33rd Annual International Conference of the IEEE EMBS
Boston, Massachusetts USA, August 30 - September 3, 2011

Electrical Detachment of Cells for Engineering Capillary-like Structures in a Photocrosslinkable Hydrogel

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***Abstract*—A major challenge in tissue engineering is the fabrication of vascular networks capable of delivering oxygen and nutrients throughout tissue constructs. Because cells located more than a few hundred micrometers away from the nearest capillaries are susceptible to oxygen shortages, it is crucial to develop microscale technologies for engineering a vascular structure in three-dimensionally thick tissues. This study describes an electrochemical approach for fabricating capillary-like structures precisely aligned within micrometer distances, the internal surfaces of which are covered with vascular endothelial cells in a photocrosslinkable hydrogel.**

I. Introduction

One of the major obstacles in engineering complex and thick tissue constructs is the requirement to fabricate vascular networks. Because oxygen is supplied only by diffusion, cells located more than a few hundred micrometers away from the surface of tissue constructs suffer from hypoxia and apoptosis. Therefore, the fabrication of spatially controlled capillaries is crucial for reconstructing three-dimensionally thick tissues. Most approaches to engineering vascularized tissues have depended on vascularization from the host vasculature after transplantation. In previous reports, attempts have involved the use of growth factor-conjugated scaffolds and genetic modification of cells to promote vascularization. In addition, recent approaches have demonstrated that *in vitro* cocultures with endothelial cells lead 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 indicated that a part of the vascular networks is anastomosed with the host vasculature. However, anastomosis processes are generally considered too slow to maintain cell survival. Necrotic cell death occurs within minutes or hours, whereas the invasion and anastomosis of host vessels to prevascularized vessels requires days or months. In addition, drawbacks of the previous approaches are the inhomogeneous distribution in engineered tissues and insufficient blood flow to supply oxygen and nutrients throughout larger tissue constructs.

In this study, we proposed a technology for the detachment of cells from culture surfaces via an electrical stimulus and demonstrated its utility for the fabrication of vascular-like structures. We previously reported that cells attached to a gold surface via self-assembled monolayers of alkanethiol molecules were detached within 5 min after applying an electrical potential [1,2]. We also previously demonstrated that, as an alternative to alkanethiol, an oligopeptide could be used for the detachment of cells to improve the biocompatibility of this approach [3]. In this study, we newly designed another oligopeptide (Fig. 1). This oligopeptide forms a dense molecular layer on a gold surface with its intermolecular electrostatic force, preventing nonspecific adsorption of proteins on the gold surface and improving the detachment efficiency of cells. We also synthesized a photocrosslinkable gelatin hydrogel that can be gelled within minutes to accelerate the fabrication process of capillary-like structures. This simple technique could potentially provide a fundamental method for engineering surgically transplantable vascularized thick tissues and organs.

II. Methods

2.1 Oligopeptide and modification of gold surface

An oligopeptide, CGGGKEKEKEKGRGDSP, consisting of a cell adhesion domain (RGD) and cysteine was designed (Fig. 1). Culture substrates prepared by sputter coating of a few nanometer-layer Cr and 40-nm-layer Au on a glass wafer were immersed in a 0.5 mM oligopeptide solution for 12 h. Cysteine has a thiol group that spontaneously adsorbs onto gold surfaces via gold-thiolate bonds. During the adsorption, the oligopeptide forms a closely packed self-assembled monolayer on the surface via the electrostatic force between the alternating charged glutamic acid and lysine in the neighboring molecules (Fig. 1). Owing to the ionic salvation in the alternating sequence, the modified surface was resistant to the nonspecific adsorption of proteins, whereas cells adhered to this surface via the RGD sequence.

