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Influence of the perfusion bioreactor on Stratified and Distributed approaches for multilayered tissue engineering on biodegradable scaffolds*

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Abstract— **Despite wide use and approval of poly lactic-co-glycolic acid (PLGA) for surgical applications, there have been very few studies on tissue constructions that mimic physiological multilayer structures by combining PLGA scaffolds with tissue engineering. In our study, we developed a bioreactor system to maintain, and to train two types of three-layered vascular-like structures. Then we examined how the perfusion conditions and different tissue engineering approaches affected the formation of the layered structure and degradation of the PLGA scaffolds. In the proposed Distributed Method, the cells were seeded layer by layer on a single scaffold, using spheroids bigger than scaffold fiber gaps and we achieved the higher cell density compared with the Stratified Method where we stacked three PLGA sheets seeded with individual vascular cell types. At the histological level, scaffold degradation was more prominent in the bioreactor compared to the same time interval *in vivo*. In addition, the faster flow accelerated the decomposition of PLGA fibers. Moreover, bioreactor perfusion culture at lower flow rates could balance cell adhesion and survival, improve the cell density and promote self-organization of multilayer structure with desirable rate of PLGA scaffolds degradation.**

I. Introduction

Congenital heart disease is the most common congenital disorder in newborns [1]. During the surgical treatment of congenital cardiovascular disease, vascular patches are often placed to correct various anatomical abnormalities. The patch materials currently dominating the market are ePTFE (expanded polytetrafluoroethylene) and bovine pericardium. However, these existing products have the limitations causing both deterioration and calcification progression over time [2]. These problems often require correction of the hemodynamic disturbance by re-operation to replace the failed material. There are number of other physiological conditions which as well can benefit from flat multilayered tissue implants, like: hernias, the meninges reconstruction, plastic surgery in chronic pleural empyema and cosmetic interventions.

For desirable heart surgery outcome, the biodegradable polymers, extracellular matrices, tissue engineered patches, or combinations of these materials are expected not to be prone to calcification and be amenable as infant growths. The potential benefits, efficacy, and limitations of the various patch materials that could replace biological tissues are being evaluated in a literature [3]. Poly Lactic-co-glycolic Acid (PLGA) is one of the biocompatible and biodegradable copolymer materials; in water-based environment, PLGA degrades by hydrolysis of its ester linkages. This material can be tailored to exhibit shape memory [4] which expect to be beneficial for creating flat/arc/tubular shape. Previously combined PLGA scaffolds with seeded cells and ECM were reported and the cells and ECM load reduced the inflammation caused by PLGA alone and prevented PLGA from earlier degradation [5]. The potential clinical applications of the artificial blood vessels through the hybridization of smooth muscle cells and endothelial cells with PLGA scaffold were investigated using a canine model [6]. The hybridized dual layered blood vessel showed neointimal formation with good patency.

Despite wide use and approval for surgical uses, there have been very few studies on tissue constructions that reproduce physiological multilayer structures by combining PLGA material and tissue engineering. Partially, because PLGA biodegradable scaffolds are gradually dissolving in the media during cell culture, and the appropriate conditions for the bioreactor system required for continuous cultivation have not been established. Therefore, parameters for keeping the balance between cell maturation and scaffold degradation should be established.

In this study, we proposed and developed a bioreactor system which can promote cell growth and maturation in two types of culture approaches to create a three-layered vascular-like structures on the circular PLGA sheets: Stratified and Distributed approaches as shown in Fig.1, examined how the perfusion bioreactor cultivation of different cell types on biodegradable material affects the formation of the layered structure, and compared the degradation of the PLGA scaffolds *in vitro* and *in vivo*.

II. Materials and Methods

A. In vitro biocompatibility

Fibrous sheets which are composed mainly of medical grade Poly Lactic-co-glycolic Acid (PLGA) (ORB-R001 / ORTHOREBIRTH Co., Ltd., Kanagawa, Japan) were used as a biodegradable scaffold. The size of the PLGA sheet was 23 mm in outer diameter and about 0.15-0.2 mm in thickness. The fibers design contains non-uniform perforating gaps, ranging in size from tens to hundreds of micrometers. To evaluate the

