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Oxidized Alginate-Cross-Linked Alginate/Gelatin Hydrogel Fibers for Fabricating Tubular Constructs with Layered Smooth Muscle Cells and Endothelial Cells in Collagen Gels

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Hydrogel fibers that possessed a cell-adhesive surface and were degradable via enzymatic reactions were developed for fabricating tubular constructs with smooth muscle cell (SMC) and endothelial cell (EC) layers, similar to native blood vessels, in collagen gels. The fibers were prepared by soaking hydrogel fibers prepared from a solution of sodium alginate and gelatin containing bovine ECs (BECs) in medium containing oxidized alginate (AO). BECs soaked in 8.0% (w/v) AO showed no reduction in viability within 3 h of soaking. Furthermore, mouse SMCs (MSMCs) adhered and proliferated on the AO-cross-linked hydrogels. Based on these results, we prepared AO-cross-linked hydrogel fibers containing BECs, covered their surface with MSMCs, and embedded them in collagen gels. We then degraded the fibers using alginate lyase to obtain channels in the collagen gels. Histological analysis of the released ECs using a specific fluorescent dye revealed the formation of tubular structures with layered BECs and MSMCs.

Introduction

One hopeful approach for treating patients with failing or malfunctioning organs or tissues is the transplantation of artificial constructs to replace their functions. Current tissue engineering strategies for fabricating such constructs require the development of adequate scaffolds that support the attachment and proliferation of seeded tissue-specific cells as well as their production of extracellular matrix. On the basis of these strategies, more than 20 tissue types composed of cells and scaffolds have been reported.¹ The majority of the reported artificial tissues have the common features of being quite thin or being constructed from cells with a low oxygen demand, because the oxygen supply to cells in the constructs is purely dependent on diffusion from the surrounding environment. The absence of a vascular network capable of distributing oxygen and other nutrients within tissue-engineered constructs is a major limiting factor in creating dense artificial tissues.²

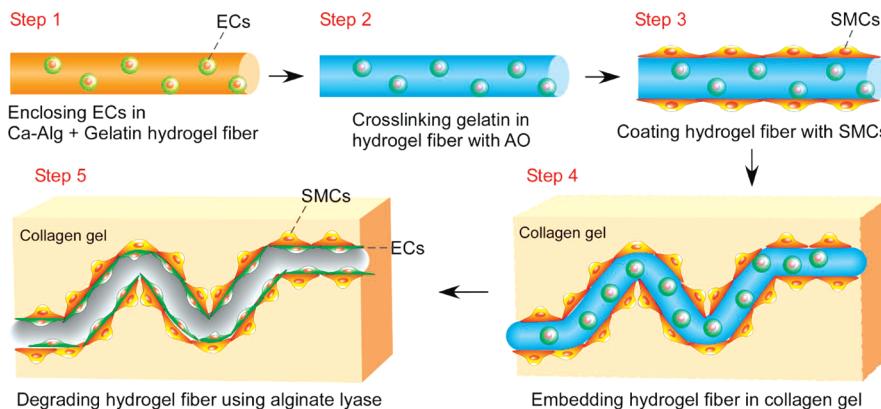
Recently, we developed a method for creating tubular constructs that are several hundreds of micrometers in diameter in collagen gels *in vitro*.³ Specifically, calcium-alginate (Ca-Alg) hydrogel fibers enclosing vascular endothelial cells (ECs) were embedded in collagen gels and subsequently degraded using alginate lyase. The ECs released from the degraded fibers into the resultant channels attached to and covered the surface of the lumen. In addition, we revealed that oxygen and nutrients could be supplied via the tubular network to living hepatic cells immobilized in the ambient collagen gels by perfusing culture medium into the channels.⁴ In these previous reports, the tubular constructs were composed of ECs alone. However, native blood vessels of the same size as the constructs consist of several cell layers, comprising mainly EC and vascular smooth muscle cell (SMC) layers.⁵ The importance of heterotypic cell–cell interactions for the stabilization and proper functioning of native vessels is widely recognized.⁶ Apart from the fabrication of

tubular constructs templated by alginate gel fibers, we successfully prepared double-layered tubular constructs consisting of ECs and SMCs in collagen gels using stainless needles as a template for the tubular constructs.⁷ Briefly, channels were fabricated in collagen gels containing SMCs by inserting stainless steel needles into collagen gels and pulling them out again. Next, the channels were filled with ECs suspended in medium. However, a crucial limitation of this method is that only straight channels can be created. In contrast, the use of flexible hydrogel fibers as templates for the tubular constructs would allow us to fabricate more desirable configurations in collagen gels.

