Sacificial Alginate-Assisted Microfluidic Engineering of Cell-Supportive Protein Microfibers for Hydrogel-Based Cell Encapsulation

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ABSTRACT: Although many types of technologies for hydrogel-based cell cultivation have recently been developed, strategies to integrate cell-adhesive micrometer-sized supports with bulk-scale hydrogel platforms have not been fully established. Here, we present a highly unique approach to produce cell-adhesive, protein-based microfibers assisted by the sacrificial template of alginate; we applied these fibers as microengineered scaffolds for hydrogel-based cell encapsulation. Two types of microfluidic devices were designed and fabricated: a single-layered device for producing relatively thick (Φ of 10−60 μm) alginate−protein composite fibers with a uniform cross-sectional morphology and a four-layered device for preparing thinner (Φ of ∼4 μm) ones through the formation of patterned microfibers with eight distinct alginate−protein composite regions. Following chemical cross-linking of protein molecules and the subsequent removal of the sacrificial alginate from the double-network matrices, microfibers composed only of cross-linked proteins were obtained. We used gelatin, albumin, and hemoglobin as the protein material, and the gelatin-based cell-adhesive fibers were further encapsulated in hydrogels together with the mammalian cells. We clarified that the thinner fibers were especially effective in promoting cell proliferation, and the shape of the constructs was maintained even after removing the hydrogel matrices. The presented approach offers cells with biocompatible solid supports that enhance cell adhesion and proliferation, paving the way for the next generation of techniques for tissue engineering and multicellular organoid formation.

INTRODUCTION

Precise engineering of three-dimensional (3D) tissue models in in vitro culture platforms is gaining extensive attention with increasing demands on effective tools for regenerative medicine and cell-based drug assays.1 Recent advancements in stem cell engineering have also accelerated the development of new approaches that can recapitulate the developmental fields of multicellular organoids.2,3 Techniques to assemble individual mammalian cells and extracellular matrix (ECM) components into large architectures with predefined shapes are hence of significant importance in maintaining the native functions and differentiated characteristics of cells. Compared to the conventional plate-based culture methods, 3D cell culture platforms offer cells physiological cues similar to those of the in vivo tissues, resulting in the maintenance of proper cell morphology, polarity, and functions.4

Among various technologies for 3D cell culture, hydrogel-based cell encapsulation is a highly efficient approach that permits the arbitrary design of the shapes of constructs. Unlike in the other 3D cell culture techniques, as represented by the multicellular spheroid formation,5,6 the cell density in the hydrogel matrix can be finely tuned. Hence, we are able to avoid the problem of hypoxia and shortage of nutrition for the cells located at the center of the constructs. Additionally, cells could be uniformly inoculated in the hydrogel matrices when the cell-containing precursor solution is transformed into the hydrogel. Various types of natural and synthetic polymers have been utilized as hydrogel matrices that can encapsulate intact cells, including collagen, gelatin, Matrigel, alginate, poly(ethylene glycol)-based polymers, and polyacrylamide.7−9 Ideally, the following characteristics are required for the hydrogels for cell encapsulation: (i) the gelation process does not necessitate a harsh change in the temperature or pH, (ii) the 3D hydrogel morphology is freely designable, and (iii) the hydrogel matrix is removable when proper cell-cell and cell−matrix interactions are formed. Hydrogel-based cell culture systems have been used to create a variety of tissue models, including vasculature tissues,10−12 skin equivalents,13,14 and liver microorganisms,15,16 with the help of, for example, bio 3D printers and microfabrication technologies. However, several challenges still remain in conventional hydrogel-based cell
encapsulation techniques. First, hydrogel matrices often prevent cell proliferation, especially when their mechanical properties are high, as in the case of alginate-based hydrogels;17 that is, the stability/durability of the hydrogels and the proliferation ability of the encapsulated cells are mostly in a trade-off relationship. The lack of proper cell-binding motifs of the hydrogel-constituting molecules would also result in insufficient cell proliferation. Additionally, the hydrogel matrices hamper the proper formation of intercellular networks, resulting in insufficient stability of the tissue morphology when the hydrogel matrices are removed. Therefore, research on new concepts that facilitate cell proliferation while maximizing the cell-supportive potential of hydrogels is of great interest.