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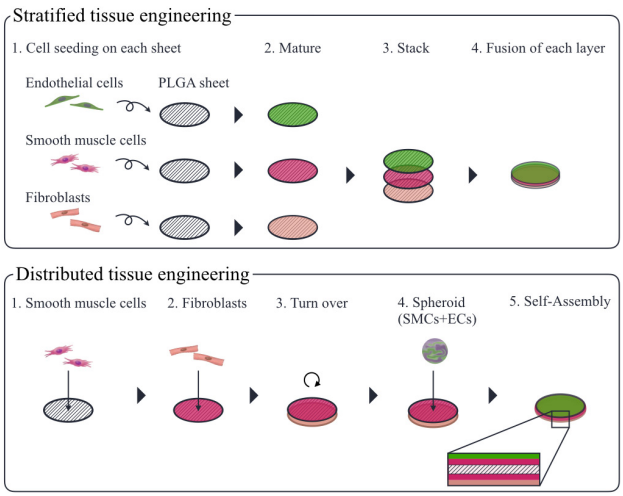

Figure 1. Proposed cultivation methods for Stratified and Distributed tissue engineering.

in vitro biocompatibility of the scaffold, fibroblasts seeded on a sterilized PLGA sheet were cultivated in DMEM media with 10 % fetal calf serum without antibiotics for 10 days, and then stained with LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probes / Thermo-Fischer) for measuring cell viability. The fluorescent microscopic images were digitized with ImageJ software, and the cell viability was estimated by averaging the values from 5 separate fields of view.

B. Animal handling and surgeries

Adult female rats were purchased from local supplier and kept under 23 ℃ 12h/12h light/dark cycle. At the time of surgery, the rats were 18 months old with mean weight 350 g. To assess the short-term biodegradability of the scaffold as a pilot experiment before the bioreactor cultivation, we implanted 15x8 mm naïve scaffold sheet subcutaneously into the rats under isoflurane anesthesia. The animals received non-steroid anti-inflammatory drugs and antibiotics for first 5 days. 2 weeks post-surgery rat was sacrificed and the PLGA sheet with surrounding tissues (skin, connective and adipose tissues) was aseptically dissected under general anesthesia. Care of the animal followed University and national guidelines including minimization of animal use. Experimental procedure was approved by Animal Care Committee of Tsukuba University.

C. Titanium frame

We developed a titanium housing frame to fit the PLGA sheet size (Fig.2). The frame allows (1) to seed the cells on PLGA sheets without lateral escape, (2) to stack the sheets in the Stratified Method, and (3) could be transferred into the bioreactor for long time cultivation. A titanium mesh with a wire diameter of 0.10 mm and an inter-wire gap of 0.15 mm

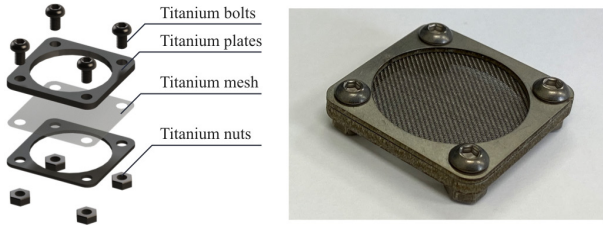

Figure 2. Titanium frame design.

was sandwiched between titanium plates. When a PLGA sheet(s) is/are placed on top of the mesh, the media was able to access both the upper and lower surfaces. The frames were sterilized by autoclavation.

D. Bioreactor design

Previously we have developed a bioreactor system to promote cell maturation and tissue self-organization [7]–[9]. Our bioreactor system consists of a control unit, an autoclavable 4-channels perfusion pump unit (WPM2-P3EA-CP, Welco), a gas exchange unit, and shared media reservoir. Every component except the control unit was sterilized by autoclaving. The size of entire system is 30 cm length, 20 cm width, and 17 cm height, and whole device was installed inside a CO2 incubator during entire culture period. The maximum discharge flow rate of the pump cassette can be changed according to the diameter of the tube to be set inside, in this case 5 ml/min. Moreover, we proposed and adapted the bioreactor system to satisfy the following required specifications: (1) design allowing access for growing tissue and inserting titanium frames after seeding cells and stacking PLGA sheets. (2) resealable scheme to prevent fluid leakage during perfusion culture (compatible with internal accessibility). (3). Imitation of the physiological shear and pulsatile flow for the flat tissues. (4) The entire system could be installed onto the inverted microscope stage for live fluorescent cell imaging using RFP and GFP.

