Hand-Maneuverable Collagen Sheet with Micropatterns for 3D Modular Tissue Engineering

Jaejung Son, Min Seo Bang, and Je-Kyun Park*  
Department of Bio and Brain Engineering, Korea Advanced Institute of Science and Technology (KAIST), 291 Daehak-ro, Yuseong-gu, Daejeon 34141, Republic of Korea

ABSTRACT: Modular tissue engineering creates a three-dimensional (3D) macroscale tissue construct from modular microscale units for complex 3D tissue reconstruction. In particular, a hydrogel sheet that is one of the module types allows easy and controllable assembly of 3D microenvironments as compared to other module types such as a microcapsule and a microfiber. However, it is difficult to manipulate a hydrogel sheet made of extracellular matrix (ECM) proteins. Thus, in this study, we propose a fabrication technique for the manipulation of thin collagen sheet containing cells with a paper support. A donut-shaped paper support was combined with the micropatterned collagen sheet by permeation of cell–collagen mixture passing through a micropatterned mold into the paper. We established a simple method for the paper support to maintain high cell viability and intrinsic cell morphology and demonstrated an endothelial module having 3D tubular networks. The endothelial module also showed that control of the pattern length resulted in a change in the tubular size and network density within a sheet. The geometrically controlled collagen sheet modules are expected to be used for improved implantation and biologically relevant drug testing.

KEYWORDS: 3D tissue, collagen, hydrogel sheet, micropattern, modular tissue engineering

INTRODUCTION

Modular tissue engineering has been introduced to create a three-dimensional (3D) macroscale tissue construct which is assembled by modular microscale units called modules to build a more physiologically relevant tissue with intrinsic architecture and native tissue complexity. As a building block of the tissue construct, a cellular module can be controlled by cell type, concentration, extracellular matrix (ECM), and so forth. Thus, these modules are necessary for implantation or in vitro drug assay and biological study at the tissue level because they can easily mimic the in vivo tissue geometries by assembling modules with various combinations.

The modules can be classified into two types, depending on the presence or absence of hydrogel. In the case of a module without hydrogel, a tissue construct with high cell density can be constructed using a cell sheet and a spheroid. However, it is better to use the hydrogel medium to control the effects of cell–ECM interactions. Because of its porosity, biocompatibility, and high water content, hydrogel is similar to natural ECM. It can be easily manufactured by various cross-linking methods such as temperature, light, and chemical methods, providing a space and mechanical support that allows cells to grow in a 3D environment. Cell-laden hydrogel module types include microcapsule, microfiber, and sheet. Among them, the sheet type is manufactured in a mesoscale area unit, and it is advantageous to have a simple and controllable 3D assembly possible.

In our previous work, cell-containing modules that mimic the microstructure of liver and pancreatic tissue were fabricated using a micropatterned alginate sheet. Simple manipulation was also possible using an end-cut pipet. However, since alginate has no cell-binding site, it can only be applied to highly aggregated cell types in a 3D environment. The use of hydrogels consisting of ECM proteins such as collagen, matrigel, and fibrin gel allows the growth of cells whose function is affected by 3D spatial organization, such as cardiocytes, fibroblasts, and endothelial cells, but it is difficult to manipulate the hydrogels because they are mechanically fragile. One way to manipulate them is to use a supporting material. Mosadegh et al. presented “cells-in-gels-in-paper” which is a rigid wax-patterned paper array containing cell–hydrogel mixture, but it was difficult to construct a controlled microgeometry. Bian et al. manipulated hydrogel sheets with a controllable architecture using the Velcro tape as a support, but it had a thick thickness (~1 mm) to be used as a
module and had the inconvenience of fixing the Velcro tape onto the mold to make a sheet.30,31

In this study, we propose a simple fabrication technique of manipulable micropatterned collagen sheets. A cell-containing micropatterned collagen sheet with a thickness of 200 μm was fabricated by the aid of paper support and observed for the possibility of manipulation and the cell morphology. In addition, we demonstrated endothelial modules with various dimensions for use in the vascularization of large 3D modular tissue using human umbilical vein endothelial cells (HUVECs). The results suggest that a thin and soft cellular hydrogel sheet can be easily manipulated and cultured for modular tissue engineering.

