used to suppress this shrinkage.¹⁶ We used a combination of PGA and NaA in this study to suppress the shrinkage of the precursor solution. PGA is a non-gelling ester derivative of alginate that has been used to adjust the physical stiffness of anisotropic hydrogel materials^{35,36} and to modify alginate hydrogels chemically to make them cell adhesive.³⁷

We tested various mixing ratios of NaA and PGA in the precursor solution to find the optimal condition for shrinkage suppression. The NaA concentration was fixed at 1.5%, which ensured the physical stiffness of the hydrogel while maintaining the relatively low viscosity of the precursor solution. Spherical hydrogel beads with an average diameter of ~ 2 mm were prepared by dropping aliquots of the precursor solution into a gelation solution (0.1 M CaCl_2 in distilled water), and the volume change before and after the gelation process was estimated by

FIG. 2. Relationship between PGA concentration and volume shrinkage of the Ca-alginate hydrogel. The concentration of sodium alginate was fixed at 1.5% (w/v).

calculating the volume of the obtained beads. The results are shown in Fig. 2. Severe shrinkage was observed when PGA was not added; the volume ratio of the formed hydrogel to precursor solution was only $\sim 35\%$ (volume shrinkage of $\sim 65\%$). Under this condition, molding-based fabrication of Ca-alginate hydrogel plates was impossible because a rugged clump of hydrogel formed on the silicon mold. By contrast, the volume shrinkage of the hydrogel was suppressed by increasing the PGA concentration. For example, a PGA concentration of 3% decreased the volume shrinkage to just $\sim 10\%$. Under this condition, we successfully prepared alginate hydrogel plates with uniform thickness and flat surfaces using the molding process, as shown in Fig. 3(a). Notably, BaCl_2 caused more severe shrinkage than CaCl_2 (data not shown), probably because the binding affinity of Ba^{2+} ions with alginate is higher than that of Ca^{2+} .³⁸ In the experiments that followed, we used the precursor solution of 1.5% NaA and 3.0% PGA to prepare Ca-alginate-based hydrogel microchannels.

Next, we examined the bonding process of Ca-alginate hydrogel plates using PLL as a chemical glue. PLL is a positively charged polyamino acid that adheres to the surface of negatively charged alginate hydrogels, forming a thin layer.³⁹ The formation of such a polyelectrolyte layer has been used to produce negative/positive multilayers; for example, alginate hydrogel beads with outer alginate-PLL-alginate membranes have been used as carriers for cell transplantation that avoids immunorejection.⁴⁰ We attempted to bond two alginate plates using this polyelectrolyte layer. One alginate plate was dipped in the PLL solution and brought into contact with a plate without the PLL coating. As shown in Fig. 3(b), PLL-coated and non-coated hydrogel plates were strongly bonded by electrostatic interaction and did not come apart when high-molecular-weight PLL (150 000–300 000 and $> 300\,000$) was used. On the contrary, when low-molecular-weight PLL (15 000–30 000) was used, the hydrogel plates were not bonded. The reason for this failure is the insufficient length of the polymer chain of low-molecular-weight PLL as the cross-linker between the plates. Most notably, the hydrogel plates did not bond when they were both coated with PLL.

We next examined whether the two hydrogel plates were completely bonded and whether enclosed microchannels were formed. DI water containing a blue dye or green fluorescent microparticles ($\Phi = 10\,\mu\text{m}$) was introduced into the hydrogel microchannels using a syringe pump, and fluid flow was generated within the channel structure (width = $200\,\mu\text{m}$ and depth = $100\,\mu\text{m}$). No fluid leakage was observed from the bonded surfaces (Figs. 3(c)–3(e)), when we applied pressure of $\sim 3\,\text{kPa}$ using a pressure-controlling apparatus (My Flow HIP-240, Arbiotech Corp., Tokyo, Japan); when higher pressure was applied, fluid leakage was observed from the joint point of the inlet/outlet tubings, because the hydrogel glue did not chemically bond the silicone tubes with the hydrogel.