The developed chamber consists of a glass dish, a sealing cap, and a base pedestal to stabilize the titanium frame inside (Fig.3A, B). The sealing cap is made of PDMS silicon and has a media inlet and an outlet on both sides connecting to the media reservoir and circulation pump of the bioreactor via the silicon tube with luer connectors. As shown in Fig.3C, if the lower surface of the cap is straight, some air bubbles are generated in the chamber due to atmospheric pressure

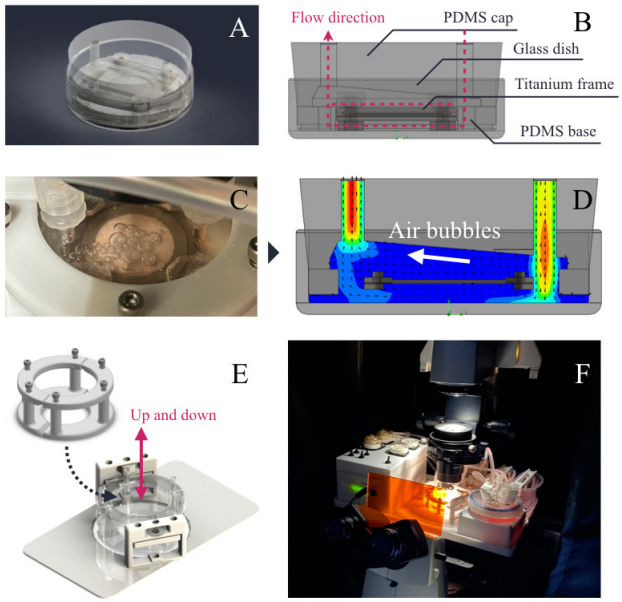

Figure 3. Bioreactor Design. (A) CAD model of the developed chamber. (B) Chamber configuration and internal structure. (C) Air accumulation on top of the PLGA scaffold. (D) Air bubble removal structure. (E) Clamp-type housing to keep the assembly sealed, yet optically accessible. (F) Whole bioreactor system with two chambers installed on the microscope stage.

fluctuations and accumulated on top of the PLGA sheet. To avoid both cell damage and uneven mass exchange, we designed the sealing cap with a slanted angle from the inlet to the outlet to flush the air bubbles toward the outlet continuously (Fig.3D).

The chamber is sealed by press-fitting the PDMS cap into the glass dish. Since the cap and the glass dish are not fixed providing access and allowing the maintenance of the tissue, a force is generated in the direction of loosening the press fit. In order to prevent leakage in such cases, we developed a clamp-type housing that can be easily attached and detached by using several compression springs (Fig.3E). The whole chamber with this housing can be lifted at the center of the bioreactor system, allowing observation of the tissue without taking it out at any time during the culture period using an inverted microscope (Fig.3F). This reduces the risk of contamination by periodical opening and closing the chamber and keeps physiological condition during the perfusion culture.

E. Cell culture with a scaffold

We used 3 types of cells: Fibroblasts (NHDFc, C-12302, PromoCell, Heidelberg, Germany), Smooth muscle cells (hASMCs, cAP-0026RFP, Angio-Proteomie, Boston, USA), and Endothelial cells (HUVECs, cAP-0001GFP, Angio-Proteomie, Boston, USA). Frozen stock of each type of the cells was melted and expanded in appropriate media: D-MEM (High Glucose with Phenol Red and Sodium Pyruvate, StemSure®, Wako) supplemented with 10 % of fetal calf serum for smooth muscle cells and fibroblasts and Endothelial media (Endothelial Cell Growth Medium, PromoCell) for endothelial cells in 75 cm^2 flasks (Primaria, Corning, NC, USA). Before co-culture cells were adapted for 1:1 mix of the above media for 72 hr. For layer formation cells were detached from the surface of their housing flasks with Trypsin/EDTA solution (Wako, Japan), and washed with its used cultured media filtered through 1.2 um membrane filter.