### MATERIALS AND METHODS

**Cell Culture.** HUVECs were maintained in endothelial basal medium supplemented with the endothelial growth medium bullet kit (EGM-2, Lonza, MD, USA). The mouse embryo fibroblast cell line (NIH3T3) was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% v/v fetal bovine serum and 1% v/v penicillin–streptomycin in a 5% CO2 incubator at 37 °C. The HUVECs and NIH3T3 cells were split every 3–4 days, and the culture medium was exchanged every other day after washing the cells with phosphate-buffered saline (Corning Cellgro, VA, USA). In particular, HUVECs were used for the experiments at passages 3 to 6.

**Preparation of a Paper Support.** A cellulose filter paper (Whatman no. 4, UK), which has a thickness of 205 μm and a pore size of 20–25 μm, was cut with a laser for fabrication of donut-shaped paper supports with an inner diameter of 4 mm and an outer diameter of 6.5 mm. The laser-cut paper support was sterilized by acetone and ethanol dipping with sonication for each 30 min and autoclaving immersed in distilled water and dried in an oven at 65 °C overnight. The steps were repeated three times.

**Micropatterned Mold.** A micropatterned poly(dimethylsiloxane) (PDMS, Dow Corning, MI, USA) mold for collagen sheet patterning was produced via a replica molding of PDMS with a silicon wafer master which was patterned with a SU-8 2100 photoresist (MicroChem, MA, USA) by standard photolithography. The mold has a thickness of 217.3 ± 4.9 μm and (a) a pattern width of 130 or 170 μm and (b) a pattern length of 100, 150, 250, or 500 μm (Figure 1). The autoclaved mold was treated with 50% v/v 2-[methoxy(polyethyleneoxy)6-9propyl]trimethoxysilane, tech-90 (PEG-silane, Gelest, PA, USA) in ethanol for 30 min and washed three times with ethanol and distilled water to prevent the hydrogel adhesion to the mold during harvest. The molds were dried in an oven at 65 °C for 1 h. All molds and paper supports were sterilized with ultraviolet light for 30 min before use.
Fabrication of a Paper-Combined Collagen Sheet. Figure 1 shows the process of fabricating paper-combined collagen sheet to manipulate a pure collagen sheet. The paper support was loaded into the PDMS mold. Then, neutralized 3 mg/mL collagen solution containing HUVECs or NIH3T3 cells at a concentration of $1 \times 10^5$ cells/mL was loaded onto the mold and wetted the paper support in the end. Other concentrations of collagen solution were made by diluting the neutralized 3 mg/mL collagen solution into phosphate-buffered saline. After 30 min of gelation, the sheet was picked up with tweezers and harvested from the mold with the medium therein.

**Collagen Staining.** The collagen sheets with different concentrations were stained with a Picro-Sirius Red Stain Kit (Abcam, UK) containing Picro-Sirus Red solution and acetic acid solution. The sheet was submerged in 800 μL of Picro-Sirius Red solution for 1 h with shaking and rinsed for 1 min in acetic acid solution two times and then rinsed for 2 min in ethanol three times.

**Cell Viability Assay.** Cell viability was assessed by a commercially available live/dead staining method. Cells embedded in the collagen sheet were stained with 4 μM calcein-AM (green; Invitrogen, CA, USA) and 2 μM ethidium homodimer-1 (red; Invitrogen) for 5 min at 37 °C and observed with an inverted microscope.