FIG. 3. Preparation of Ca-alginate hydrogel microdevices. (a) Hydrogel plate peeled off from the mold, (b) two hydrogel plates bonded via PLL coating, (c) hydrogel microchannel with inlet/outlet tubing and an aqueous solution with a blue dye, (d) bright field microscopic image of region A of the microchannel in image (c), and (e) fluorescent micrograph showing flowing 10- μm fluorescent microparticles.

Stacking multiple hydrogel plates is one of the most efficient approaches for constructing layered hydrogel-based microchannels. In the preparation of agarose hydrogel microchannels, for example, a thermally melted hydrogel plate can be bonded to prepare enclosed channel structures, but maintaining the initial microchannel dimensions is difficult owing to the partial melting and re-gelation. In addition, the heating process damages cells embedded in the hydrogel matrix. Several reports on the fabrication of alginate-based microchannel structures describe the use of physical compression to bond the hydrogel plates because alginate hydrogel is thermally stable. However, specific devices or jigs are required, and the pressures must be applied throughout the experiments.¹⁶ In addition, the partial dissolution-based bonding process introduces difficulty in maintaining the original microchannel dimensions.¹⁷ Compared with the processes described in these previous studies, the presented method is capable of inducing stable chemical bonding with a polyelectrolyte membrane without complicated devices and operations, and also provides a strategy for suppressing volume shrinkage.

B. Preparation of gelatin hydrogel microchannels

Gelatin is an irreversibly denatured form of collagen and is widely used as a natural biomaterial for tissue engineering, pharmaceuticals, and biomedical applications. Gelatin-based hydrogels have important characteristics, including high cell adhesion and reversible sol/gel transition. However, gelatin hydrogel melts at 37°C—the optimal temperature for mammalian cell

FIG. 4. Preparation of gelatin-based hydrogel microdevices. (a) Gelatin hydrogel plate peeled off from the mold, (b) two flat gelatin hydrogel plates bonded via cross-linking with MTG, (c) and (d) straight and serpentine microchannels attached with inlet/outlet tubing, with introduced flow, (e) bright field microscopic image of the microchannel (width of 500 μm), and (f) fluorescence micrograph showing 10- μm microparticles flowing through a straight microchannel.

cultivation—without chemical stabilization such as cross-linking. To date, gelatin-based hydrogel microchannels have been developed by several researchers. For example, capillary or microchannel structure-embedding gelatin hydrogels have been prepared using photocrosslinkable gelatin.²⁸ However, to the best of our knowledge, lamination-based microchannel fabrication using gelatin hydrogel plates has not been reported, although such methods would enable the preparation of microchannel structures with arbitrary designs. Herein, we present a new strategy for producing gelatin-based hydrogel microchannels using enzymatic reaction-based bonding of hydrogel plates. We employed MTG, an enzyme with wide application in the food industry and biochemistry,^{41,42} to catalyze the cross-link reaction between the glutamine and lysine groups of proteins. Unlike mammalian transglutaminases, MTG does not require Ca^{2+} ions for cross-link reactions. We used MTG both to bond the gelatin hydrogel plates and to improve the thermal stability of gelatin hydrogel at 37 °C.

The prepared gelatin-based microfluidic devices are shown in Fig. 4. By adopting the molding process, we obtained hydrogel plates of gelatin with microchannel structures after 30 min of incubation at 4 °C (Fig. 4(a)). Next, the prepared gelatin hydrogel plates were brought into contact after dropping an aliquot of MTG solution between the plates and then incubated at 20 °C for up to 24 h for cross-linking. We examined two parameters that may affect the properties of the gelatin hydrogel microdevice: the MTG concentration in the gelatin matrix (0.1~50 U/ml) and the incubation period for crosslinking at 20 °C (0~24 h). We evaluated the effects of these