We have tested two methods of forming a 3-layer structure on a scaffold according to the Fig.1 schemes. In the Stratified Method, three separate sheets were used, and each sheet was seeded independently with an individual type of cell. Then three sheets were stacked together into single the titanium frame. In the Distributed Method, cells were seeded sequentially on a single sheet. Smooth muscle cells were first seeded as a middle, growing through the scaffold layer, and then sandwiched between fibroblasts and endothelial cells layers on both sides. Since endothelial cells do not have strong adhesive properties, we prepared them in form of the mixed spheroids with smooth muscle cells. 100µl of the mixed cell suspension (10^4-10^5 cells/ml) was placed into each well of 96-well-plates (MS-9096V / SUMITOMO BAKELITE CO., LTD.) and left overnight in CO_2 incubator at 37 ℃. The average diameter of formed spheroids was about 120 µm (Fig.6A), and they were seeded on top of the smooth muscle cell side of the PLGA sheet. Each sheet was matured in static culture for 1 week.

F. Maturation in the bioreactor

Titanium frames with PLGA sheets and attached cells were placed into two individual chambers of the bioreactor, a sealing cap was press-fitted, air removed, and the culture process started at the same time. The chambers were perfused with individual flow rates of 0.5 ml/min and 5.0 ml/min, respectively, while the faster flow rate was achieved by gradually increasing from the initial 0.5 ml/min to the maximum speed during 3 days to prevent the detachment of non-trained cells. Both the Stratified Method and the Distributed Method were cultured for 2 weeks, and live cell imaging was performed by fluorescence observation during the culture period in the bioreactor and after the culture was completed.

G. Histological analysis

Samples of tissues formed in bioreactor and *in vivo* were immediately fixed with 4 % paraformaldehyde in phosphate buffer saline for 20 min at 37 ℃, stored in 0.5 % paraformaldehyde in the same buffer. The fixed tissues were sent to external processing (Biopathology Institute Co. Ltd., Oita, Japan) for sectioning and staining. HE stained images were taken with BZ-9000 BioRevo microscope (Keyence).

III. Results

A. In vitro biocompatibility

Fibroblasts attached well to PLGA fibers, spread along the individual fibers, sometimes forming clusters and connections between the individual fibers. Staining with Calcein AM revealed that most of the cells remain metabolically active (Fig.4). Percentage of live cells estimated as 92.3 ± 3.35 % by co-staining nuclei with Ethidium Homodimer-1.

B. Stratified tissue engineering

Before stacking three PLGA sheets, attachment of endothelial cells and smooth muscle cells on the scaffolds were additionally confirmed (Fig.5A, B). Both cell types were mainly aligned along the fibers, filling the gaps between the fibers with some cells. Sheets were assembled into the holding frames according to the scheme (Fig.1) After maturation by the bioreactor, the PLGA sheet stack was extracted out of the chamber, cut, and the cross section was observed by live fluorescent microscopy (Fig.5C). Although the three sheets consolidate in the original order, we could see that some endothelial cells and smooth muscle cells have detached and fallen into the fibroblast layer. Presumably, those cells detached during the scaffold decay or cell divisions and felt into the lower levels by gravity, where reattached. The bonds between the sheets were rather weak.

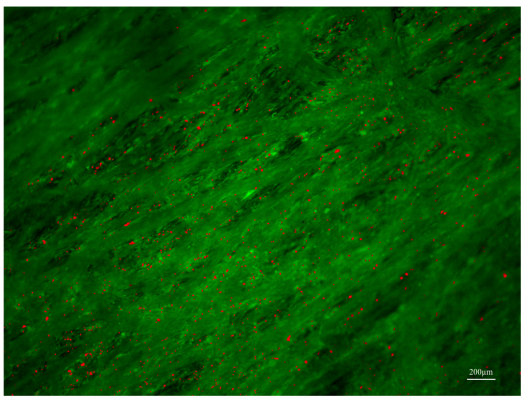

Figure 4. Survival of cells on a PLGA sheet stained with Calcein-AM and Ethidium.