The objective of the present study was to develop alginate-based gel fibers that enable the development of tubular constructs with EC and SMC layers and desirable configurations. Our methodology for this purpose is summarized in Scheme 1. In step 1, we prepare a hydrogel fiber composed of Ca-Alg and gelatin (Ca-Alg/gelatin) from a mixture of sodium alginate (Na-Alg) and gelatin based on the previously described method for fabricating cell-enclosing Ca-Alg gel fibers.⁸ In step 2, we soak the fiber in a solution containing oxidized alginate (AO) to develop a cell-adhesive surface by cross-linking the gelatin with AO. In step 3, SMCs are seeded onto the AO-cross-linked fiber (Alg/gelatin/AO) for the formation of a SMC layer. In step 4, the fiber is embedded in a collagen gel with the desired configuration. In step 5, the embedded fiber is degraded using alginate lyase to form a channel in the collagen gel. AO derived from alginate is expected to be degradable using alginate lyase. As a result of these processes, we can prepare a channel with a SMC layer on the surface of the collagen gel and an EC layer on the SMC layer.

Alginates are naturally occurring polysaccharides that have been studied for a wide variety of biomedical applications.^{9–11} This interest in alginates is primarily due to their high biocompatibility and low toxicity, as well as the relative ease with which they can undergo gelation with divalent cations under mild conditions suitable for biomacromolecules and living

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Scheme 1. Schematic Illustration of the Fabrication of a Tubular Construct with Layered ECs and SMCs in a Collagen Gel

cells.¹² The major drawbacks of alginates are their significantly low cell-adhesiveness and poor support of cell proliferation. In the present study, gelatin was mixed with Na-Alg to enhance the cell-adhesiveness of the resultant constructs. However, gelatin gels obtained via thermo-induced gelation alone gradually dissolve at temperatures around 40 °C, meaning that gelatin gels obtained by thermo-induced gelation alone gradually dissolve at the temperature used for culturing mammalian cells. To suppress this dissolution, several chemical modifications, such as the use of glutaraldehyde and carbodiimides, have been investigated.¹² In general, such modifications are difficult to apply to constructs containing living cells due to the toxicity of the cross-linking agents. In the present study, we used AO to suppress the dissolution of gelatin from Ca-Alg/gelatin hydrogel fibers. AO is obtained by periodate oxidation of Na-Alg.¹³ Balakrishnan et al. reported that AO can function as a cross-linking agent for proteins such as gelatin during the preparation of hydrogels.¹⁴ Although the cell viability immediately after enclosure was not reported, the viability of hepatocytes enclosed in a gel obtained by mixing gelatin and AO in the presence of a small amount of sodium tetraborate was found to increase as the culture period increased.¹⁴ In our system, we continuously prepared EC-enclosing fibers by extruding a suspension of ECs into a coflowing CaCl_2 solution, because gel preparation by mixing gelatin and AO directly was not applicable due to the gradual progression of cross-linking reactions that hinder the continuous production of hydrogel fibers. Therefore, we first prepared Ca-Alg/gelatin hydrogel fibers from a mixture of Na-Alg and gelatin and then cross-linked the entrapped gelatin using AO. In the present study, we evaluated the cytotoxic effects of AO on the viability of ECs and adhesiveness of SMCs in Alg/gelatin/AO hydrogels. In addition, we attempted to prepare tubular constructs with cell layers of ECs and SMCs in collagen gels.

Materials and Methods

Materials. Na-Alg with a high content of guluronic acid (molar ratio of manuronate acid to guluronic acid of 0.65) and a molecular weight of 70000 Da was kindly donated by Kimica (Tokyo, Japan). Gelatin (Bloom 300; type A; MW: 100000) and alginate lyase from *Flavobacterium* sp. were purchased from Sigma (St. Louis, MO). Sodium periodate and collagen type I were purchased from Wako Chemical Co. (Osaka, Japan) and Nitta Gelatin (Osaka, Japan), respectively. Bovine carotid artery ECs (BECs) and aortic SMCs from p53 knockout mice (p53LMAC01 cells; MSMCs) were obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan). BECs and MSMCs were cultured in Eagle's minimum essential medium (MEM; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) and Dulbecco's

modified Eagle's medium (DMEM; Sigma), respectively. Both media were supplemented with 10% (v/v) fetal bovine serum (FBS), 100 mg/L penicillin and 58.8 mg/L streptomycin. The cells were cultured in a humidified atmosphere at 37 °C under 5% CO₂. All the solutions containing Na-Alg, AO, and gelatin were sterilized by filtration through a membrane with 0.20 μm pores before using.