To date, efforts have been made to produce cell-sized, microengineered materials that were used as cell-adhesive solid supports in 3D tissue culture platforms.18,19 Microparticles made of ECM components, such as collagen, have been implemented into 3D cellular aggregates that can modulate the cell density, form capillary-like void spaces, and provide proper cell–ECM interactions.20,21 Fiber-shaped, micrometer-sized materials have also been prepared, which were particularly suitable as cell-adhesive matrices for directing cell growth and orientation on the surface22,23 and as cell-encapsulating matrices to generate linear cell assemblies.15,24 We speculated that these microengineered materials would be especially beneficial as cell-supportive scaffolds if they were embedded into hydrogel-based cell culture platforms. However, previous strategies on microfiber production processes were constrained by several major limitations. One of the widely used techniques for producing micro/nanofibers is electrospinning,25−28 but the fibers obtained by this technique are interconnected and entangled on the fiber mats, making it difficult to obtain freely standing, dispersible fibers that could be implemented into hydrogels. Microfluidic spinning processes to produce fibers have also been proposed,29,30 but in general, production of cell-sized microfibers, ideally with a diameter of several micrometers, is difficult. Moreover, most of these previous approaches were only applicable to producing specific protein-based materials, mainly collagen, and the production of microfibers made of various types of protein molecules has not been fully developed. Facile and universally available approaches that can produce micrometer-sized protein microfibers are hence greatly anticipated.

Here, we propose a highly unique and efficient strategy to produce protein-based microfibers that can be uniformly encapsulated in hydrogel matrices together with intact cells. We recently introduced versatile microfluidic platforms to produce alginate-based hydrogel microfibers,15,31−33 which can precisely control the fiber diameter and the cross-sectional anisotropy. In this study, this process was extended to the production of protein microfibers by using the alginate matrix as the sacrificial material (Figure 1). We employed two types of microfluidic devices, one for producing relatively thick, homogeneous hydrogel fibers and the other for producing thinner fibers through the formation of patterned fibers. Both of these devices were first used to produce composite microfibers consisting of calcium alginate (Ca-alg) and proteins. The protein molecules of the fibers were then chemically cross-linked to form double-network matrices, followed by the selective removal of the sacrificial alginate by treating the fibers with citrate, which chelates Ca2+ ions. In this study, we prepared microfibers composed of several types of proteins, and the obtained fibers, especially those made of cross-linked gelatin, were used as microscaffolds that were implemented into the hydrogel matrices together with cells. We clarified the effects of the fiber diameter on the cell proliferation characteristics and the maintenance of the tissue shape after hydrogel removal in order to show the feasibility of using the presented approach across the wide applications associated with hydrogel-based cell culture and tissue engineering.

**Figure 1.** Schematic showing the production processes of protein microfibers. Two types of microfluidic devices were used: (a) Single-layered device to produce homogeneous Ca-alginate and gelatin fibers and (b) four-layered device to produce patterned microfibers, which were finally transformed into thick (10−60 μm) and thin (~4 μm) gelatin fibers, respectively. (c) Post microfluidic processes to prepare fragmented protein microfibers. Fibers made of several types of proteins (gelatin, albumin, and hemoglobin) were produced.
### MATERIALS AND METHODS

**Materials.** Sodium alginate (Na-alg; viscosity of 300–400 mPa s at 1% solution at 20 °C), gelatin (from bovine bone; pH of a 50 g L⁻¹ solution at 35 °C of 5.5–6.0, catalog number of 076-02765), calcium chloride (CaCl₂) dihydrate, 25% glutaraldehyde solution, and trisodium citrate dihydrate were obtained from Fujifilm Wako Pure Chemical (Osaka, Japan). Dextran (from *Leuconostoc* spp., Mₕ of 450,000–650,000), Dulbecco’s modified Eagle’s medium (DMEM), penicillin–streptomycin solution, trypsin/ethylenediaminetetracetic acid (EDTA) solution, alginate lyase (from *Flavobacterium* multi-vorum), and hemoglobin (from bovine blood) were obtained from Sigma-Aldrich (MO, USA). Polydymethylsiloxane (PDMS) prepolymer (Silpot 184) was obtained from Dow Corning Toray (Tokyo, Japan). Bovine serum albumin (BSA) was obtained from Rockland Immunochemicals (PA, USA). Polydimethylsiloxane (Shiga, Japan). Fetal bovine serum (FBS), green fluorescent microbeads (diameter of 0.5 μm), and alamarBlue cell viability reagent were obtained from Thermo Fisher Scientific (MA, USA). These materials were used as received. All other chemicals were of analytical grade.