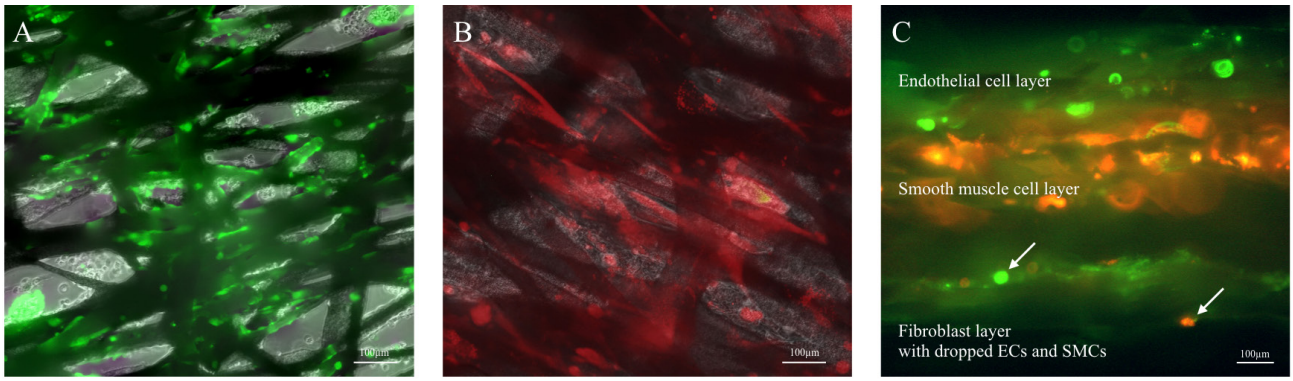

Figure 5. Fluorescence images in Stratified tissue engineering. (A) GFP endothelial cells and PLGA sheet before stacking. (B) RFP smooth muscle cells and PLGA sheet before stacking. (C) Three stratified sheets after maturation in the bioreactor. White arrows: dropped endothelial cells and smooth muscle cells.

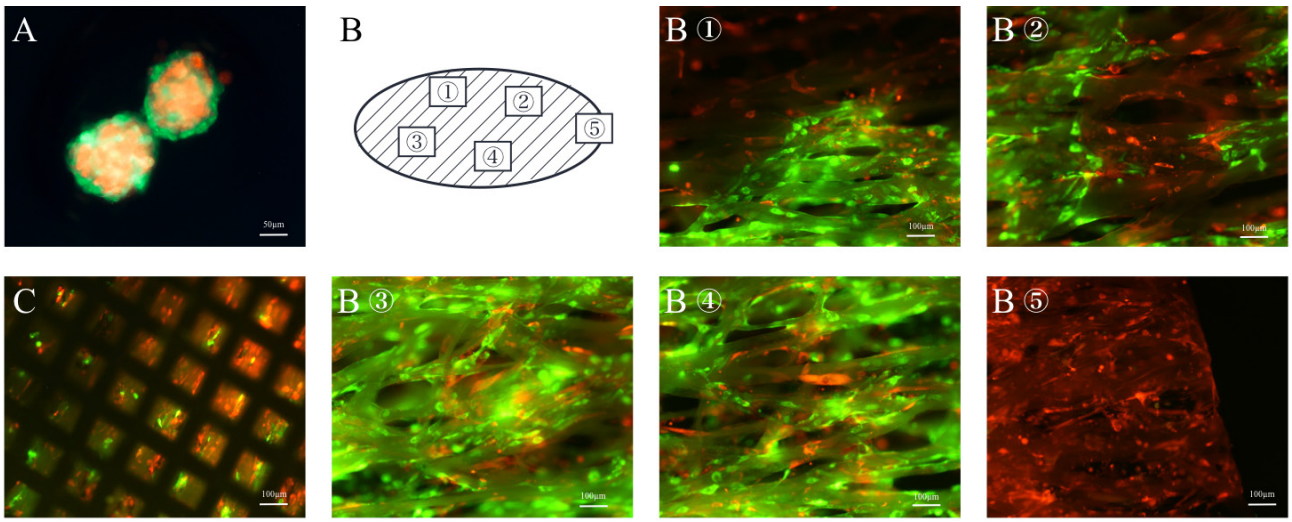

Figure 6. Fluorescence images in Distributed tissue engineering. (A) Mixed spheroids of GFP HUVECs and RFP smooth muscle cells. (B) Cell distribution at different positions after spheroid disintegration on PLGA scaffolds. (C) The inverse side of PLGA sheet taken from the back of the titanium mesh.

C. Distributed tissue engineering

Fig.6B ①~⑤ display the cell distribution at different positions on the scaffold. Initially seeded RFP-SMC layer spread almost evenly throughout the scaffold. Fibroblast layer cannot be seen directly by fluorescent microscopy and analyzed later after histological staining. The GFP labeled endothelial cells seeded in mixed spheroids vary in fluorescent intensity depending on the location, although their layer was mainly distributed on top of SMCs layer. Fig. 6C is the inverse live view of PLGA sheet taken through the back of the titanium mesh during cultivation in bioreactor, and it is suggesting that some rare endothelial cells have passed through the gaps between the fibers and moved to the opposite side of the scaffold or actively migrated.