2.2 Cyclic voltammetry

Manuscript received October 9, 2001. (Write the date on which you submitted your paper for review.) This work was supported in part by the U.S. Department of Commerce under Grant BS123456 (sponsor and financial support acknowledgment goes here). Paper titles should be written in uppercase and lowercase letters, not all uppercase. Avoid writing long formulas with subscripts in the title; short formulas that identify the elements are fine (e.g., "Nd–Fe–B"). Do not write "(Invited)" in the title. Full names of authors are preferred in the author field, but are not required. Put a space between authors' initials.

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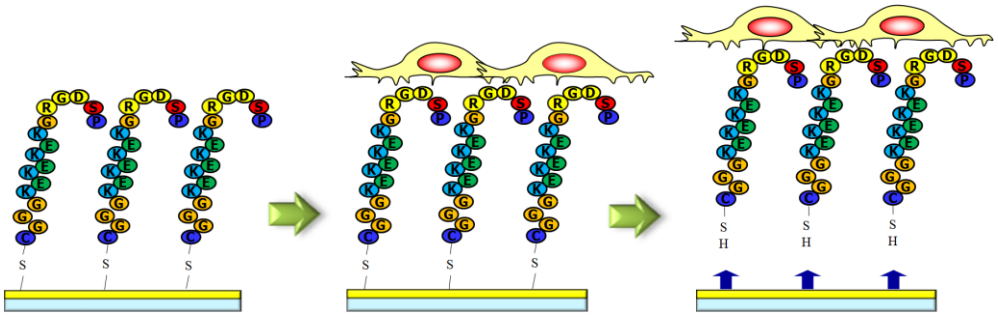

Fig. 1 Electrochemical cell detachment along with the reductive desorption of the zwitterionic oligopeptide. The oligopeptide includes cysteine for bonding to gold, positively charged lysine and negatively charged glutamic acid, and the RGD sequence to promote cell adhesion.

Cyclic voltammetry was employed to determine the reductive potential for desorption of the oligopeptide adsorbed on the gold surface. Immediately before cyclic voltammetry testing, an electrolyte solution containing 0.5 M KOH was deoxygenated by bubbling nitrogen gas for 20 min. The oligopeptide-modified gold substrate, a Ag/AgCl reference electrode, and a platinum auxiliary electrode were set in the electrolyte solution and connected to an electrochemical measurement system. In this study, all potential values refer to those measured with respect to the Ag/AgCl electrode. A cyclic voltammogram was recorded at the scanning rate of 20 mV/s from 0 to -1.0 V.

2.3 Photocrosslinkable gelatin hydrogel (GelMa)

The primary amine group of gelatin was reacted with methacrylic anhydride to add a methacrylate group and create a photocrosslinkable gel. After dialysis using 12–14-kDa cutoff tubing with distilled water to remove methacrylic acid, GelMa was lyophilized to a white porous foam. 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 buffer saline solution. The prepared GelMa solution was poured into the culture chamber and gelled via light exposure (365 nm, 6.9 mW/cm^2) for 90 sec.

2.4 Fluorescence recovery after photobleaching (FRAP)

To examine whether cell-cell adhesion molecules remained intact after the transfer of a two-dimensionally connected cell monolayer to GelMa, the transport of fluorescent substances between cells was measured by FRAP. Fluorescein diacetate is a nonpolar compound that readily diffuses into cells. Intracellular esterase hydrolyzes fluorescein diacetate to fluorescein, which is a highly polar molecule that scarcely diffuses out from cells. Thus, cells treated with fluorescein diacetate were stained with fluorescein by means of this process. The cells were then washed with phosphate buffer saline solution 3 times. Fluorescein in the cells was bleached under an argon laser light source, and fluorescence recovery in the bleached cells was monitored by a confocal laser-scanning microscope for 10 min.

2.5 Culture chamber

The chamber for perfusion culture was fabricated with a poly(methyl methacrylate) plate (thickness, 2 mm) using a computer-aided laser machine (Figs. 2 and 3). The volume of the chamber was 1.5 mL. The chamber had 3 pairs of holes 600 μm in diameter at intervals of 500 mm to guide the gold rods.