Synthesis of AO. AO was synthesized based on a previously reported method.¹⁴ Briefly, 2.0 g of Na-Alg was dispersed in 10 mL of ethanol. Next, 10 mL of deionized water containing 1.08 g of sodium periodate was added to the solution and stirred at room temperature in the dark for 6 h. The resultant polymer solution was dialyzed against deionized water using a dialysis membrane (MWCO: 12000) for 4 days with several changes of the deionized water. The absence of periodate was confirmed by mixing the final dialysate with the same volume of 1% silver nitrate solution and ensuring the absence of any precipitate. The resultant polymer solution was filtered using an ultrafiltration membrane (MWCO: 30000) and lyophilized. The percentage of oxidized uronic acid residues to the number of uronic acid residues in Na-Alg was quantified using a previously reported method¹⁵ and found to be 20.3%.

Cytotoxicity of AO toward BECs. In our method, BECs were enclosed in Ca-Alg/gelatin hydrogel fibers and then immersed in FBS-free medium containing AO. Therefore, the cytotoxicity of AO toward BECs was evaluated by placing the cells in FBS-free medium containing AO. After culture on tissue culture dishes, the BECs were collected by trypsinization and dispersed in FBS-free MEM containing 8% (w/v) AO at 1.0×10^6 cells/mL at room temperature. The suspension was allowed to stand for 1, 3, or 5 h. Then, viabilities of the cells were determined using a Trypan blue exclusion method. The results were normalized by cells cultured without AO.

Adhesion of MSMCs to Hydrogels. In our method, SMCs were seeded onto Alg/gelatin/AO fibers obtained by soaking Ca-Alg/gelatin fibers in medium containing AO for 1 h. To examine the adhesion of SMCs, three kinds of hydrogel sheets (Ca-Alg, Ca-Alg/gelatin, and Alg/gelatin/AO) were prepared. For Ca-Alg hydrogels, Ca²⁺-free Krebs Ringer Hepes-buffered solution (CF-KRH; pH 7.4) containing 1.0% (w/v) Na-Alg was sandwiched between paper filters soaked in 100 mM CaCl_2 . The resultant hydrogel sheets were approximately 0.5 mm in thickness. Circular hydrogels were punched out from the sheets and placed in six-well tissue culture dishes. For Ca-Alg/gelatin hydrogels, 1 mL of CF-KRH containing 0.5% (w/v) Na-Alg and 5.0% (w/v) gelatin was poured into the wells of six-well tissue culture dishes. After cooling for 30 min at 4 °C, 100 mM CaCl_2 solution was poured onto the hydrogels and allowed to stand for 1 h. For Alg/gelatin/AO hydrogels, 1 mL of FBS-free MEM containing 8.0% (w/v) AO was poured onto the Ca-Alg/gelatin hydrogels and allowed to stand for 1 or 3 h. All the resultant hydrogels were sequentially rinsed with Ca²⁺-, Mg²⁺-free phosphate buffer solution (CMF-PBS), 100 mM CaCl_2 solution and MEM. MSMCs suspended in MEM were seeded onto each hydrogel at 5.2×10^4 cells/cm². After 4 h of culture, the cells adhering to the hydrogels were trypsinized and counted using a hemocytometer.

Production of Hydrogel Fibers. Ca-Alg/gelatin fibers were prepared based on a previously described method for the production of Ca-Alg fibers.⁸ Briefly, CF-KRH containing 0.5% (w/v) Na-Alg and 5.0% (w/v) gelatin was extruded from an inner stainless steel needle of 270, 480, or 940 μm in diameter into 100 mM CaCl₂ solution (4 °C, pH 7.4) flowing in the same direction in a coaxial glass tubule of 3.5 mm inner diameter and 17 cm in length. To produce a cell-enclosing hydrogel fiber, a solution containing BECs at 1.0×10^7 cells/mL was extruded from an inner needle of 480 μm in diameter. The resultant Ca-Alg/gelatin hydrogel fiber was soaked in FBS-free MEM containing 8.0% (w/v) AO for 1 h at room temperature. The resultant fiber cross-linked with AO was then rinsed with CMF-PBS and 100 mM CaCl₂ solution.

