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Fabrication of perfusable vasculatures by using micromolding and electrochemical cell transfer

Tatsuya Osaki, Takahiro Kakegawa, Naoto Mochizuki, and Junji Fukuda, Member, IEEE

Abstract—**Fabrication of vascular networks for the delivery of oxygen and nutrients is a critical issue when engineering 3-dimensional tissues and organs. This study describes an approach that involves micromolding and electrochemical cell transfer and can be employed to fabricate endothelial cell-lined vascular-like structures that are precisely aligned at micrometer intervals in a photocrosslinkable gelatin hydrogel. Subsequent perfusion culture induces the migration and sprouting of endothelial cells in the hydrogel and facilitates luminal structure formation.**

I. Introduction

The generation of perfusable microvascular networks is a crucial obstacle to engineering complex 3-dimensional tissues and organs, such as the liver and pancreas. Because oxygen is supplied by diffusion, cells located more than a few hundred micrometers away from the surface of tissue constructs suffer from hypoxia and apoptosis. Therefore, fabrication of spatially controlled capillaries is essential in tissue engineering and regenerative medicine.

Most previous vascularized-tissue engineering approaches have relied on vascularization from host vasculature after transplantation. These methods have utilized growth factor-conjugated scaffolds and genetic modification of cells to promote vascularization. Recent approaches have demonstrated that *in vitro* coculture with endothelial cells leads to the formation of vascular networks in thick tissues and that such prevascularization improves the subsequent *in vivo* performance of the graft tissues. These studies showed that a part of the vascular network anastomosed with the host vasculature. However, the drawbacks of these previous approaches are the inhomogeneous distribution of the network and insufficient blood flow to supply oxygen and nutrients throughout larger tissue constructs, thereby limiting their use to small and thinner tissues. Another approach is to fabricate uniformly aligned and perfusable microchannels in a cell-encapsulated hydrogel. In this approach, endothelial cells are subsequently seeded onto the microchannels. Although this results in endothelial cell-lined perfusable vascular structures, the oxygen and nutrient supply must be stopped for more than a few hours for cell adhesion in the seeding processes, which could cause necrotic cell death especially when parenchymal cells are seeded in a hydrogel at a high cell density.

Tatsuya Osaki, Takahiro Kakegawa, Naoto Mochizuki, and Junji Fukuda are associated with the Graduate School of Pure and Applied Sciences, University of Tsukuba, Japan and Faculty of Engineering, Yokohama National University, Japan. (Corresponding author: Tel: +81-29-853-4995, Fax: +81-853-4490, E-mail: fukuda@ims.tsukuba.ac.jp).

In this study, we demonstrate the benefits of our electrochemical cell transfer approach for rapid fabrication of vascular-like structures. We have previously reported that cells attached to a gold surface via self-assembled monolayers of alkanethiol molecules and oligopeptides are detached within 5 min along with electrochemical desorption of the molecular layers (Fig. 1) [1, 2]. Transferring cells from gold-coated rods in a hydrogel was also useful for the fabrication of vascular-like structures in which the internal surface was covered with human umbilical vein endothelial cells (HUVECs) (Fig. 2). When the structures were fabricated in collagen gel, subsequent perfusion culture induced sprouting of endothelial cells and vascular network formation. However, the mechanical strength of the collagen gel was not sufficient to support the constructs over a few weeks. When a photocrosslinkable gelatin hydrogel (GelMa) was utilized instead of collagen, the appropriate stiffness was achieved; however, the HUVECs did not migrate into the hydrogel. Therefore, there is the trade-off between the mechanical strength of a hydrogel and the migration of cells.

To reduce this problem, in this study, we cocultured HUVECs with fibroblasts on gold rods and fabricated vascular-like structures composed of 2 cell types to accelerate the sprouting of HUVECs in a sufficiently stiff hydrogel. This was then utilized to fabricate thicker vascularized tissues by using multiple 3-dimensionally aligned needles. This simple approach could potentially be a fundamental method for engineering surgically transplantable, vascularized, thick tissues and organs.