**Immunofluorescent Staining.** Cells embedded in the sheet were fixed by 4% formaldehyde solution for 10 min at room temperature, and the fixed cells were stained with 40× diluted rhodamine-phalloidin (Invitrogen), 100× diluted Alexa Fluor 488-conjugated anti-CD31 (Abcam), and 20× diluted Alexa Fluor 488-conjugated anti-VE-cadherin antibodies (Invitrogen). Then, they were also stained with 2000× diluted Hoechst 33258 (Invitrogen) for 15 min for counterstaining. The cells were visualized by an inverted microscope and a confocal microscope using z-stack imaging (with a z-axis interval in 1 μm).

## RESULTS AND DISCUSSION

**Fabrication and Harvest of Freestanding Paper-Combined Collagen Sheets.** Freestanding collagen sheets combined with a paper support could be harvested easily from the PDMS mold in an aqueous solution. PEG-silane coating reduced the collagen–PDMS interaction as well as maintained the hydrophilic PDMS surface during the drying process, allowing the stable harvest of collagen sheets with various conditions. Figure 2A–D demonstrates the harvest of collagen sheets embedding 6 μm fluorescent beads from the PDMS molds with a pattern length (as shown in Figure 1) of 100, 250, and 500 μm. As the pattern length became longer, the sheet became more fragile. However, the harvest yield from the mold was not significantly different. The sheets with various collagen concentrations of 1–3 mg/mL could also be harvested from the mold although the sheet at 1 mg/mL showed 70% harvest yield, which was 20% lower than other concentrations (Figure 2E–H). Thus, the sheet-based collagen module has shown that it can be fabricated with various patterns and concentrations.

**Manipulation of a Paper-Combined Collagen Sheet.** The paper supports allow handling of the micropatterned freestanding collagen sheets having a thickness of 200 μm. In an aqueous solution, the paper-combined collagen sheet was well spread out because it was anchored to the paper support (Figure 3A). However, the sheet without the paper support was easily folded depending on the movement of the aqueous solution (Figure 3B). Furthermore, the paper support made it possible to manipulate the collagen micropatterns in sheet form (Figure 3C), although the micropatterns without the paper support were irreversibly folded or tangled and stuck to a handling tool such as tweezers (Figure 3D).

The thickness of the collagen sheets was determined by the thickness of the paper support because the thinner micropatterned part of the sheet than the paper support made easy to tear. The filter paper used as a paper support had a thickness of 205 μm and a pore size of 20–25 μm, which is suitable for typical cell size (10–20 μm). The pore size of the paper support can make an effect on the particle or cell distribution within the collagen sheet. Figure S1 shows the distribution of fluorescent particles having a size of 15 μm within the collagen sheets combined with paper supports that have two different pore sizes of 20–25 and 2.5 μm. The paper with the smaller pore size than the particle size can cause the unbalanced particle distribution between paper support and collagen micropatterns. Thus, if a thinner filter paper with a pore size of 20–25 μm is used, the thickness of a collagen sheet can be reduced to be 155 μm.

**Establishment of the Sterilization Method of Paper Support.** To ensure that the cells could be cultured on a paper-combined collagen sheet, the viability of the cells was checked by a live/dead staining assay. NIH3T3 cells were much less viable when cultured in a collagen sheet combined with a paper support, which was autoclaved without being immersed in water prior to fabrication of the cellular sheet (Figure 4B), than when no paper support was provided (Figure 4A). The difference in viability was apparent only 1 day after culture. However, when the cells were cultured in a collagen sheet combined with a paper support autoclaved with immersion in water (Figure 4C,D), they showed good viability as when no paper support was present. During sterilization, immersion of the paper support in water cleaned the burned edges of the paper support, which was cut with a laser cutter, so that it was able to restore cell viability. In addition, treatment with acetone and ethanol before sterilization facilitated cell proliferation (Figure 4D). The qualitative filter paper used contains a resin to improve high wet strength, which can be removed with organic solvents such as acetone dissolved by ethanol before the sterilization with water.