D. Histological analysis

HE-stained images of the tissue at each flow rate by Stratified and Distributed Methods are shown in Fig.7. The scaffolds (Fig.7E, F) were sandwiched between filter papers on both sides to stabilize the specimen preparation. We marked the remaining PLGA sheet material with yellow on cross sections (transparency 30 %). We calculated the average area of fibers in 5 images after bioreactor maturation at each different flow rate: the area of PLGA scaffolds at a flow rate of 5.0 ml/min compared to 0.5 ml/min decreased by 56 % in the Stratified method and by 45 % in the Distributed method ($p<0.01$). The cell density was highest when the cells were cultured with a single sheet scaffold at 0.5 ml/min, and cell order was preserved the best compared to other experimental conditions.

A representable HE-stained image of the surrounding tissue of the PLGA sheet implanted in the rat body is shown in Fig.8, and the PLGA area was also marked in yellow. Most of the scaffold *in vivo* remained undissolved compared with scaffolds after maturing in the bioreactor. Although cell adhesion was seen on the entire surface, infiltration of the scaffold with cells was minimal.

IV. Discussion

There are a few studies on PLGA degradation rate [10]–[12] including computation modelling [13], however, most of the researchers are still rely on empirical data from scaffold manufacturer or their own previous experience. In our study, scaffold degradation was more prominent in bioreactor

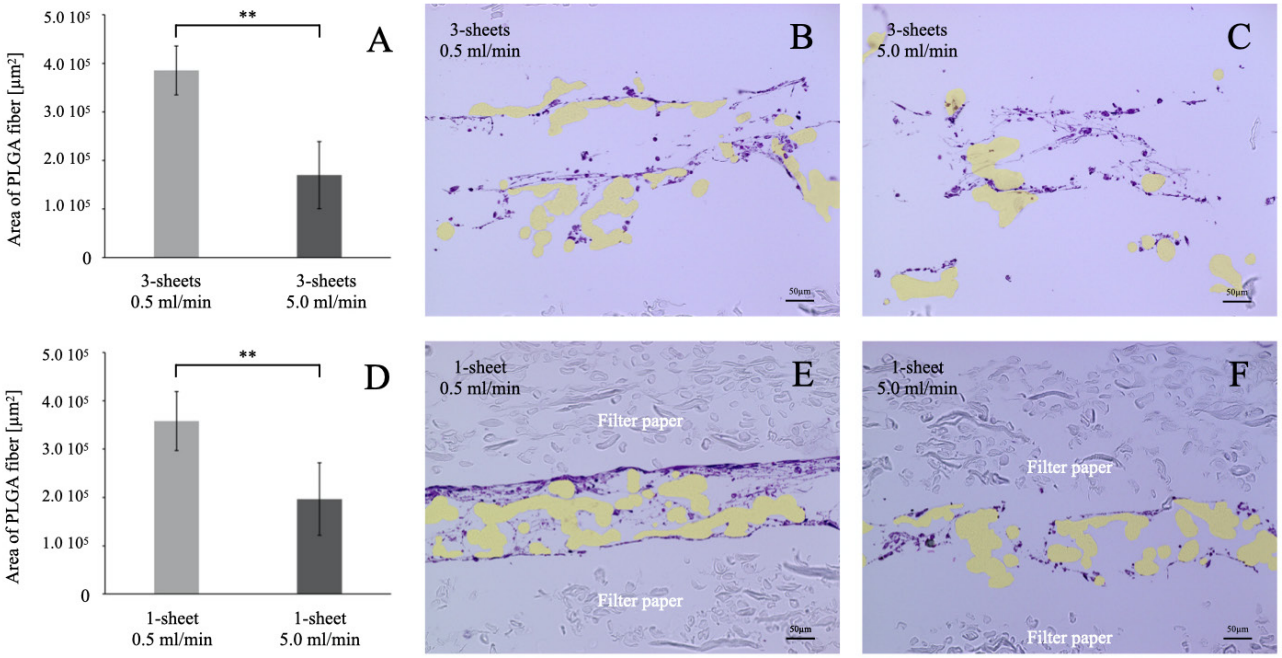

Figure 7. HE-stained tissue image at each flow rate by both methods, and calculation of scaffold area. (A) Area of PLGA fibers (yellow) at different flow rates in the Stratified method (B, C). (D) Area of PLGA fibers (yellow) at different flow rates in the Distributed method (E, F). **p < 0.01 by two-tailed t-test with an alpha level of 0.05. The error bar relates to the standard deviation of the mean.