FIG. 5. Stability and bonding property of gelatin hydrogel plates with various MTG concentrations in the gelatin solution and the incubation time after bonding. The MTG concentration in the aqueous solution for bonding was constant (10 U/ml). (a) Stability of the microdevice incubated at 37 °C. The open circle indicates that the shape of the hydrogel structure was completely preserved after incubating at 37 °C for 1 h. (b) Bonding property of two gelatin hydrogel plates. The open circle indicates that the two plates were completely bonded and were not separated even partially when they were tried to be separated.

factors on the stability of the hydrogel matrix at 37 °C and the bonding properties between the plates. As shown in Fig. 5(a), when the MTG concentration was too low, the obtained hydrogel plate was unstable at 37 °C. On the contrary, high MTG concentration resulted in a failure to bond (Fig. 5(b)), likely because most of the glutamine and lysine groups were cross-linked in the first incubation step for gelation and few remained for bonding. From this result, we confirmed that the MTG concentration in the gelatin matrix should be 2–5 U/ml. Under this condition, the incubation time for cross-linking was at least ~7 h to ensure both perfect bonding and matrix cross-linking.

Using the optimal conditions (MTG concentration of 2 U/ml, overnight incubation), we prepared gelatin hydrogel microchannels with various morphologies, as shown in Figs. 4(c) and 4(d). To examine applicability to continuous fluidic experiments, we introduced a buffer solution with a blue dye or green fluorescent particles into the gelatin microchannels using a syringe pump (Figs. 4(d)–4(f)). As a result, we observed the stable loading of fluid flows within the microchannels without leakage from the bonded surface, confirming the formation of microchannels applicable to microfluidic experiments. This kind of hydrogel-based microfluidic device would be highly useful in the fabrication of a bulk-scale cell-embedding cultivation scaffold incorporating capillary networks, which enables the efficient supply of oxygen and nutrition. In addition, gelatin-based hydrogels are highly cell-adhesive and bio-active, and hence, it would enable the efficient cell culture while preserving cellular functions when cells are seeded on the microchannel surface or within the hydrogel matrices.

C. Cell culture within hydrogel microchannels

Next, we conducted cell culture experiments within the two types of prepared hydrogel microchannels. First, we employed Ca-alginate hydrogel microchannels. Alginate hydrogel is mechanically strong but generally non-cell adhesive; and thus, specific treatments such as PGA-based modification³⁷ and fibronectin coating via a PLL membrane⁴³ have been applied to modify the hydrogel surface to be cell adhesive. The PLL membrane is likely applicable to direct cultivation of several types of cells (e.g., PC-12, a pheochromocytoma cell line); but in this study, we used another strategy with the cell adhesive peptide-conjugated alginate polymer. We prepared a triple-layer polyelectrolyte membrane composed of alginate, PLL, and GRGDSP peptide-conjugated alginate. The inner layer of GRGDSP-alginate enables stable cell adhesion and cultivation. Fig. 6(a) shows microscopic images of NIH-3T3 cells within an alginate hydrogel microchannel just after seeding and after 4 days of cultivation. The initial cell positions were localized at the center of the hydrogel microchannel, because a relatively thick layer of GRGDSP alginate was formed near the corners of the microchannel and the cross-sectional

FIG. 6. Cell culture experiments within the microfluidic devices. (a) NIH-3T3 cells cultured within a Ca-alginate hydrogel microchannel under static culture conditions. The inner surface of the microchannel was coated with GRGDSP peptide-conjugated alginate. Cell nuclei were stained blue with Hoechst 33342. (b) HUVECs cultured under perfusion culture conditions with a flow rate of $10 \mu\text{L}/\text{min}$. Cell nuclei and cytoskeleton were stained blue and green, respectively. (c) Distribution of cell nucleus orientation of HUVECs cultured within gelatin hydrogel microchannels under two different flow-rate conditions. The orientation of cell nuclei indicates the angle between the flow direction and the longest axis of the nucleus. On average, ~ 230 cells were evaluated for each condition.

shape was rounded (data not shown). After 4 days of cultivation, cells formed a confluent monolayer at the center of the microchannel. Although we did not continuously introduce culture medium into the microchannel, cells adhered, and proliferated within the microchannel, maintaining high cell viability ($>95\%$, by dye-exclusion test), because the permeable hydrogel matrix of the microchannel enabled efficient delivery of oxygen and nutrition to the cells. Cells did not spread on the entire surface of the microchannel, possibly because the cell adhesion on the RGD-alginate layer was not so strong. We expect that cells can be cultivated within the alginate matrix because the presented process required no heating.