**Culture of Cell-Laden Micropatterned Collagen Sheets with a Paper Support.** After establishing the sterilization method, we cultured HUVECs within the paper-combined collagen sheets, which had a pattern length of 250 μm to fabricate an endothelial module which can be applied to any modular tissues. During the day, round-shaped cells elongated densely along the patterns of the sheet, and the
collagen patterns were sharply decreased in width due to the cell traction force exerted on the collagen fiber (Figure 5A,B). Although the pattern width gradually decreased from 126.1 to 43.4 μm during the 3 days (Figure 5C), the decrease was not large after the first day of the culture because the daily reduction amount from the first day to the third day was 71.1, 2.2, and 9.4 μm (Figure 5D). Unlike HUVECs, HepG2 human hepatoma cells formed clusters within the collagen patterns, and the aggregates finally fill the patterns after 4 days of culture, although the width of collagen did not decrease significantly (Figure S2). This means that the freestanding collagen sheet can provide a 3D culture environment suitable for each cell type. We also observed the viability and the distribution of HUVECs within the two parts of the paper-combined collagen sheet, which are the micropattern part and the paper support part. Both parts provided a viable 3D environment for HUVECs. However, the micropattern part led HUVECs to form a controlled endothelial network (Figure 5E,G), but the cells in the paper part proliferated randomly with only a partial connection (Figure 5F,H). Furthermore, the paper part hindered daily observation of the cells in the bright field since the transmittance of light is significantly lower than that of hydrogel alone. As shown in Figure 5F,H, the dye remains within the paper, so it may be difficult to observe clearly when fluorescence imaging is performed.

However, it was not possible to manipulate the sheet without the paper via the collagen sheet mixed with more rigid alginate because it showed different morphology of cells in comparison with the pure collagen sheet (Figure S3). After 5 days of incubation, the sheet containing a higher concentration of alginate (1% w/v) prevented HUVECs from elongating, but the sheet with a lower concentration of alginate (0.4% w/v) increased the elongating cells similarly to pure collagen. On the other hand, the more collagen the sheet contained, the easier it was to fold because the rigidity of collagen is much lower than alginate, so it was difficult to manipulate like a collagen sheet without the paper support. Thus, paper-combined collagen sheets provided easily manipulable but more physiological-relevant 3D culture environments for spindle-shaped cells such as endothelial cells and fibroblasts.

Characterization of Micropatterned Cell-Laden Collagen Sheet for the Demonstration of 3D Endothelial Module. A HUVEC-laden collagen sheet was demonstrated as an endothelial module for modular tissue construction. HUVECs showed a 3D tubular structure along the collagen micropatterns (Figure S4). The aligned cells also expressed vascular endothelial cadherin (VE-cadherin) that is localized at cell–cell contact sites and the cluster of differentiation 31 (CD31) that is an endothelial differentiation marker (Figure 6A,B). The connected cells appeared to remodel the collagen structure and condensed into a tubular structure centering the long axis of the patterns. In addition, the endothelial modules with various network densities and tubular sizes could be fabricated depending on the length of micropatterns. The tubular shapes were confirmed in all of the sheets having three pattern lengths of 100, 250, and 500 μm, which had the different branch numbers of 200, 60, and 20 in the same area of 12.6 mm². Figure 6C–E shows the 3D reconstructed images of three endothelial modules with different pattern lengths, and Figure 6F–H shows the cross-sectioned images of the tubular structure within the collagen patterns. After 6 days of culture, the final thickness of all sheets became about 20 μm. However,
Figure 6. Characterization of HUVEC-laden collagen sheets as a 3D endothelial module. (A, B) Green immunofluorescent images of HUVEC-laden sheet with VE-cadherin (A) and CD31 (B) staining and blue nuclei counterstaining. (C–E) 3D reconstructed confocal images of HUVEC-laden sheets with a designed pattern length of 100 μm (C), 250 μm (D), and 500 μm (E). (F–H) Cross-sectioned images of a pattern of (C–E). (I) A graph of pattern width and aspect ratio according to the designed pattern length (n = 3). Red: actin; blue: nuclei. Scale bars are 50 μm (A, B), 100 μm (C–E), and 25 μm (F–H).