II. Methods

A. Oligopeptide design for electrochemical cell transfer

An oligopeptide, CGGGKEKEKEKGRGDSP, was designed to mediate cell adhesion and subsequent electrochemical cell detachment from the gold surface. The oligopeptide consists of a cell adhesion domain (RGD) and a cysteine (C). Cysteine has a thiol group that spontaneously adsorbs onto gold surfaces via a gold-thiolate bond. We expect that these oligopeptides form a closely packed self-assembled monolayer on the surface via the electrostatic forces of the alternating charged glutamic acid (E) and lysine (K) residues between neighboring oligopeptide molecules. Due to the ionic salvation of this alternating sequence, the modified surface is resistant to nonspecific adsorption of proteins. In contrast, cells adhere to the surface via the RGD sequence. The gold-thiolate bold can be cleaved by applying a negative potential, and then the cells adhering

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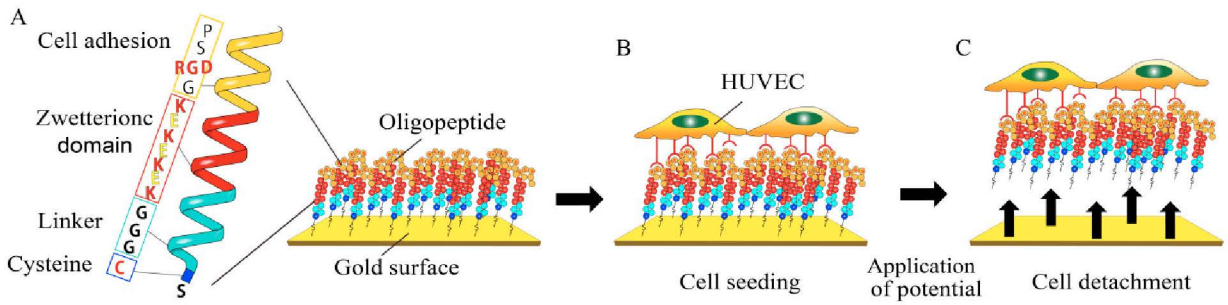

Figure 1. Electrochemical detachment of cells along with reductive desorption of zwitterionic oligopeptide. (A) Oligopeptide layer attached to the gold surface. (B) Cells adhering to the gold surface via the oligopeptide layer. (C) Electrochemical detachment of the cells along with the oligopeptide layer.

to the surface will detach along with the desorption of the oligopeptide.

A gold-coated surface was modified by immersing it in a 0.5 mM oligopeptide solution for 12 h. The amount of adsorbed oligopeptide was determined with a quartz crystal microbalance. The electrical potential required for cleavage of the gold-thiolate bond was previously determined by cyclic voltammetry measurements to be −1.0 V (vs. Ag/AgCl). After seeding cells on the oligopeptide-modified surface, the relationship between potential application time (−1.0 V vs. Ag/AgCl) and the number of remaining cells on the gold surface was determined.

B. *Photocrosslinkable gelatin hydrogel*

Due its advantages of quick gelation and biocompatibility, photocrosslinkable gelatin methacrylate hydrogel (GelMa) has been employed for tissue engineering. The primary amine group of gelatin was reacted with methacrylic anhydride to make it photocrosslinkable. After dialysis in 12–14-kDa cutoff dialysis tubing, the GelMa was lyophilized to yield white porous foam. A GelMa solution for cell encapsulation was prepared by mixing 5% (w/v) GelMa and 0.5% (w/v) of a photoinitiator (Irgacure 2959) in phosphate-buffered saline. The solution was gelled by light exposure (380–405 nm, 6.9 mW/cm^2) for 90 s.

C. *Multicapillary needle*

A multicapillary needle composed of 9 needles was used (Fig. 2). The needles were 500 μm in diameter and spatially aligned in a 3 × 3 arrangement with 500-μm interspaces. The multicapillary needle was sputter-coated with gold and modified with the oligopeptide as described above.

D. *Culture chamber*

A culture chamber (Fig. 2) was fabricated using a computer-aided laser machine with a poly(methyl methacrylate) plate (thickness, 2 mm). The volume of the chamber was approximately 1.5 mL. To guide the needles, 9 pairs of holes were bored through the plates for the inlets and outlets.