**Fabrication of Microfluidic Devices.** We employed two types of microfluidic devices to prepare composite microfibers: single-layered PDMS microdevices and four-layered poly-(methyl methacrylate) (PMMA) microdevices. The PDMS microdevices were fabricated using soft lithography and replica molding processes as previously reported. Briefly, SU-8 structures were patterned on Si wafers, and the PDMS prepolymer was cast on the mold. After peeling off the PDMS plate from the mold and making inlet/outlet holes, this plate was bonded with a flat glass slide via O₂-plasma activation. The microchannel was equipped with five inlet channels and a straight gelation channel. The width, depth, and length of the gelation channel were 400 μm, 115 μm, and 50 mm, respectively.

Four-layered PMMA microdevices were fabricated by laminating one flat and three micromachined PMMA plates. First, the PMMA plates (30 × 70 × 1.5 mm) were micromachined by using a numerical control (NC) machine (Micro MC-2; PMT Corp., Fukuoka, Japan) with an end mill (diameter of 100–900 μm). Through-holes were also made using the NC machine with a drill mill (diameter of 100–300 μm). Inlet and outlet holes (diameter of 2 mm) were formed using a drill machine. Then, four PMMA plates were thermally bonded with an applied pressure of ~2 × 10⁵ Pa for 10 min at 120 °C and 5 min at 130 °C. The microdevice was composed of a top layer with a gelation channel (width of 400 μm, depth of 300 μm), a second layer with a focusing nozzle (a through-hole with an exit diameter of 300 μm), a third layer with a micronozzle array structure and inlet distribution channels, and a flat bottom layer. There were six inlets for this device.

**Production of Protein Microfibers.** An aqueous solution of 1% Na-alg and protein (3% gelatin, 20% albumin, or 10% hemoglobin) in deionized (DI) water was used as the precursor solution to produce composite microfibers. Aqueous solutions of 10% dextran with and without 0.1 M CaCl₂ were used as the gelation agent solution and the buffer solution, respectively. These solutions were introduced into the microchannel using syringe pumps (KDS 200; KD Scientific, MA, USA). For the single-layered device to produce thick and homogeneous microfibers, the precursor solution, the buffer solution, and the gelation agent solution were continuously introduced from inlet 1, inlets 2/2’, and inlets 3/3’, respectively. The flow rates of the precursor solution, the buffer solution, and the gelation agent solution were 20, 5–10, and 100 μL min⁻¹, respectively, unless otherwise noted. The formed composite microfibers were recovered by using a roller (diameter of ~85 mm, rotated at 0–40 rpm with the corresponding recovery speed of 0–10.7 m min⁻¹), which was partially dipped in a bath containing the recovery solution (the gelation agent solution without dextran). To produce BSA and hemoglobin fibers, 2.5% glutaraldehyde was added to this solution. The obtained fiber bundle was detached from the roller and immersed in 2.5% glutaraldehyde solution overnight at 4 °C to completely cross-link the protein molecules in the microfibers. Then, the Ca-alg hydrogel was removed by treating the fiber in 0.1 M trisodium citrate solution for ~10 min. The fibers were cut into ~1 mm long fragments using microtome blades.

To prepare much thinner fibers, we first produced patterned microfibers using the four-layered microdevice. The buffer solution and the gelation agent solution were introduced from inlets 5 and 6 at flow rates of 40–50 and 400–500 μL min⁻¹, respectively. The precursor solution I (an aqueous solution of 2% Na-alg) was introduced from inlets 1, 2, and 3, with flow rates of 60, 30, and 30 μL min⁻¹, respectively. The precursor solution II (an aqueous solution of 1% Na-alg and 3% gelatin) was introduced from inlet 4 with a flow rate of 15 μL min⁻¹. The patterned microfibers were recovered by using the roller, and the fiber bundle was treated in the same manner as the homogeneous fibers described above.