Development of Channels with MSMC/BEC Layers. A culture device composed of an acrylic box (internal dimensions, 30 × 6 × 17 mm) and two injection needles (970 μm in diameter) cut at 90° to their axial direction and fixed to the box was designed in our laboratory.³ A BEC-enclosing Alg/gelatin/AO fiber of approximately 500 μm in diameter was passed through the two needles. MEM was poured into the box and allowed to stand for 30 min in an incubator (37 °C, 5% CO₂). After removing the medium, 0.5 mL of MEM containing MSMCs at 1.0×10^7 cells/mL was poured into the box, and the cells were allowed to adhere to the surface of the gel fiber. After 1.5 h, the medium was removed and an additional 0.2 mL of MEM containing MSMCs was poured into the box and allowed to stand for 1.5 h to allow coverage of the fiber surface. The two-step procedure of adding MSMCs was performed for developing the surface with more MSMCs. To suppress cell adhesion to the surface of the acrylic box, the surface was coated with agarose gel before these procedures were carried out. After rinsing with MEM, fresh MEM was poured into the box and incubated for 3 h in an incubator. At this point, almost the entire surface of the fiber was covered by MSMCs. Next, 1 mL of FBS-free MEM containing collagen type I (2.4 mg/mL) was poured into the box. The configuration of the fiber in the collagen solution was manually altered using tweezers, and the device was placed in an incubator. After gelation of the collagen solution, 1 mL MEM containing 20% (v/v) FBS, resulting in 10% (v/v) FBS in the system, and 25 ng/mL basic fibroblast growth factor was layered onto the collagen gel. The fibroblast growth factor was added for enhancing growth of BECs. After 24 h of incubation, the medium on the collagen gel was replaced with medium containing alginate lyase at 0.4 mg/mL. At 1 day after the procedure, the medium was exchanged for medium without alginate lyase. The resultant channel in the collagen gel was rinsed with medium via the two needles fixed to the box.

Histological Examination. BEC and MSMC layer formation was evaluated using 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (Dil-Ac-LDL, Biomedical Technologies, Stoughton, MA), a specific marker for ECs, and Hoechst 33342 (Dojindo, Kumamoto, Japan), a DNA-specific fluorescent dye. Briefly, medium containing 10 $\mu\text{g}/\text{mL}$ Dil-Ac-LDL was injected into the channel after 1 week of culture (i.e., at 6 days after degradation of the fiber). After 4 h of incubation, the channel was rinsed with CMF-PBS. Next, the collagen gel containing the tubular construct was removed from the culture device and soaked in 10% neutral-buffered formalin. The resultant specimen was rinsed with CMF-PBS and soaked in CMF-PBS containing Hoechst 33342 at 10 $\mu\text{g}/\text{mL}$ for 1 h. Cross-sections of the channel were obtained with a cryostat and observed using a fluorescence microscope. Hematoxylin-eosin (H&E) staining was performed on paraffin-embedded specimens.

Statistical Analysis. Data are expressed as means ± standard deviations. Comparisons among groups were carried out using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. Values of $p < 0.05$ were considered to indicate statistical significance.

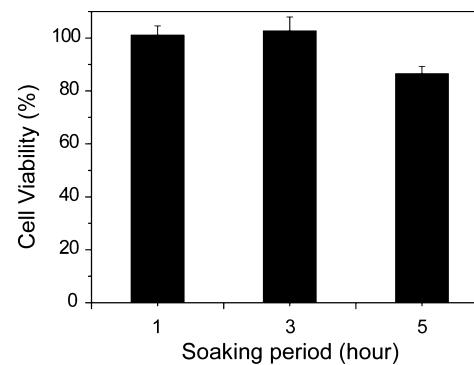


Figure 1. Viability of BECs as a function of the soaking period in FBS-free MEM containing 8% (w/v) AO. Data represent means ± standard deviations ($n = 3$).

