



## Original article

## Engineering thick cell sheets by electrochemical desorption of oligopeptides on membrane substrates



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## ABSTRACT

We developed a gold-coated membrane substrate modified with an oligopeptide layer that can be used to grow and subsequently detach a thick cell sheet through an electrochemical reaction. The oligopeptide CRRGDWLC was designed to contain a cell adhesive domain (RGD) in the center and cysteine residues at both terminals. Cysteine contains a thiol group that forms a gold–thiolate bond on a gold surface. Cells attached to gold-coated membrane substrates via the oligopeptide layer were readily and noninvasively detached by applying a negative electrical potential to cleave the gold–thiolate bond. Because of the effective oxygen supply, fibroblasts vigorously grew on the membrane substrate and the thickness of the cell sheets was ~60 μm at 14 days of culture, which was 2.9-fold greater than that of cells grown on a conventional culture dish. The cell sheets were detached after 7 min of electrical potential application. Using this approach, five layers of cell sheets were stacked sequentially with thicknesses reaching >200 μm. This approach was also beneficial for rapidly and readily transplanting cell sheets. Grafted cell sheets secreted collagen and remained at the transplanted site for at least 2 months after transplantation. This simple electrochemical cell sheet engineering technology is a promising tool for tissue engineering and regenerative medicine applications.

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## 1. Introduction

Regenerative medicine and tissue engineering have great potential to fulfill some unmet needs in medicine [1]. Since only a small percentage of cells are integrated into host organs when cells are directly injected as a single-cell suspension, various tissue engineering approaches have been developed to reconstruct tissues and organs *in vitro* prior to transplantation [2]. Scaffold-based tissue engineering is a widely used approach and has been shown to be beneficial in successful clinical trials [3–5]. However, some previous studies noted mismatches between scaffolds and native matrices, spatial and temporal differences between cell growth, and the degradation of scaffolds [6,7].

Another promising tissue engineering approach is using a building-up process with cellular building blocks without scaffolds, such as cell sheets, spheroids, and cell-dense microgels [8–10]. Among these, the cell sheet-based approach has been applied in clinical trials. For example, autologous oral mucosal epithelium cell sheets were transplanted for corneal reconstruction; these sheets promoted the recovery of weakened vision [11,12]. Myoblast cell sheets improved cardiac function in a patient with dilated cardiomyopathy [13]. Compared to scaffold-based approaches, the cell sheet approach may be more suitable for some tissues because reconstructed tissues should possess tissue-specific functions such as transparency for the cornea and flexibility for the heart. The manipulation and transplantation of cell sheets have inherently relied on innovative strategies to cleave cell-to-culture substrate connections while preserving cell-to-cell connections. Thus, a thermo-responsive surface has been extensively studied for cell sheet-based approaches [12]. This sophisticated technology can be used to harvest cell sheets through simple temperature reduction. One potential difficulty of this technology is that a relatively long

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time is required to detach a cell sheet (30–60 min) [14,15]. Shortening this period is very important for minimizing the exposure of cells to non-physiological conditions. In particular, it becomes increasingly important when the processes are repeated to stack cell sheets and fabricate transplantable multilayer cell sheets. Although as another approach multiple cell sheets can be detached simultaneously, detached cell sheets readily shrink and fold, and thus, a supporting layer such as a hydrogel layer has to be added to maintain the shape of detached cell sheets. The hydrogel layer however possibly hinder cell–cell contact between cell sheets when stacked together. Therefore, the stacking of cell sheets should be conducted individually, and in this case, rapid cell sheet detachment could be significant.

Other cell detachment approaches using external stimuli such as electrochemical, photochemical, or magnetic cues have been reported [16,17]. Electrochemically responsive molecular layers have been used for dynamic and spatially-controlled cell detachments [18–20]. Polyelectrolyte thin films have been used for detachment of cell sheets by changing a local pH and dissociating the films electrochemically [21]. We have also reported that cells could be rapidly detached from a gold surface along with desorption of alkanethiol molecular layer [22,23]. This reaction led to the detachment of cells within 5 min. We further demonstrated that cell sheets can be detached from the surface in the same manner [24]. However, cell sheets cultured on the bottom surface of a culture dish suffer from a shortage of oxygen. This is obvious considering the diffusion coefficient, solubility in culture medium, and consumption rate of oxygen in cells [25]. The limited supply of oxygen hinders the growth of cells and the rapid formation of a thicker cell sheet, making stacking processes of cell sheets laborious and time-consuming.

In this study, to alleviate these limitations, we examined whether our electrochemical cell detachment approach could be applied to a porous membrane substrate. We expected that an improved supply of oxygen and nutrients through the membrane would lead vigorous proliferation and the formation of thicker cell sheets, facilitate the handling of cell sheets, and reduce the repetition of stacking processes to obtain transplantable thick cell sheets. We also expected that the use of membrane substrates would provide pathways for the culture medium during the detachment of cell sheets, creating a robust approach for the detachment of cell sheets independent of their dimensions. In this study, we demonstrated that the proposed electrochemical approach is a rapid and reliable tool for preparing transplantable thick cell sheets for tissue engineering applications.