**Characterization of the Microfibers.** To evaluate the mechanical properties of the gelatin fibers, the Young’s moduli and the compression breaking stresses of the obtained composite hydrogels before/after removal of the Ca-alg matrix were evaluated using a rheometer (RheoStress 6000; Thermo Fisher Scientific). Disc-shaped gelatin/Ca-alg hydrogels (diameter of 10 mm, height of 5 mm) were used as the substrate instead of the microfibers. The compression test was performed by pushing the hydrogel with a steel rod (Φ of 2 mm) with a flat tip end at a speed of 0.5 mm min⁻¹. We obtained the data on the Young’s moduli from the 0–5% displacement regions of the stress–strain curves and the data on the breaking stress simultaneously. The obtained data from three individual samples were averaged.

We performed several tests to investigate whether the Ca-alginate hydrogel matrix was removed. First, the dry weights of the fibers before/after removing the alginate matrix were measured. Additionally, the Fourier transform infrared (FTIR) spectra were measured using a FTIR spectrophotometer (FTIR-8400S, Shimadzu, Kyoto, Japan); planar disc-shape hydrogels (diameter of 10 mm, height of 5 mm) were prepared and dried for 24 h at 50 °C for use in this experiment. The following four samples were tested: (1) Ca-alginate, (2) gelatin, (3) alginate–gelatin (before removal of alginate), and (4) alginate–gelatin (after removal of alginate) hydrogels.

Additionally, the cross-sectional morphology of the patterned fibers was observed by preparing thin sections. Briefly, patterned fibers incorporating 0.5 μm green fluorescent micro particles in the gelatin/Ca-alg composite regions were prepared and embedded in O.C.T. compound (Sakura Finetek, Tokyo, Japan). Thin frozen sections were prepared using a cryostat (LEICA CM1510S; Leica Biosystems, Wetzlar, Germany).
Germany), and the cross-sectional morphology was observed using a fluorescence microscope.

**Cell Preparation and Culture.** HepG2 cells (a human hepatoma cell line, provided by RIKEN BRC, Ibaraki, Japan) and Swiss-3T3 cells (mouse fibroblasts, Riken BRC) were cultured in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin and maintained at 37 °C in a 5% CO₂ atmosphere in a CO₂ incubator. Cells at ~80% confluence were harvested by trypsin/EDTA treatment and used for the experiments. For the cell adhesion test on the prepared gelatin fibers, cells (1 × 10⁵ cells cm⁻²) and fiber fragments (diameter of ~20 μm, length of ~1 mm) were mixed and seeded on a non-cell-adhesive culture plate (Hydrocell 6 multi-well plate, 9.5 cm² per well; CellSeed, Tokyo, Japan) and cultured.

**Cell Culture in Hydrogel Sheets with Gelatin Microfibers.** Two types of gelatin microfibers, with average diameters of ~4 and ~20 μm, were employed. These fibers were washed with an aqueous solution of 0.1% BSA in DI water twice and then cut into ~1 mm long fragments, and the fiber pellet in saline was formed by gentle centrifugation. The fiber pellet, a suspension of HepG2 cells (conc. of 3 × 10⁷ cells mL⁻¹) in saline, and 2% Na-alg in saline were mixed with a volumetric mixing ratio of 2:1:1. Considering the water content of the fiber pellet (~50%), the final concentration of Na-alg was ~0.7%, and the cell concentration was 1 × 10⁷ cells mL⁻¹, when the volume of the gelatin fibers was neglected. For preparing control samples without encapsulating the fibers, the concentrations of Na-alg and cells were set to be equal to these values. Then, a small aliquot of the precursor solution (16 μL) was dropped onto a glass slide. This slide was placed onto another glass slide with an aqueous solution of 0.1 M CaCl₂ (volume of 100 μL) using thin glass plates (thickness of 0.15 mm) as spacers between these two slides. After completing the gelation of the precursor (1–2 min), the formed hydrogel sheet was recovered and washed with saline and then placed onto a 6-well plate with 2 mL of the cell culture medium. Cells were cultured in a CO₂ incubator, and the culture medium was exchanged every other day. After day 1, a culture medium with a lower FBS concentration (1%) was used.