2.6 Fabrication of the capillary-like structures

The schematic for the fabrication of capillary-like structures is presented in Fig. 2. Thin gold rods were prepared by sputter coating layers of Cr and Au on a glass stick 600 μm in diameter. Similar to the glass wafer surface, the surface of the gold rod was modified with the oligopeptide. The modified gold rod was then rinsed with pure water and sterilized with 70% ethanol. The gold rods were placed in a

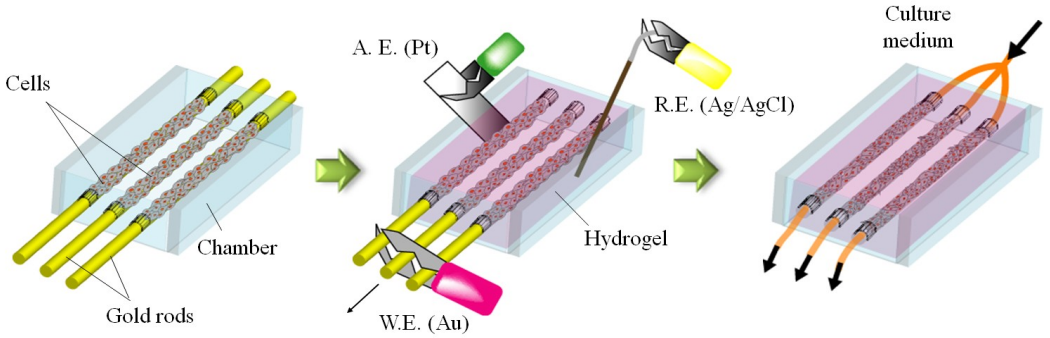

Fig. 2 Scheme for the fabrication of the capillary-like structures in GelMa by using the electrical detachment of cells from the gold rods.

cell-non-adherent 35-mm dish, and human umbilical vein endothelial cells (HUVECs) were seeded at a density of 3.0×10^5 cells in 2-mL of culture medium. The cells were attached to the gold rods and grown to reach confluence for 3–4 days. The gold rods with cells were fixed in the culture chamber, and 1.2 mL of the GelMa solution was then poured into the chamber, which was then closed with a lid to prevent evaporation. After the gelation of GelMa by UV irradiation (365 nm) for 90 sec, the rods were carefully extracted by applying a potential of -1.0 V for 5 min with respect to the Ag/AgCl reference electrode. Then, the chamber was connected to a microsyringe pump, and the culture medium was perfused at a rate of 10 μL/min. In the coculture experiments, human hepatoblastoma cells (Hep G2) were previously mixed in the GelMa solution at a density of 1.0×10^4 cells/mL and poured into the chamber to fabricate liver-like tissue constructs.

III. Results and Discussion

3.1 Cyclic voltammetry

Cyclic voltammetry results indicate that the peak potential for reductive desorption of the oligopeptide was approximately -0.85 V (Fig 4). Because cells and cell sheets may exhibit electrical resistance, we decided to use a potential of -1.0 V in the following experiments, which is larger than the peak potential, to apply sufficient potential for desorption while preventing electrolysis.

3.2 Cell detachment

HUVECs adhered to the modified gold surface were detached with the reductive desorption of oligopeptide within 5 min. To quantitatively evaluate the detachment of cells, we counted the number of cells remaining after each minute of applying -1.0 V (Fig. 5). The number of cells was reduced over time in a linear manner, and almost all the cells were detached within 4 min of applying the potential.