Results and Discussion

Polysaccharide derivatives prepared via periodate oxidation of biocompatible polysaccharides have attracted attention as cross-linking agents to obtain hydrogels for biomedical applications due to their lower cytotoxicity than conventional chemical cross-linking agents such as glutaraldehyde and carbodiimides.^{14–17} In the present study, AO was used as a cross-linking agent to suppress the dissolution of gelatin incorporated into EC-enclosing hydrogel fibers prepared from a mixture of alginate and gelatin (Scheme 1, step 2), because gelatin gels obtained via a thermal process alone show a reversible nature at the temperature used for mammalian cell culture. During the cross-linking process, the enclosed cells were exposed to AO diffusing through the gel mixture. It was feared that soaking BECs in a solution of AO would hinder their viability, because cytotoxicity of oxidized hyaluronic acid, carboxymethylcellulose, and dextran toward mesothelial cells and macrophages *in vitro* was previously reported.^{16,17} In the present study, we found that AO showed time-dependent cytotoxicity (Figure 1). Specifically, no reductions in BEC viability were observed after 1 and 3 h of soaking in AO, whereas 5 h of soaking in AO significantly reduced the viability by approximately 15% ($p < 0.01$). The reduction in viability after 5 h of soaking was interpreted as a consequence of the increased cross-linking between proteins on the surface of the cells and AO. The differences in our results for cytotoxicity and those previously reported for oxidized polymers can be attributed to the long soaking period (2 or 3 days) in the oxidized polymer solutions in the previous studies.^{16,17} According to our finding that BECs maintained their viability within 3 h of soaking in 8% (w/v) AO solution, we soaked Ca-Alg/gelatin hydrogels in 8.0% (w/v) AO solution for less than 3 h to cross-link the gelatin in the following experiments.

We investigated the adhesion and proliferation of SMCs on Alg/gelatin/AO hydrogels using MSMCs to evaluate the possibility of SMC layer formation on the hydrogel fibers (Scheme 1, step 3). As clearly shown in Figure 2a, a cross-linked hydrogel prepared by soaking a Ca-Alg/gelatin hydrogel in AO solution for 1 h showed cellular adhesiveness toward MSMCs. The cells attached to and spread on the hydrogel after 4 h of culture. In contrast, the majority of cells seeded onto a hydrogel without AO cross-linking (i.e., Ca-Alg/gelatin hydrogel) remained round and did not spread (Figure 2b). Similar to the case for the low cell-adhesiveness of unmodified alginate gels,¹⁸ almost all the MSMCs seeded onto Ca-Alg hydrogels floated in the medium without adhesion (data not shown). A quantitative analysis of the differences in the degrees of cell adhesion is shown in Figure

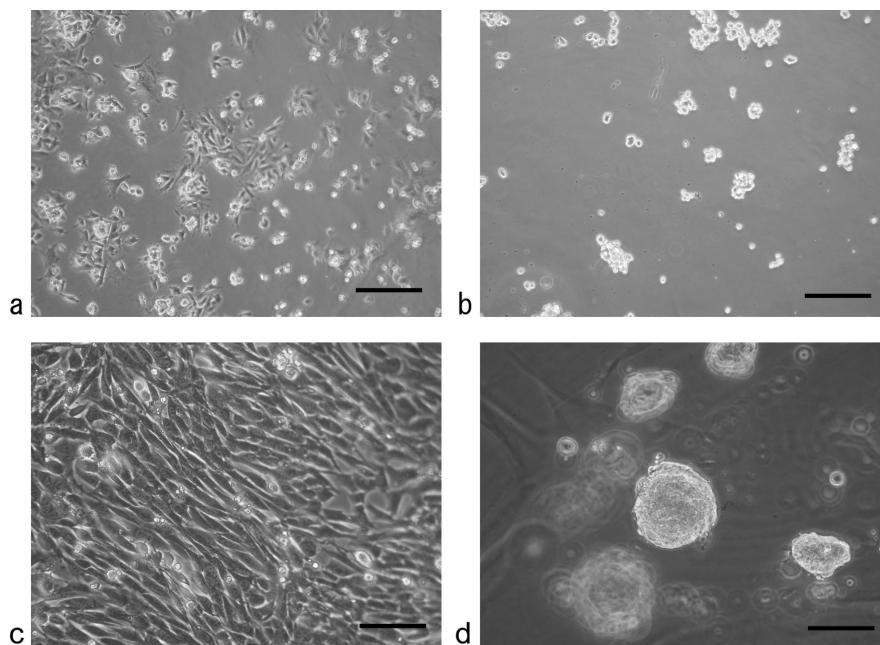


Figure 2. Photographs of MSMCs seeded on (a, c) an Alg/gelatin/AO hydrogel prepared by soaking in FBS-free MEM containing 8% (w/v) AO for 1 h and (b, d) a noncross-linked Ca-Alg/gelatin hydrogel. Incubation periods: (a, b) 4 h; (c, d) 48 h. Bars: (a, c) 200 μ m; (b, d) 100 μ m.