**Characterization of Cells in Hydrogels with Gelatin Microfibers.** To evaluate the proliferation ability of the encapsulated HepG2 cells, alamarBlue assay was performed on days 1 and 5. Three hours after exchanging the culture medium with a fresh medium with 10% alamarBlue reagent, the medium was collected, and the alamarBlue reduction ratio was evaluated by measuring the fluorescence intensity (excitation of 550 nm and emission of 590 nm) using a microplate reader (MTP-810Lab, Corona Electric, Ibaraki, Japan). The obtained data were statistically analyzed by one-way analysis of variance with Bonferroni post hoc test using IBM SPSS Statistics 22 software (IBM Japan, Tokyo, Japan). The cell viability was also investigated by the trypan blue dye exclusion test. Additionally, to investigate the formation of intercellular networks in the hydrogel matrix, the hydrogel removal test was performed. Alginate lyase was added to the culture medium at a concentration of 1 unit mL⁻¹ to enzymatically digest the hydrogel sheet. After digestion, the number of dispersed cells dropped out of the hydrogel sheet was evaluated.

**RESULTS AND DISCUSSION**

**Production of Homogeneous Fibers Using the Single-Layered Device.** We first produced homogeneous microfibers composed of Ca-alg and gelatin using the single-layered microfluidic device. The introduction of the buffer solution from inlets 2/2′, between the precursor and the gelation agent solutions, was essential to prevent clog formation at the junction and stabilize the fiber production process. Figure 2a,b shows the micrographs of the flows of the solutions in the microchannel. Because of the low Reynolds’s...
number in the microchannel (estimated to be less than 1), the pattern of the parallel laminar flows was stably maintained. At the middle point of the gelation channel (~20 mm from the junction), the precursor solution was already transformed into a solid hydrogel fiber because of the rapid gelation of alginate by the diffusion-based supply of the Ca\(^{2+}\) ions. From the outlet, composite microfibers made of Ca-alginate and gelatin were continuously recovered (Figure 2c). The obtained fibers were sufficiently strong that we were able to recover the fiber by using a roller. We controlled the fiber diameter either by adjusting the flow rate of the precursor solution or by changing the rotation speed of the roller (Figure 2d,e). Thick fibers were formed under high flow-rate conditions of the precursor solution and/or the low rotation speed of the roller. By using this device, the fiber diameter could be tuned in a range from 15 to 50 μm.

Next, to transform the obtained composite fibers into those composed only of proteins, we chemically cross-linked gelatin molecules by glutaraldehyde and then removed the Ca-alg matrix by citrate. Because the cross-linking reaction with glutaraldehyde occurred through the amine moieties of gelatin, the alginate polymer, which does not contain amine, would not be cross-linked. Consequently, the alginate matrix would be removed by the simple treatment of the fibers with citrate. Figure 2f shows the micrographs of the fibers, which were cross-linked by glutaraldehyde, before and after removing alginate by citrate treatment. The citrate treatment did not cause apparent change in the fiber diameter and morphology, but the fibers became fragile after removing the alginate polymer.

**Characterization of the Gelatin Microfibers.** For cell culture applications, the surface of the microfibers should be rendered cell-adhesive. Alginate is known as a highly anti-cell-adhesive polymer, and hence, we investigated whether the alginate matrices were properly removed by the citrate treatment. First, the weights of the dried fibers (average diameter of ~20 μm) before and after removing the alginate polymer were measured and compared. As a result, the dry weight of the fiber after the citrate treatment was decreased to ~66% of that of the gelatin-crosslinked fiber, and this result was consistent with the roughly estimated value for the complete removal of alginate (~75%) from the composite fiber.