## 2. Methods

### 2.1. Materials and reagents

The materials used for the fabrication of culture substrates were as follows: membrane substrates (Cell Culture Insert #353493; pore size, 0.4  $\mu\text{m}$ ; pore density,  $1.0 \times 10^8$  pores/ $\text{cm}^2$ ) and 6-well culture plates (#353046) from BD Biosciences (Franklin Lakes, NJ, USA); synthetic oligopeptide, CRRGDWLC, from Sigma–Aldrich (St. Louis, MO, USA); type I collagen from Nitta Gelatin (Osaka, Japan); mesh support (Beschitin) from Unitika, Ltd. (Osaka-Shi, Japan).

The reagents used for cell culture and tests were obtained from the following commercial sources: Swiss 3T3 murine fibroblasts (RCB1642) and normal human neonatal skin fibroblasts (NB1RGB) from the Riken Cell Bank (Ibaraki, Japan); Dulbecco's modified Eagle medium, minimum essential medium  $\alpha$ , fetal bovine serum, and phosphate buffer saline (PBS) from Invitrogen (Carlsbad, CA, USA); penicillin-streptomycin solution from Millipore (Billerica, MA,

USA); immortalized human umbilical vein endothelial cells constitutively expressing green fluorescent protein (GFP-HUVECs), a generous gift from Dr. J. Folkman, Children's Hospital, Boston; endothelial basal medium-2 (EBM-2, CC-3156), and SingleQuots growth supplement (CC-3162) from Cambrex Bio Science Baltimore, Inc. (Baltimore, MA, USA); fluorescent diacetate (FDA) and ethidium bromide (EB) from Wako Pure Chemical Industries (Osaka, Japan); DAPI from Vector Laboratories Inc., USA; Dapi-Fluoromount-G from SouthernBiotech (Birmingham, AL, USA); PKH26 linker kit from Sigma–Aldrich; rhodamine phalloidin from Cytoskeleton, Inc. (Denver, CO, USA); enzyme-linked immunosorbent assay (ELISA) kit for mouse vascular endothelial growth factor (VEGF) (ELM-VEGF-001) from RayBiotech, Inc. (Norcross, GA, USA); QIAmp DNA Mini Kit from Qiagen (Hilden, Germany); OTC compound (Tissue-Tek) from Miles Laboratories (Elkhart, IN, USA); rabbit anti-human collagen monoclonal IgG from Abcam Inc. (Cambridge, UK); Alexa Fluor 488 conjugated anti-rabbit IgG polyclonal antibody (Invitrogen). All other chemicals were purchased from Wako Pure Chemical Industries, unless otherwise indicated.

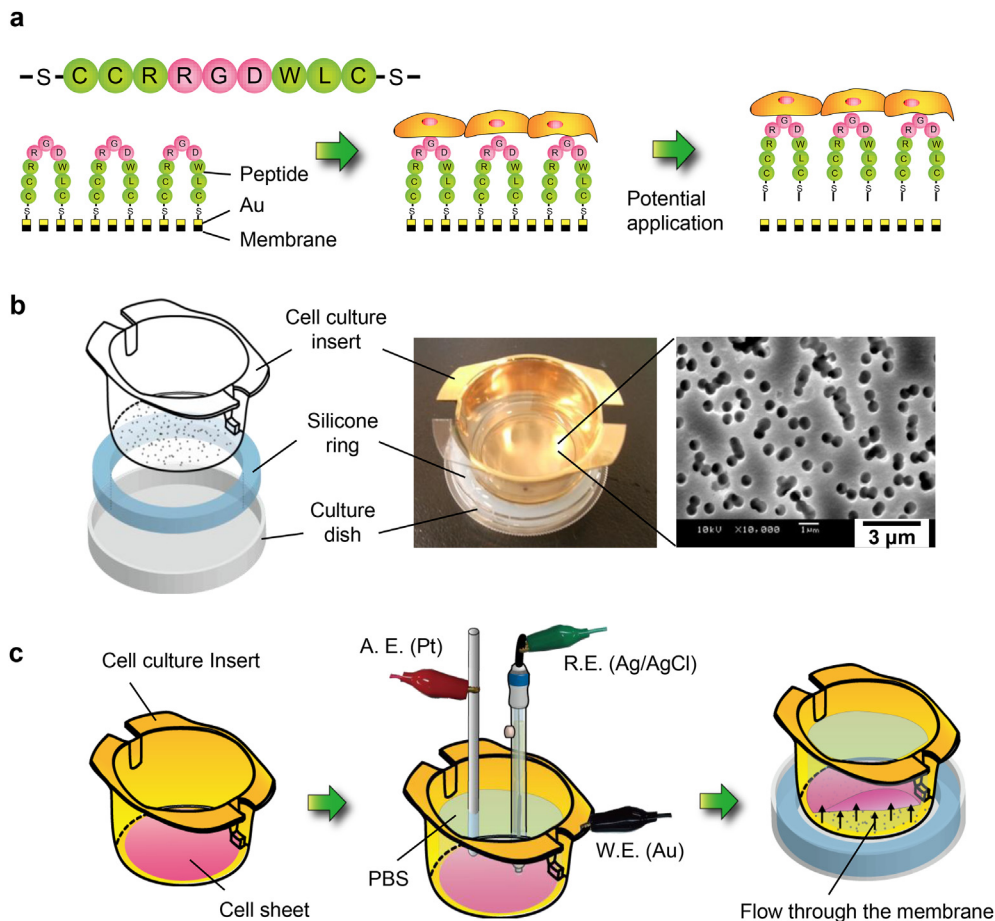
### 2.2. Modification of membrane substrates with gold and oligopeptides