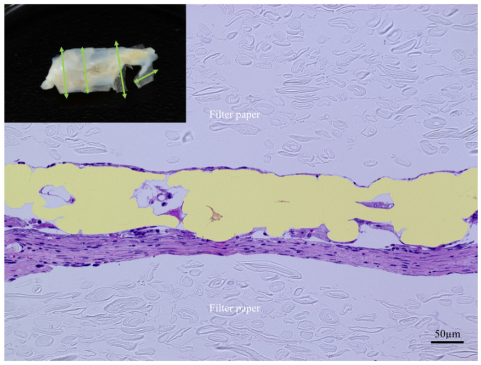

Figure 8. HE-stained image of the surrounding tissues of the PLGA sheet after implantation in the rat, Yellow: remaining scaffolds.

compared to the same time interval *in vivo*. It could be explained by higher mass exchange in bioreactor and more aggressive cell infiltration during seeding of the cells compared to slower migration of cells in a body. In addition, the fast flow accelerated the decomposition of PLGA fibers. A supply of media at lower (0.5 ml/min) rate could maintain cell survival, adhesion and the layered structure without over-degrading the fibers in the single scaffold, but a slightly slower flow rate might be acceptable as long as it can maintain cell viability.

Earlier works demonstrated that cell viability was inversely related to degradation rate of PLGA scaffolds and was dependent on the depth from the seeding (upper) surface toward the lower surface. Their pH measurement showed time-dependent decrease in pH in the PLGA scaffolds, corresponding to PLGA degradation, and closely related to cell viability [11]. These effects were not significant in our experiments, perhaps due to pH stabilizing effects of bioreactor cultivation and prompt removal of the degradation products from cells' vicinity.

Loading of the cells onto surface with non-flat topology containing empty areas in vertical projection led in our experiments to differences in resulting cell densities between the scaffold areas and to escape a portion of the cells through the scaffold. Each next cell layer tended to form more non-even areas. Previously, studying PLGA-based scaffolds manufactured using textile technologies researchers concluded that final cell density was rather little dependent on the initial seeding cell density and in all cases was lower than cell density in conventional 2D culture [14]. Since the Stratified method used three sheets, the density of cells covering each sheet was relatively low. As a result, a larger surface area was in direct contact with the media, which dissolved the fibers excessively, causing mainly endothelial cells to drop out from its position on the scaffold. Although the porosity of the scaffold is important for the tissue engineering, the increase in surface area due to increased porosity has to be considered when perfusion culture is performed *in vitro* using biodegradable materials.

Fluorescent microscopy results indicate that the spheroids with the diameter bigger than the average gap size in the scaffold were useful for improving the cell density at the initial seeding, however, the lateral distribution of cells on the scaffold reflected how even the distribution during seeding was. Robotizing the seeding and the incubator loading is expected to solve these problems in future. Regarding the layers' distribution, the endothelial cells from the mixed spheroids moved and consolidated on top of the smooth muscle cells and, at lower extend, escaped to the lower part of the scaffold presumably through the gaps. Further investigation how the setting of fiber gap patterns or the gap sizes modification affect the layer formation would lead to

more precise control of the self-organization in the multilayered patch construction. Ultimately, knowing the dynamics of the scaffold degradation, of even facilitating the degradation enzymatically [15] could lead to the transition from scaffold-based tissue engineering to scaffold-free synthetic tissue formation.

V. Conclusion

To examine how the perfusion conditions and different tissue engineering approaches affected the formation of the layered structure and degradation of the PLGA scaffolds, we developed a bioreactor system to fabricate and maintain two types of three-layered vascular-like structures. The bioreactor maintained a sealed physiological environment and allowed for live cell imaging using fluorescent protein labeling during entire culture process. Stratified approach showed neatly divided multilayer structure, but the fibers dissolved excessively causing cells to drop out from its position on the scaffold. Distributed approach achieved the higher cell density, and at lower flow rate displayed better balance between cell adhesion and their survival with desirable rate of PLGA scaffolds degradation, promoting self-organization of multilayer structure.

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