We also performed FTIR measurement of the gelatin/Ca-alg hydrogel matrices before and after the citrate treatment. The result is shown in Figure 3a. The absorption at 3300–3400 cm\(^{-1}\) corresponds to the O–H and N–H stretching,\(^{35,36}\) which decreased and shifted to higher wavenumbers for the three samples containing gelatin, compared to the pure Ca-alg hydrogel. The Ca-alg hydrogel showed specific adsorption at ~1605 cm\(^{-1}\) (asymmetric stretching vibration of COO\(^{-}\)),\(^{35–37}\) ~1080 cm\(^{-1}\) (C–C stretching band), and ~1030 cm\(^{-1}\) (C–O stretching band),\(^{35–37}\) which were not observed for the gelatin hydrogel and decreased for the composite material before/after the citrate treatment. On the other hand, peaks at ~1541 cm\(^{-1}\) (amide II) and ~1639 cm\(^{-1}\) (amide I) appeared for the materials containing gelatin, although they were not clearly separated for the composite material after citrate treatment. This may be caused by the presence of a certain amount of residual alginate after citrate treatment.

The mechanical properties of the hydrogel materials before and after citrate treatment were also examined (Figure 3b,c). By the citrate treatment, both the Young’s modulus and the breaking stress were decreased, which indicated that the matrix after the citrate treatment, mainly composed of cross-linked gelatin, became soft and fragile compared to the relatively solid double-network composite hydrogels.

Finally, an adhesion test of mammalian cells was performed. Here, we employed fibroblasts (3T3 cells), which exhibit relatively high adhesion characteristics. The results are shown in Figure 3d. Before removing alginate, only a limited number of cells adhered on the fiber surface, mainly due to the non-cell-adhesive nature of alginate. In marked contrast, cells adhered on the fiber surface after removing the alginate matrices; after 4 h of cultivation, the fibers were completely covered by the cells, indicating that the chemical property of the fibers was dramatically changed. Although the results of FTIR suggested that alginate was not completely removed, we concluded that the alginate matrices were mostly removed so that the fiber surface was rendered sufficiently cell-adhesive and could be applicable to cell culture experiments. To date, methods to produce porous hydrogels using sacrificial alginate beads/fibers have been proposed,\(^{37,38}\) and composite fibers made of alginate and collagen/gelatin have been reported.\(^{15,24,28}\) However, to the best of our knowledge, the selective removal of alginate from the double-network hydrogel matrices has not been fully developed, with only a few exceptions producing cross-linked hyaluronan–fibrin hydrogels using phosphate as the reagent to remove alginate.\(^{39}\) The presented approach is thus a useful combination that produces...
purely protein-based fibers by removing the sacrificial alginate polymer network from the composite material.

**Production of Fibers Using Different Proteins.** The presented approach could be extended to the production of other protein-based microfibers. To prove this concept, we next attempted to produce fibers made of albumin and hemoglobin, both of which are water soluble. When an aqueous solution of 1% Na-alg and 20% albumin (BSA) was used as the precursor, composite microfibers were successfully formed. Unlike gelatin, which is not soluble in water at room temperature, albumin could be dissolved in water, and hence, we needed to add the cross-linker in the recovery solution in the roller bath. When the initial BSA concentration was 20% (Figure 4a), we were able to obtain cross-linked albumin fibers with a diameter of several tens of micrometers. On the other hand, when the albumin concentration was decreased to, for example, 10%, microfibers were formed, but they were fragile, and their shape was unstable after the citrate treatment (data not shown). Under a much lower albumin concentration condition (e.g., 5%), the fibers were dissolved at the time of citrate treatment. These results indicated that a certain amount of albumin was lost from the fiber by diffusion during the cross-linking reaction. We were also able to produce microfibers made of hemoglobin from a precursor solution with 1% Na-alg and 10% hemoglobin (Figure 4b); we obtained red-brown colored fibers using the same procedure. Previous studies have demonstrated the production of microfibers made of these proteins, especially, electrospun albumin fiber mats for use in tissue engineering applications.40–42 Compared to the electrospun fibers, the presented strategy enabled us to produce dispersible fibers with controlled diameters, which are particularly suitable for implementation into hydrogel-based cell culture platforms.