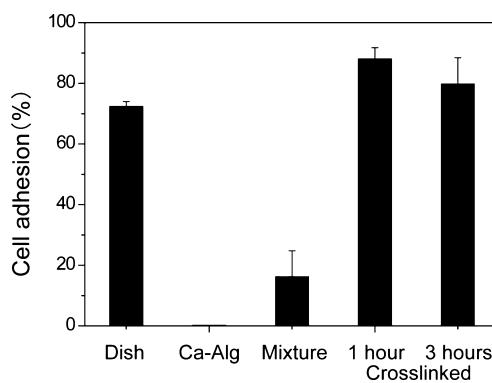


Figure 3. Percentages of adherent cells on tissue culture dishes (Dish), Ca-Alg hydrogels, noncross-linked Ca-Alg/gelatin hydrogels (mixture) and Alg/gelatin/AO hydrogels at 4 h after seeding. Data represent means \pm standard deviations ($n = 3$).

3. More than 80% of the seeded cells attached to the two hydrogels cross-linked by soaking in AO solution for 1 and 3 h after 4 h of culture. There was no significant difference between the values regardless of the soaking period for cross-linking ($p > 0.05$). In contrast, only 16% of cells adhered to noncross-linked Ca-Alg/gelatin hydrogels. Furthermore, even the cells that adhered at 4 h after seeding detached from the surface and formed cellular aggregates during the subsequent culture period (Figure 2d). The low cell adhesiveness and detachment of cells on the noncross-linked hydrogels were interpreted as a consequence of gelatin dissolution into the medium at 37 °C during the cell culture. These results are consistent with previous results for hydrogels prepared from agarose and gelatin via a thermal gelation process alone.¹⁹ Specifically, feline kidney cells seeded onto agarose/gelatin hydrogels exhibited adhesion after 4 h of incubation, but no adherent cells remained on the hydrogels after 24 h of incubation at 37 °C. The adhered cells on the AO-cross-linked hydrogels proliferated and covered the surface after 48 h of culture (Figure 2c). These results indicate that the gelatin in the Ca-Alg/gelatin hydrogels was successfully cross-linked by soaking the hydrogels in AO solution for 1 h. In addition, the cross-linking was effective for developing a hydrogel surface on which MSMCs could adhere and proliferate.

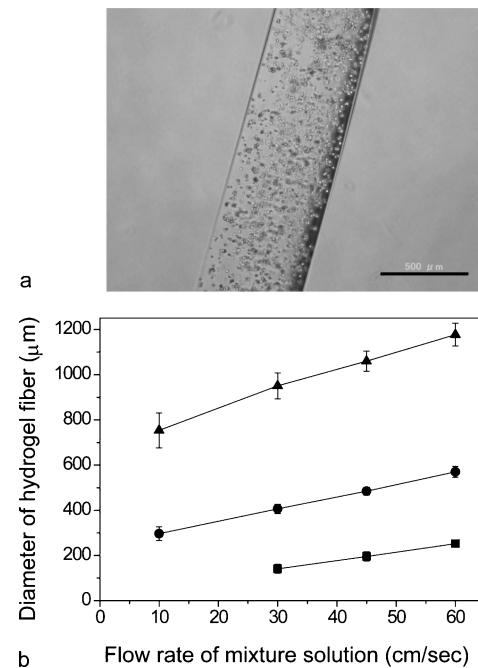


Figure 4. (a) Photograph of a BEC-enclosing Ca-Alg/gelatin fiber. Bar: 500 μ m. (b) Diameters of Ca-Alg/gelatin hydrogel fibers as a function of the flow rate of the Na-Alg/gelatin mixture and the diameter of the needle (270, 480, and 940 μ m in diameter) from which the mixture was extruded. The flow rate of the 100 mM CaCl₂ solution was fixed at 33.9 cm/s.

Based on the above-described results, we attempted to develop hydrogel fibers that enclosed BECs and had their surface covered with MSMCs. We investigated whether we could prepare Ca-Alg/gelatin hydrogel fibers by extruding a solution of Na-Alg and gelatin containing BECs into a coflowing CaCl₂ solution cooled at 4 °C based on a previous method developed for preparing cell-enclosing hydrogel fibers from Na-Alg solution alone.⁸ As shown in Figure 4a, we successfully obtained cell-enclosing hydrogel fibers from the Na-Alg/gelatin solution that ranged in diameter from 150 to 1200 μ m by changing the flow rate of the Na-Alg/gelatin solution and the diameter of the