Next, we cultured vascular endothelial cells within the gelatin hydrogel microchannels. HUVECs were introduced into the microchannels, and incubated for 12 h to ensure adhesion on the microchannel surface. Then, the perfusion of the culture medium was started and continued for several days. Microscopic images of HUVECs cultured for 3 days at a perfusion flow rate of $10 \mu\text{L}/\text{min}$ are shown in Fig. 6(b). We confirmed that cells adhered to the gelatin hydrogel, proliferated, and formed a confluent monolayer on the microchannel surface. We stained cell nuclei and cytoskeleton with Hoechst 33342 (blue) and phalloidin (green), respectively, and calculated the distribution of cell nucleus orientation at different flow rates of the medium perfusion. As shown in Fig. 6(c), the orientation of cell nuclei was almost random under a low flow rate ($4 \mu\text{L}/\text{min}$); the average angle $\pm$ standard deviation (SD) of the longest axis of the cell

nuclei against the flow direction was $44.4^\circ \pm 29.0^\circ$. By contrast, the ratio of cell nuclei with larger angles decreased under a high flow rate (10 $\mu\text{l}/\text{min}$); the average angle $\pm$ SD was $22.1^\circ \pm 19.8^\circ$. The difference between these values was significant ($p < 0.01$, t-test). The high flow rate controlled the orientation of vascular endothelial cells as a result of flow-induced shear stress on the endothelial cells. The shear stress applied on cells can be calculated by using the following equation: $\tau = 6Q\mu/wh^2$, where τ is the shear stress, Q is the volumetric flow rate, μ is the fluid viscosity of the cell culture medium, and w and h are the width and height of the microchannel, respectively.⁴⁴ When the flow rate was 10 $\mu\text{l}/\text{min}$, the shear stress was estimated to be ~ 0.1 dyne/cm². Although this value was slightly lower than the shear stress applied on endothelial cells in small resistance vessels under physiological conditions (0.2–20 dyne/cm²),⁴⁵ we confirmed that the orientation of endothelial cells in the presented hydrogel microchannels was controlled by changing the perfusion flow rate. This experiment clearly demonstrated that basic cell culture experiments are possible using these hydrogel-based microfluidic devices, enabling a variety of biological applications including studies of cancer cell metastasis, cell-based assay systems for drug development, and preparation of vascular tissue models. Compared to collagen hydrogels, which are often used as substrates for hydrogel-based microchannels, the use of gelatin as a microchannel material offers the benefits of high physical stability and low cost, thus enabling facile use and wide application. The cell cultivation technique within the hydrogel microchannels would be useful for preparing vascular tissue models for biochemical research and fabricating 3D tissue models embossing blood vessel networks.

IV. CONCLUSIONS

In this study, two processes were developed to prepare hydrogel-based microfluidic devices with the natural biopolymers, alginate and gelatin, composing the hydrogel matrix. The presented bonding strategies are simple and reproducible and enable the facile fabrication of enclosed microchannel structures without necessitating complicated operations or devices. Because hydrogel-based microfluidic devices are gaining increasing attention as platforms for cell cultivation in a 3D environment, the presented approaches will be useful in a variety of biological experiments, including cell-based drug screening assays, physiological studies of cells in *in vivo*-mimicking environments, and the construction of organ-on-a-chip systems.