**Formation of Thin Gelatin Fibers Using the Four-Layered Device.** The microfluidic process was capable of producing gelatin fibers as thin as ~15 μm, but it was difficult to further decrease the fiber diameter to less than ~10 μm using the single-layered device, mainly because of the insufficient mechanical stability of the fibers at the stage of fiber recovery. For fibers applicable to cell culture experiments, wide controllability in the fiber diameter would be the key to maximizing the cellular functions. In addition, it is desirable to increase the volumetric production throughput, especially for the thinner fibers. Here, we proposed another microfluidic device (a four-layered device) to produce thinner microfibers in parallel (Figures 1b and 5a). From the peripheral 16 micronozzles, the precursor solutions I and II, without and with gelatin, respectively, were introduced with an alternate pattern, and the precursor solution I for the core flow was introduced from the larger central nozzle. These flows were then united in the focusing nozzle, introduced into the gelation channel, and finally gelled, resulting in the formation of patterned fibers with eight parallel gelatin-containing regions on the periphery. This process does not decrease the entire diameter of the fibers, making the roller-based recovery possible. Additionally, the volumetric production throughput of the fibers would not be compromised, but even increased, for the thinner fibers.

**Figure 5a** shows the flow patterns of the precursor solutions in the four-layered microfluidic device. The precursor II solution (with Na-alg and gelatin) was selectively visualized by adding green fluorescent microparticles. In the focusing nozzle, the flows of the precursor solutions were united and focused into a narrow stream and then introduced into the gelation channel on the upper layer, keeping the alternate flow pattern. As shown in **Figure 5b,c,** we successfully created patterned microfibers, which contained eight distinct parallel regions of the Ca-alg/gelatin composite, with the entire fiber diameter of 60–70 μm. From the cross-sectional observation, the gelatin containing green-colored regions (width of ~4 μm) were separated (**Figure 5d**), and their shapes were not cylindrical but slightly flattened. After chemical cross-linking of gelatin and subsequent citrate treatment, the eight parallel regions were completely separated (**Figure 5e**), generating thin microfibers with a relatively high uniformity in diameter. The

![Figure 4. Production of microfibers of different proteins using the single-layered microfluidic device: (a) albumin and (b) hemoglobin. These micrographs show fibers after cross-linking and citrate treatment. The flow rates of the precursor, buffer, and gelation agent solutions were 20, 5, and 100 μL min⁻¹, respectively.](https://pubs.acs.org/doi/10.1021/acsomega.0c02385/abstract)

![Figure 5. Production of thinner microfibers of gelatin using the four-layered microfluidic devices. (a) Schematic of the microchannel design and the behaviors of the flow in the channel. Green fluorescent microparticles were added only in the precursor solution containing Na-alg and gelatin, introduced from inlet 4. (b,c) Bright field and fluorescence microscopic images of the formed patterned fibers. (d) Cross-sectional image of the microfiber. (e) Micrograph showing the formed thin fibers (~4 μm) after treating the patterned fibers with citrate.](https://pubs.acs.org/doi/10.1021/acsomega.0c02385/abstract)
throughput of the fiber production (input flow rate of the precursor solution II of 15 μL min⁻¹) was comparable to that of the single-layered microdevice (typically 10 μL min⁻¹), which is a great advantage of this device in effectively preparing thin fibers.

**Cell Culture in Hydrogels with the Prepared Gelatin Microfibers.** To verify the applicability of the presented fibers as cell-supportive scaffolds that enhance cell proliferation in hydrogel-based cell culture, here we again employed alginate hydrogels. Alginate-based hydrogels have been utilized for cell encapsulation, transplantation, and tissue engineering applications because of their highly cell-compatible characteristics.¹⁷ Alginate hydrogel enables encapsulation of intact cells without causing severe damage to the cells. Additionally, the hydrogel matrices could be easily removed by treatment with citrate or alginate lyase, as demonstrated above, after the cell organization was completed or tissues were matured. However, as mentioned above, the main challenge is the negative effect of the alginate hydrogel on the encapsulated cells because it hampers the growth of highly proliferating cells.¹⁷ We expected that the cell-adhesive gelatin microfibers would have positive effects on the cell proliferation and intercellular network formation. Here, we employed HepG2 cells, which are often used as model cells to simulate the drug metabolisms in the liver. Figure 6a shows the preparation procedure for a disc-shaped alginate hydrogel sheet containing HepG2 cells and microfibers. The diameter and the thickness of the sheet were ∼8 mm and ∼150 μm, respectively. This thin configuration ensures the diffusion-based delivery of nutrition and oxygen to the cells located deep inside the hydrogel matrices.