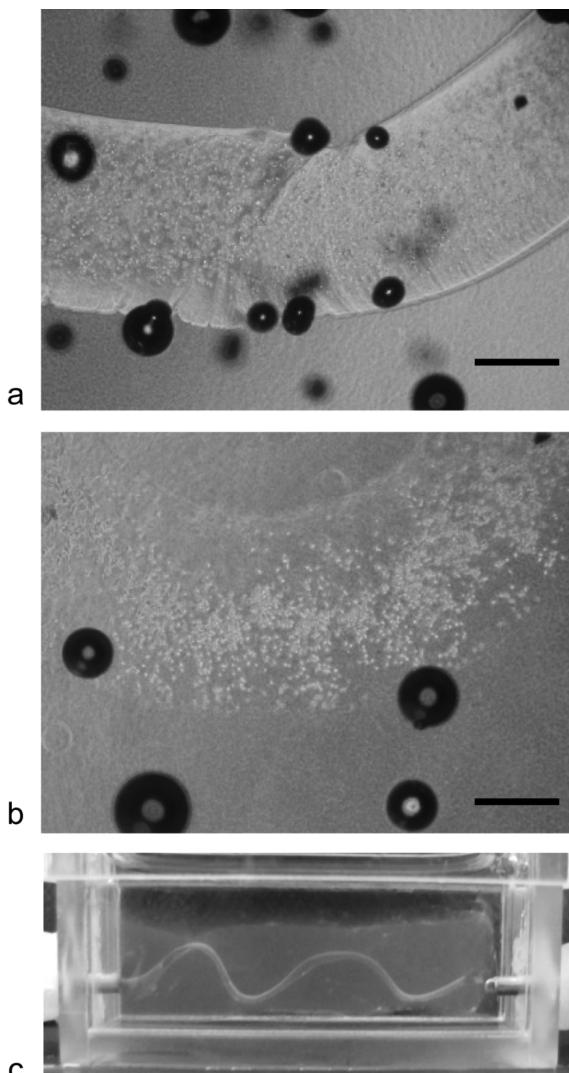


Figure 5. Photographs of a BEC-enclosing Alg/gelatin/AO hydrogel fiber embedded in a collagen gel (a) before and (b, c) after soaking in medium containing alginate lyase for 24 h. (c) The resultant channel has a wavy shape after the degradation. Bars: (a, b) 500 μ m.

needles from which the solution was extruded (Figure 4b). In the following experiments, we used fibers of approximately 500 μ m in diameter and cross-linked them by soaking in FBS-free MEM containing 8% (w/v) AO for 1 h. For these fibers, we studied their degradability with alginate lyase after embedding them in collagen gels, since there are no available reports regarding the enzymatic degradability of oxidized polysaccharide derivatives. The interface between the hydrogel fiber and the ambient collagen gel was visible before soaking in the medium containing alginate lyase (Figure 5a). The interface became difficult to distinguish after 24 h of incubation in the medium containing the enzyme (Figure 5b). We created a flow of medium into the resultant channel by injecting medium from the inlet needle, and confirmed the formation of a channel by observing medium flowing out from the outlet needle. This result clearly demonstrates that the degradability of Alg/gelatin/AO fibers using alginate lyase is the same as that of fibers prepared from Na-Alg alone via cross-linking with Ca^{2+} .³ In addition, we successfully embedded fibers in collagen gels with not only a straight configuration but also a wavy configuration due to their flexibility (Figure 5c). The degradability using alginate lyase and flexibility of Alg/gelatin/AO fibers are hopeful findings for our final goal of developing dense tissues with vascular