variation in the pattern length had an influence on the final pattern width and the aspect ratio of the cross section of the tubular structure. The pattern length of 100, 250, and 500 μm provided the final pattern width of 125.7, 77.4, and 55.5 μm and the aspect ratio of 6.2, 3.8, and 2.7, respectively (Figure 6I). Thus, the tubular structure with a long pattern length showed a round cross section. Although the formation of the tubular structure resembled an early process of vasculogenesis, previous study revealed that collagen remained within the lumens of the endothelial cords formed from the micropatterned construct. The potential limitation is expected to be overcome via biological approaches such as the activation of matrix metalloproteases and the acceleration of vacuole formation or engineering approaches in which a sacrificial layer is inserted into the sheets.32

The endothelial modules are expected to be used for functional tissue fabrication by assembly of the modules. Figure S5A,B demonstrates stacking of the collagen sheets with a pattern length of 100 μm containing HUVEC and HepG2 cells. After 4 days of culture, the stacked collagen sheets could be manipulated as a single construct without any adhesives. HUVEC-laden sheets were also stacked alternately with cell-free collagen sheets (Figure S5C,D). As shown in Figure S5D, three collagen layers were sandwiched between four HUVEC layers, showing the interconnected HUVECs between the layered sheets. The endothelial cells were migrated into the cell-free layers, and all layers were connected each other by the cells, indicating that stacked collagen sheets can be used for vascularized 3D tissue construction. Several researches demonstrated that geometrically controlled vasculature helped rapid anastomosis with the host vasculature and improved the function of the engineered tissue after implantation. Thus, the endothelial cell-laden collagen sheet is also expected to contribute rapid in vivo vascularization of customized modular tissues assembled with functional cell-laden collagen sheets.

■ CONCLUSIONS

In summary, we successfully fabricated a hand-maneuverable thin collagen module with ~200 μm thick by combining a paper support with a micropatterned collagen sheet. The paper support allowed the manipulation of a thin pure collagen sheet (1–3 mg/mL) with a diameter of 4 mm because the cell–collagen mixture soaked through the paper as well as the micropatterned mold and was cross-linked together for 30 min. In addition, to culture highly viable cells within the paper-combined sheet, we established the sterilization method of the paper support by dipping in acetone and ethanol and autoclaving within distilled water. Using the freestanding sheet, we cultured NIH3T3 cells and HUVECs in 3D microenvironments with various dimensions, and they proliferated and elongated well along the micropatterns. In particular, HUVECs formed a tubular network along the patterns regardless of the dimension and the designed pattern length induced the change of the tubular size as well as the network density. Although we demonstrated an endothelial module only, the manipulable and assemblable sheet modules that have various cell types, pattern sizes, and collagen concentrations can be used for 3D tissue reconstruction for functionally improved implantation and biologically relevant drug assays.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsbiomater.8b01066.

Materials and methods for the fabrication of a collagen–alginate sheet without paper support and lamination of cellular collagen sheets; bead distribution depending on the pore size of a paper support (Figure S1); daily culture images of HepG2 cells within a paper-combined collagen sheet (Figure S2); collagen–alginate sheet fabrication for manipulation of the HUVEC culture module (Figure S3); sectioned images of the 3D tubular structure of HUVECs within the collagen sheets (Figure S4); future possibility of endothelial cell-laden collagen sheets for modular tissue construction (Figure S5) (PDF)
AUTHOR INFORMATION

Corresponding Author
*Tel +82-42-350-4315; Fax +82-42-350-4310; e-mail jekyun@kaist.ac.kr.

ORCID

Je-Kyun Park: 0000-0001-4522-2574

Notes

The authors declare no competing financial interest.

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