E. *Fabrication of perfusable vasculatures*

A schematic of the steps in the fabrication of capillary-like structures is presented in Fig. 2. The oligopeptide-modified multicapillary needle was sterilized with 70% ethanol. HUVECs were then seeded on the surfaces of the needle and cultured in EGM-2 supplemented with VEGF. After the cells were grown to confluence, the needle was fixed in the culture chamber and 1 mL of GelMa solution was poured into the chamber (Fig. 2B). The solution was gelled by UV irradiation (6.9 mW/cm^2) for 90 s (Fig. 2C). After applying a potential of −1.0 V (vs. Ag/AgCl) for 5 min, the needle was extracted from the chamber. Then, the chamber was connected to a pump, and culture medium was perfused at a rate of 10 μL/min per channel.

F. *Cell orientation and polarity*

To evaluate whether the HUVECs that were transferred onto the internal surface of microchannels could respond to the shear stress generated by medium flow, phase-contrast images were taken on day 0 and day 14 after cell transfer. The images were then binarized and converted to a power spectrum through 2-dimensional Fourier transform. The

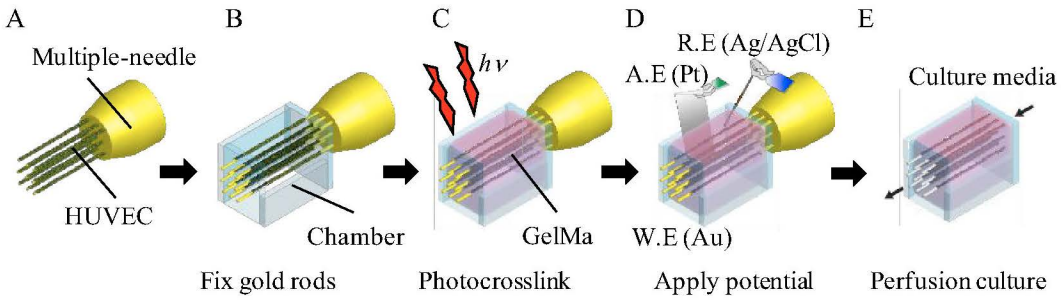

Figure 2. Fabrication of vascular-like structures in GelMa by using electrochemical cell detachment from multiple gold needles.

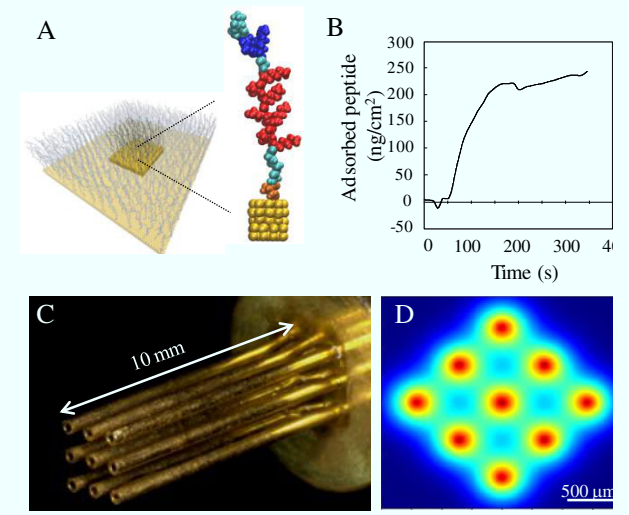

Figure 3. (A) Expected configuration of oligopeptides adsorbed on the gold surface. (B) Adsorbed amount determined by QCM. (C) Multicapillary needle. (D) Oxygen concentration in hydrogel calculated by convection-diffusion equation.

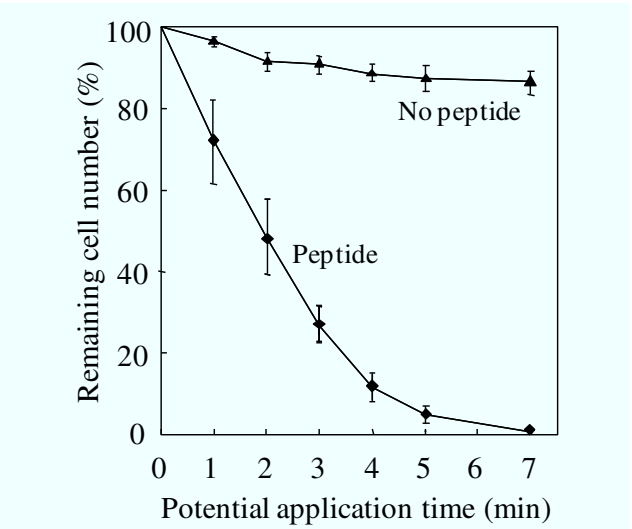

Figure 4. Change in the number of HUVECs remaining on the gold surface. Almost all the cells detached within 5 min of application in the presence of the oligopeptide (◆), whereas few cells detached from the surface in the absence of the oligopeptide (▲).

power spectrum was then converted to a histogram in polar coordinates to obtain orientation intensity, which is the ratio of the short to long axes of the histogram.