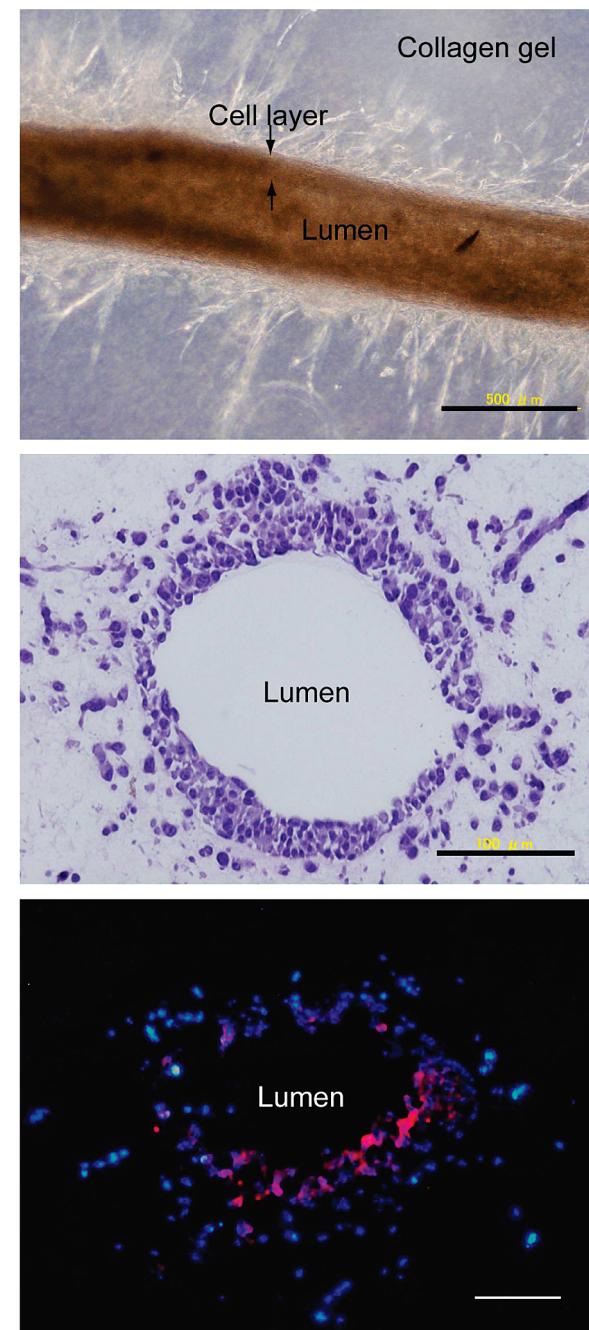


Figure 6. Photographs of (a) a tubular construct with a cell layer in a collagen gel and (b) an H&E-stained cross-section of the tubular construct. (c) Fluorescence microscopic image of a cross-section of the cell layer after treatment with Dil-Ac-LDL (pink) and Hoechst 33342 (blue) at 6 days after fiber degradation using alginate lyase. Bars: (a) 500 μ m; (b) 100 μ m; (c) 100 μ m.

networks to supply oxygen and nutrients to cells within the constructs. To develop such tissues, hydrogel fibers embedded in collagen gels containing living mammalian cells with configurations that enable maximal supply of oxygen and nutrients to the cells are required.

Finally, we attempted to develop channels with MSC and BEC layers by degrading BEC-enclosing Alg/gelatin/AO fibers covered by MSCs using alginate lyase. At several days after the fiber degradation, cell layer formation became visible in the culture device. At 6 days after the fiber degradation, the thickness of the cell layer was about 50 μ m (Figure 6a) and it was comprised of 6–7 layers of cells (Figure 6b). Cross-sections

of the channel stained with Dil-Ac-LDL, a specific marker for ECs, and Hoechst 33342, a DNA-specific fluorescent dye, revealed the existence of a cell layer stained by both dyes on the luminal surface (Figure 6c), indicating the existence of a BEC layer on the luminal surface. These results demonstrate that we can successfully prepare a tubular construct with a BEC layer on MSMCs in a collagen gel by using a flexible Alg/gelatin/AO fiber and degrading it with alginate lyase.

Conclusions

AO was used to immobilize gelatin in cell-enclosing hydrogel fibers prepared from a mixture of alginate and gelatin containing living cells via extrusion of the mixture into a coflowing CaCl_2 solution cooled at 4 °C for the formation of a cell-adhesive surface. The cross-linking using AO was performed by soaking the Ca-Alg/gelatin hydrogel fibers in medium containing 8.0% (w/v) AO. The cytotoxicity of AO due to contact with the dissolved polymer was time-dependent, and no reduction in viability was detected within 3 h of soaking. MSMCs seeded onto hydrogels cross-linked by 1 and 3 h of soaking in AO solution adhered to and proliferated on the hydrogels. Based on these results, we prepared BEC-enclosing AO-cross-linked Ca-Alg/gelatin fibers covered with MSMCs and embedded them in collagen gels. The fibers were successfully degraded with alginate lyase dissolved in medium at 37 °C. The resultant channels had a structure comprised of layered MSMCs and BECs on the luminal surface. Our results indicate that AO-cross-linked alginate/gelatin hydrogel fibers are good candidates for fabricating tubular constructs with layered heterogeneous cell structures in collagen gels containing living cells with the aim of developing dense artificial tissues.

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