3.3 Cell-to-cell connections

FRAP analysis was performed to investigate whether intercellular connections via gap junctions were altered with the electrical detachment of the cells. Gap junctions are important for chemical communication between cells via the transmission of small second messengers such as inositol triphosphate and calcium ion. Fig. 6(A) shows that fluorescence intensity recovered 500 sec after the photobleaching in all 3 conditions. Fig. 6(B) provides a detailed explanation of the changes in the intensity. As control experiments, we conducted FRAP analysis on cells that were directly seeded on a glass substrate or GelMa. The recovery in a cell on GelMa was faster than that in a cell on a glass substrate. This is probably because cells on GelMa, in comparison to cells on glass, had a relatively round shape due to the difference in stiffness of the substrates and thus had a larger contact area between cells. The dynamics of the recovery in the cells transferred from the gold surface to GelMa was similar to that in a glass substrate. These results

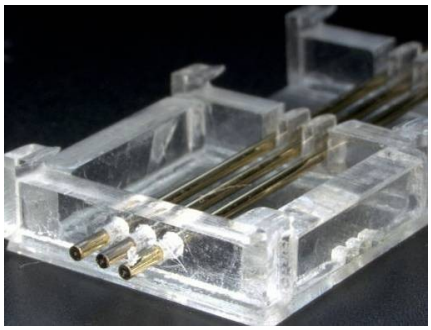

Fig. 3 The gold rods (diameter: 600 μm) fixed in the culture chamber at 500-μm intervals.

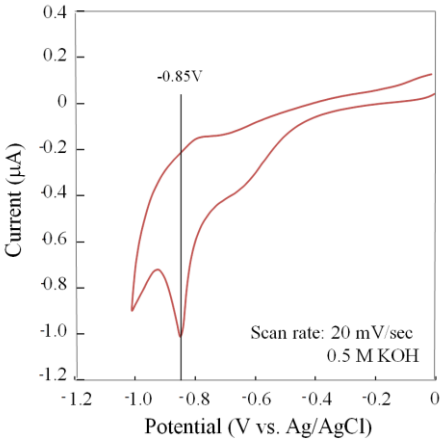

Fig. 4 Cyclic voltammogram obtained during the reductive desorption of the oligopeptide. Cyclic voltammograms were recorded at a scanning rate of 20 mV/s with respect to the Ag/AgCl reference electrode.

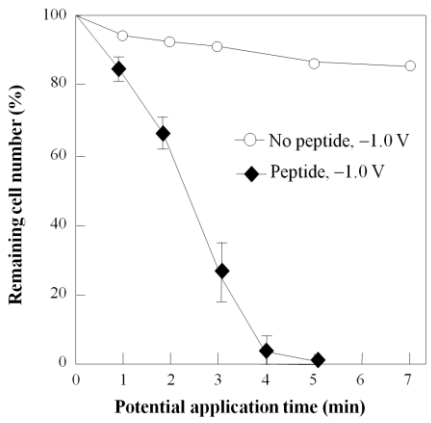

Fig. 5 Changes in the number of HUVECs remaining on the gold surface. Almost all of the cells were detached within 4 min of applying the potential in the presence of the oligopeptide (•), whereas few cells detached from the surface in the absence of the oligopeptide (◦).