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- ¹D. Kloß, M. Fischer, A. Rothermel, J. C. Simon, and A. A. Robitzki, *Lab Chip* **8**, 879 (2008).
- ²A. Ivascu and M. Kubbies, *J. Biomol. Screen.* **11**, 922 (2006).
- ³V. L. Tsang and S. N. Bhatia, *Adv. Drug Deliv. Rev.* **56**, 1635 (2004).
- ⁴J. Lee, M. J. Cuddihy, and N. A. Kotov, *Tissue Eng. Part B, Rev.* **14**, 61 (2008).
- ⁵J. A. Burdick and G. Vunjak-Novakovic, *Tissue Eng. Part A* **15**, 205 (2009).
- ⁶S. Levenberg, N. F. Huang, E. Lavik, A. B. Rogers, J. Itskovitz-Eldor, and R. Langer, *Proc. Natl. Acad. Sci. U.S.A.* **100**, 12741 (2003).
- ⁷L. Y. Wu, D. Di Carlo, and L. P. Lee, *Biomed. Microdev.* **10**, 197 (2008).
- ⁸Q. Zhang, T. Liu, and J. Qin, *Lab Chip* **12**, 2837 (2012).
- ⁹A. D. van der Meer, V. V. Orlova, P. ten Dijke, A. van den Berg, and C. L. Mummery, *Lab Chip* **13**, 3562 (2013).
- ¹⁰S. S. Verbridge, A. Chakrabarti, P. DelNero, B. Kwee, J. D. Varner, A. D. Stroock, and C. Fischbach, *J. Biomed. Mater. Res. Part A* **101**, 2948 (2013).
- ¹¹J. Fukuda, A. Khademhosseini, Y. Yeo, X. Yang, J. Yeh, G. Eng, J. Blumling, C. F. Wang, D. S. Kohane, and R. Langer, *Biomaterials* **27**, 5259 (2006).
- ¹²T. Anada, T. Masuda, Y. Honda, J. Fukuda, F. Arai, T. Fukuda, and O. Suzuki, *Sens. Actuators B* **147**, 376 (2010).
- ¹³H. Ota, T. Kodama, and N. Miki, *Biomicrofluidics* **5**, 34105 (2011).
- ¹⁴M. Iwase, M. Yamada, E. Yamada, and M. Seki, *J. Robot. Mechatron.* **25**, 682 (2013).
- ¹⁵R. Glicklis, J. C. Merchuk, and S. Cohen, *Biotechnol. Bioeng.* **86**, 672 (2004).
- ¹⁶N. W. Choi, M. Cabodi, B. Held, J. P. Gleghorn, L. J. Bonassar, and A. D. Stroock, *Nature Mater.* **6**, 908 (2007).
- ¹⁷M. Cabodi, N. W. Choi, J. P. Gleghorn, C. S. D. Lee, L. J. Bonassar, and A. D. Stroock, *J. Am. Chem. Soc.* **127**, 13788 (2005).
- ¹⁸T. Takei, S. Sakai, T. Yokonuma, H. Ijima, and K. Kawakami, *Biotechnol. Prog.* **23**, 182 (2007).