Cell polarity is an important characteristic of cells, particularly endothelial cells, which function in a spatially oriented manner. However, the electrochemical transfer inverts cell polarity for a short period (<5 min). To assess the recovery of cell polarity after transfer to the hydrogel, changes in ICAM-1 localization were monitored using confocal laser microscopy. ICAM-1 is a transmembrane protein that recruits leukocytes to the site of inflammation in the body; therefore, it is only expressed on the apical side of vascular endothelial cells.

G. *Coculture of HUVECs with fibroblasts*

HUVECs and normal human dermal fibroblasts (NHDFs) were mixed and seeded on an oligopeptide-modified gold rod (ϕ, 600 μm). Cells were then cultured for several days until the surface was completely covered. After the cell layers were electrochemically transferred to the hydrogel, the effects of NHDFs on the sprouting and luminal formation of HUVECs were evaluated after 3 and 7 d of perfusion culture using confocal laser microscopy.

III. Results and Discussion

A. *Electrochemical cell detachment*

HUVECs adhered to the modified gold surface were detached by reductive desorption of the oligopeptides within 5 min. To quantitatively evaluate the detachment of cells, we counted the number of cells remaining after each minute while applying the -1.0 V potential (Fig. 4). The number of cells decreased over time in a linear manner, and almost all the cells were detached within 5 min while applying the potential. In contrast, HUVECs adhered to an unmodified gold surface were not detached by applying a negative potential. These results suggest that the mechanism underlying cell detachment involves the electrochemical desorption of the oligopeptide layer.

B. *Fabrication of perfusable vasculatures*

Vascular-like structures, with inner surfaces covered with HUVECs, were fabricated in the GelMa through electrochemical cell detachment from the multicapillary needle (Fig. 5A, B). In the subsequent perfusion culture, the HUVECs on the surface remained alive and the microchannel structures were maintained without shrinkage or deformation of the hydrogel for at least 3 weeks (Fig. 5C, D). However, no sprouting or luminal formation was observed.

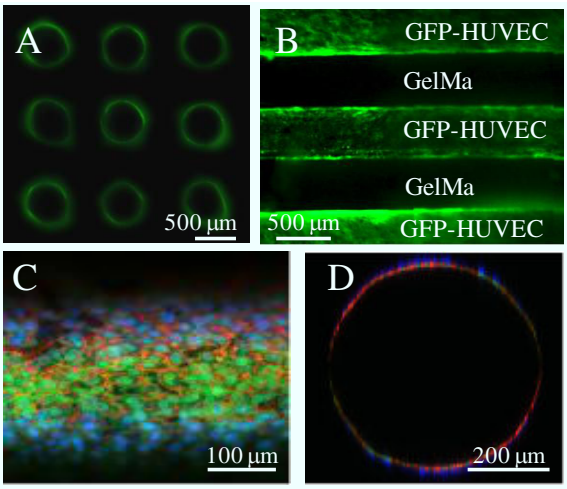

Figure 5. Fabricated vascular-like structures spatially aligned in a 3 × 3 arrangement with 500-μm interspaces. (A) Cross section. (B) Side view. (C, D) Vascular-like structure after 3 weeks of perfusion culture. Actin and nuclei are stained red and blue, respectively.