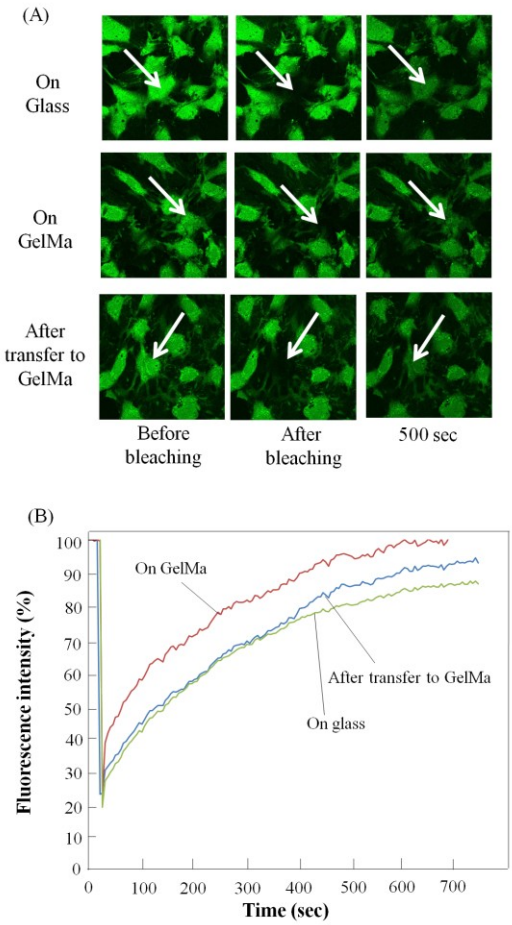

Fig. 6 FRAP analysis of intercellular interaction. (A) Representative images in the recovery of fluorescence in a cell. (B) Changes in the fluorescence intensity in a cell after bleaching with a laser.

indicate that the electrical transfer of cells to GelMa does not disturb the binding of connexin, the intercellular adhesion molecule responsible for the formation of gap junctions.

3.4 Fabrication of capillary-like structures in the hydrogel

HUVECs were attached to the surfaces of gold rods (diameter, 500 μm) via the ologopeptide, and were grown to reach confluence. The rods with cells were inserted into the GelMa solution. After the gelation of the hydrogel by UV irradiation, the rods were carefully extracted by applying a potential of -1.0 V, resulting in the formation of capillaries covered with the cells in the hydrogel (Fig. 7(A)). Then, the culture medium supplemented with VEGF and FBS was perfused through the capillaries at a flow rate of 10 μl/min. During the perfusion culture, the vascular-like structures were maintained for at least 3 weeks. In the cocultures of HUVECs and Hep G2 cells, Hep G2 cells grew in the hydrogel between the capillary-like structures (Fig. 7(B and C)). According to the image analysis, the number of Hep G2 cells was 10-fold greater than the number of inoculated cells, indicating that oxygen and nutrients were supplied to Hep G2 cells through the HUVEC layers.

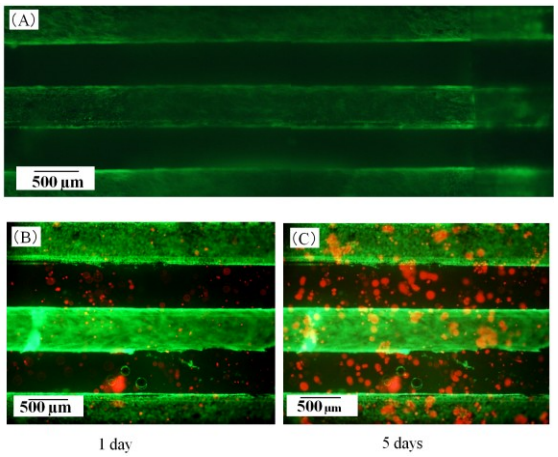

Fig. 7 Capillary-like structures in the hydrogel. (A) Entire length of capillary structures (10 mm) in the culture chamber. (B and C) Coculture of HUVECs (green) and Hep G2 (red). After 1 day (B) and 5 days (C) of perfusion culture.

IV. Conclusions

This study demonstrated that an electrochemical approach could be used to fabricate capillaries in which their internal surfaces were covered with HUVECs in a hydrogel. In this approach, HUVECs were attached to a gold surface via an oligopeptide and were detached by applying a negative potential that reductively cleaved the gold-thiolate bonds. Gold rods covered with HUVECs were inserted into a photocrosslinkable gelatin hydrogel and then extracted by applying a potential of -1.0 V, resulting in the formation of capillary-like structures. Hep G2 cells mixed in the gelatin hydrogel grew vigorously. Long-term culture will lead to migration and luminal formation of HUVECs in the gelatin hydrogel and may potentially result in the formation of highly organized liver-like structures. This approach could be a new tool for providing vascular structures capable of delivering oxygen and nutrients in thick tissue constructs.

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