- ¹⁹Y. Zheng, J. Chen, M. Craven, N. W. Choi, S. Totorica, A. Diaz-Santana, P. Kermani, B. Hempstead, C. Fischbach-Teschl, J. A. Lopez, and A. D. Stroock, *Proc. Natl. Acad. Sci. U.S.A.* **109**, 9342 (2012).
- ²⁰Y. Ling, J. Rubin, Y. Deng, C. Huang, U. Demirci, J. M. Karp, and A. Khademhosseini, *Lab Chip* **7**, 756 (2007).
- ²¹G. Huang, S. Wang, X. He, X. Zhang, T. J. Lu, and F. Xu, *Biotechnol. Bioeng.* **110**, 980 (2013).
- ²²M. P. Cuchiara, A. C. B. Allen, T. M. Chen, J. S. Miller, and J. L. West, *Biomaterials* **31**, 5491 (2010).
- ²³H. Takehara, A. Nagaoka, J. Noguchi, T. Akagi, T. Sakai, U. I. Chung, H. Kasai, and T. Ichiki, *Biomicrofluidics* **7**, 54109 (2013).
- ²⁴S. Sakai, S. Yamaguchi, T. Takei, and K. Kawakami, *Biomacromolecules* **9**, 2036 (2008).
- ²⁵K. H. K. Wong, J. G. Truslow, A. H. Khankhel, K. L. S. Chan, and J. Tien, *J. Biomed. Mater. Res. Part A* **101A**, 2181 (2013).
- ²⁶C. F. Buchanan, E. E. Voigt, C. S. Szot, J. W. Freeman, P. P. Vlachos, and M. N. Rylander, *Tissue Eng. Part C* **20**, 64 (2014).
- ²⁷W. Lee, V. Lee, S. Polio, P. Keegan, J. H. Lee, K. Fischer, J. K. Park, and S. S. Yoo, *Biotechnol. Bioeng.* **105**, 1178 (2010).
- ²⁸N. Sadr, M. Zhu, T. Osaki, T. Kakegawa, Y. Yang, M. Moretti, J. Fukuda, and A. Khademhosseini, *Biomaterials* **32**, 7479 (2011).
- ²⁹J. S. Miller, K. R. Stevens, M. T. Yang, B. M. Baker, D. H. Nguyen, D. M. Cohen, E. Toro, A. A. Chen, P. A. Galie, X. Yu, R. Chaturvedi, S. N. Bhatia, and C. S. Chen, *Nature Mater.* **11**, 768 (2012).
- ³⁰B. T. Stokke, O. Smidsrød, P. Bruheim, and G. Skjåk-Bræk, *Macromolecules* **24**, 4637 (1991).
- ³¹G. T. Grant, E. R. Morris, D. A. Rees, P. J. C. Smith, and D. Thom, *FEBS Lett.* **32**, 195 (1973).
- ³²D. C. Duffy, J. C. McDonald, O. J. A. Schueller, and G. M. Whitesides, *Anal. Chem.* **70**, 4974 (1998).
- ³³M. Yamada, R. Utoh, K. Ohashi, K. Tatsumi, M. Yamato, T. Okano, and M. Seki, *Biomaterials* **33**, 8304 (2012).
- ³⁴A. Miyama, M. Yamada, S. Sugaya, and M. Seki, *RSC Adv.* **3**, 12299 (2013).
- ³⁵M. Yamada, S. Sugaya, Y. Naganuma, and M. Seki, *Soft Matter* **8**, 3122 (2012).
- ³⁶A. Kobayashi, K. Yamakoshi, Y. Yajima, R. Utoh, M. Yamada, and M. Seki, *J. Biosci. Bioeng.* **116**, 761 (2013).
- ³⁷Y. J. Kwon and C. A. Peng, *Biotechniques* **33**, 212 (2002).
- ³⁸Y. A. Mørch, M. Qi, P. O. Gundersen, K. Formo, I. Lacik, G. Skjåk-Bræk, J. Oberholzer, and B. L. Strand, *J. Biomed. Mater. Res. Part A* **100A**, 2939 (2012).
- ³⁹X. Huang, N. Lin, R. Hang, X. Wang, X. Zhang, and B. Tang, *Carbohydr. Polym.* **92**, 1064 (2013).
- ⁴⁰M. R. Garfinkel, R. C. Harland, and E. C. Opara, *J. Surg. Res.* **76**, 7 (1998).
- ⁴¹K. Yokoyama, N. Nio, and Y. Kikuchi, *Appl. Microbiol. Biotechnol.* **64**, 447 (2004).
- ⁴²T. Tanaka, N. Kamiya, and T. Nagamune, *FEBS Lett.* **579**, 2092 (2005).
- ⁴³E. Kang, Y. Y. Choi, S. K. Chae, J. H. Moon, J. Y. Chang, and S. H. Lee, *Adv. Mater.* **24**, 4271 (2012).
- ⁴⁴D. Yoshino, N. Sakamoto, K. Takahashi, E. Inoue, and M. Sato, *J. Biomech. Sci. Eng.* **8**, 233 (2013).
- ⁴⁵Z. Cai, J. Xin, D. M. Pollock, and J. S. Pollock, *Am. J. Physiol. Renal Physiol.* **279**, F270 (2000).