Figure 6b–d shows the microscopic images of the cell-encapsulating hydrogel sheets on day 1. We tested two types of gelatin microfibers with different diameters (∼4 and ∼20 μm). The cell viabilities in these hydrogels, examined by trypan blue dye-exclusion assay, were higher than ∼80%. From these images, we confirmed that the fibers and cells were evenly and uniformly distributed throughout the entire region of the hydrogels, not localized on the bottom side of the hydrogel. Next, we performed an alamarBlue assay to quantitatively analyze the number of viable cells in the hydrogel matrices, which indicates the relative cell proliferation abilities. As shown in Figure 7, the cellular activity was enhanced for the thinner microfibers, which would be possibly due to the increased opportunity of the cells to adhere on the solid surface of the thin fibers, compared to the thick fibers or the control experiment without using fibers. These results indicated that our approach of cell encapsulation with microfibers would be effective to support cells and enhance cell proliferation. The relatively low value of the fold change of the cell activity would be due to the low cell supportive characteristics of alginate hydrogel,¹⁷ especially for the condition without using the fibers.

Finally, we attempted to remove the hydrogel matrix and examined whether the resulting microfibers improved the stability of the tissue shape. Here, we did not use citrate, which possibly compromises the cadherin-based cell–cell adhesion⁴⁶,⁴⁷ by chelating Ca²⁺ ions. Instead, we enzymatically digested alginate polymer using alginate lyase. After 1 or 5 days of cultivation, the hydrogel matrices were removed. The results are shown in Figure 8. For the control hydrogel sheets without encapsulating fibers, the tissue shape was not maintained after removing the hydrogel matrices, even after 5 days of cultivation. Cells were completely dispersed in the medium, indicating that cells did not efficiently form multicellular structures and intercellular networks. For the hydrogel sheets incorporating thick (20 μm) gelatin microfibers, the disclike shape of the construct was not maintained on day 1. On day 5, although a certain number of cells were lost, the shape of the construct was maintained, indicating that the cells gradually proliferated. In contrast, for the hydrogels with thin (4 μm) fibers, the shape of the constructs was maintained even after hydrogel removal on day 1, and the ratio of the lost cells on day 5 was minimized. These results clearly suggested that the incorporation of the single micrometer-sized thinner microfibers into the hydrogels is a reasonable strategy for maximizing the cell proliferation and intercellular network formation. This feature is especially beneficial in a situation where the hydrogel matrices should be removed at a desirable time point when the
tissue models are matured or properly engrafted at the desired sites for regenerative therapy. In this study, we just employed the gelatin microfibers for alginate hydrogel-based cell encapsulation, but the presented approaches could be extended to a variety of hydrogels with other protein-based microfibers for tissue engineering, cell-based drug evaluation, and cellular physiological studies. Additionally, the fibers would also be useful as scaffolds for 3D organization of cells into large constructs without using hydrogels, as in the case of previously demonstrated techniques using protein-based microparticles.20,21

■ CONCLUSIONS

Here, we proposed a versatile strategy for preparing protein-based microfibers using alginate as the sacrificial matrix. The use of the microfluidic systems enabled us to precisely tune the fiber diameters, and we were able to produce fibers as thin as ~4 μm with the help of the patterned microfibers prepared using the multilayered microfluidic device. In the cell culture experiments, we validated the effectiveness of the microfibers, especially the thinner ones, on the promotion of the proliferation and network formation of the mammalian cells encapsulated and cultured in hydrogel matrices. Our approach using cell-sized, cell-adhesive micrometer-sized materials to enhance cell proliferation is unique and would provide a useful means to solve the problems in conventional cell culture using hydrogels. The presented fiber-based approach would provide useful information in biofabrication of tissue models for regenerative therapy, drug development, and cellular physiological studies.

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Notes
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