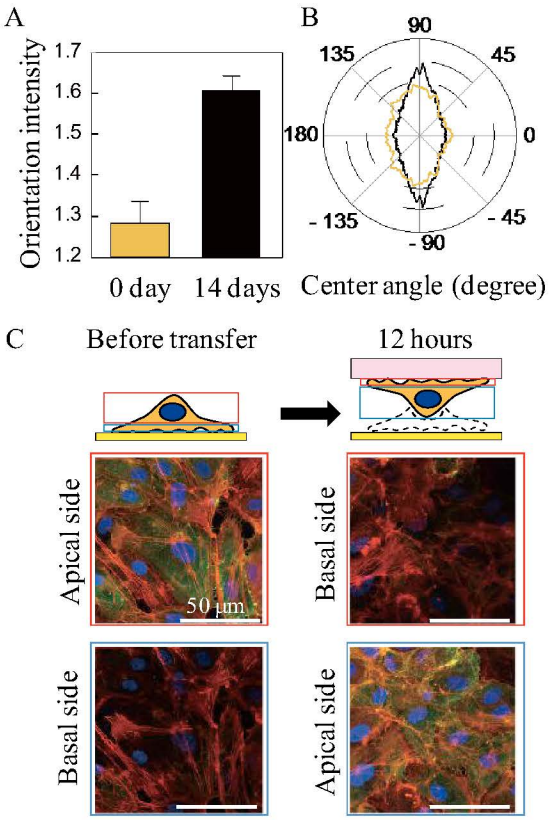

Figure 6. Cell orientation and polarity. (A) Orientation intensity at 0 and 12 d of perfusion culture. (B) Orientation angle on day 0 (yellow) and day 14 (black) of perfusion culture. (C) Confocal microscopic images showing localization of ICAM-1 before and after transferring cells from the gold surface to GelMa. The blue and red frames surrounding pictures correspond to the boxes in the cross-sectional diagrams at the top.

C. *Cell orientation and polarity after cell transfer*

Orientation intensity and angle increased significantly after 14 d of perfusion culture (Fig. 6A and B), indicating that HUVECs transferred to the hydrogel retain their native function and respond to shear stress generated by culture medium flow.

ICAM-1, the apical side indicator, was only expressed on the top of the HUVEC layers on the modified gold electrode (Fig. 6C). Immediately after the transfer of the cells, ICAM-1 was localized at the bottom of the layer. However, after 12 h of culture, ICAM-1 was localized in its proper locations (Fig. 6D).

D. *Effects of NHDF on HUVEC sprouting*

Two cell types could be transferred in the same manner onto the inner surface of the microchannels (Fig. 7A). The effects of NHDF on the sprouting of GFP-HUVECs were observed over 3 d of culture, and further induction of luminal structures was observed after 7 d (Fig. 7B-D). This was apparently different from that cells cultured without NHDF (Fig. 6), which was consistent with the literature.

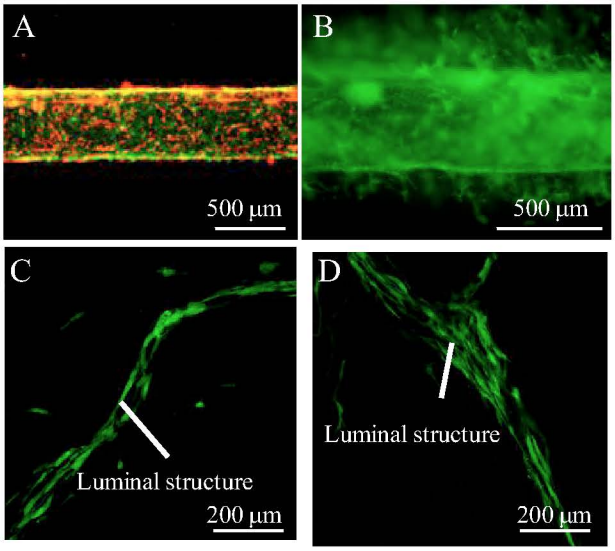

Figure 7. Effects of NHDFs on sprouting and formation of the luminal structure of HUVECs. (A) HUVECs (green) and NHDFs (red) cocultured on the internal surface of the microchannels. (B) Sprouting of HUVECs after 3 d of culture. (C, D) Formation of luminal structure at 7 d of culture.

IV. Conclusion

An electrochemical approach was used to fabricate perfusable, spatially aligned, vascular-like structures. Because of the rapid cell transfer process, the fabrication process was completed within 10 min, from pouring the GelMa solution to beginning the culture medium perfusion. We further demonstrated that coculture with fibroblasts facilitates sprouting and the formation of a luminal structure of HUVECs in a GelMa with sufficient strength. The combination of this micromolding approach and electrochemical cell transfer could open up a new avenue for the fabrication of vasculature networks for delivering oxygen and nutrients in 3-dimensional, thick tissues.

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