We designed the oligopeptide, CRRGDWLC, which contains an RGD domain in the center and cysteine residues at both terminals (Fig. 1a) [23]. Cysteine contains a thiol group that spontaneously adsorbs onto a gold surface via a gold–thiolate bond, while RGD interacts with cell-surface integrins expressed on various cell types, including fibroblasts. The adsorbed amount of the oligopeptide was estimated to be 0.18 nmol/ $\text{cm}^2$ , although the oligopeptides potentially forms disulfide bonds with each other and create long molecular chains [24]. The modification process is therefore very simple and does not require the use of organic chemistry reactions. A commercial membrane substrate (Cell Culture Insert) was sputter-coated with a few nm layer of chromium and a ~40-nm layer of gold (Fig. 1b). The oligopeptide spontaneously bound onto the gold surface by immersion into 0.5  $\mu\text{M}$  aqueous oligopeptide solution for 12 h at 4  $^\circ\text{C}$ . The substrate was then rinsed with pure water and sterilized with 70% ethanol.

### 2.3. Detachment of single cells from the membrane substrate

Scanning electron microscopy was utilized to acquire cell images, as it was nearly impossible to observe cells using a phase-contrast microscope when the membrane substrate was coated with gold. For observation by scanning electron microscopy, cells on the substrate were washed with PBS three times and fixed with a mixed solution of 2.5% glutaraldehyde and 2% formaldehyde in PBS for 1 h at room temperature. Next, the substrate was washed with PBS and fixed with 1% osmium tetroxide in PBS for 1 h at 4  $^\circ\text{C}$ . The substrate was washed with purified water and dehydrated with a graded ethanol series from 30% to 90% and absolute ethanol substitution three times at room temperature. The solution was further substituted with 100% *t*-butanol, which was frozen at 4  $^\circ\text{C}$  and dried by vacuum freeze-drying. The cells were observed under a scanning electron microscope (ED-SEM; JEOL, Tokyo, Japan) operated at 20 kV.

The membrane substrate modified with the oligopeptide was placed in a 6-well culture plate. Fibroblasts ( $1.0 \times 10^5$  cells/mL) in 2.0 mL of culture medium were seeded in the upper side of the membrane substrate and 2.5 mL of culture medium without cells was added to the lower side of the membrane substrate in the 6-well culture plate. The cells were cultured for 1 day at 37  $^\circ\text{C}$  with 5%  $\text{CO}_2$  in a humidified incubator. Next, the culture media on both



**Fig. 1.** Electrochemical detachment of cells from a gold-coated membrane substrate. (a) Principle of our setup. A gold-coated membrane substrate was modified with the oligopeptide, CRRGDWLC. Cells attached on the membrane substrate via the oligopeptide layer were detached along with electrochemical desorption of the layer. (b) SEM image of gold-coated membrane substrate with pores of  $\sim 0.4 \mu\text{m}$  in diameter. (c) Procedures for the detachment of cell sheets. Working electrode (W.E., gold-coated membrane), reference electrode (R.E., Ag/AgCl electrode), and auxiliary electrode (A.E., Pt wire).

sides were aspirated and replaced with PBS solution. The gold-coated membrane substrate was connected to a potentiostat (HA-151; Hokuto-Denko, Meguro-ku, Japan) as a working electrode (Fig. 1c). An Ag/AgCl reference electrode (#012167; BAS Inc., Tokyo, Japan) and a Pt auxiliary electrode were also set in the PBS solution and connected to the potentiostat. After applying a potential of  $-1.0 \text{ V}$  for 5 min, the substrates were gently washed with PBS and the detached cells were counted using a hemocytometer under a microscope to quantitatively analyze electrochemical detachment.

#### 2.4. Growth and metabolic activities of fibroblasts on the membrane substrate

Fibroblasts ( $5.0 \times 10^5$  cells/mL) in 2 mL of the culture medium were seeded on the oligopeptide-modified membrane substrate. The cells were then cultured for 14 days and changes in the number of cells were determined by extracting DNA using a commercial kit (QIAmp DNA Mini Kit, Qiagen). For histological staining, cell sheets were fixed with 3.7% formaldehyde in PBS, embedded in paraffin, and sectioned. Hematoxylin and eosin (H&E) staining was performed following standard procedures. Images were acquired using a digital microscope (BZ-X710, Keyence, Osaka, Japan). To evaluate the respiration activity of cell sheets, the consumption of glucose and the production of lactate were quantified using electrochemical

glucose (Nipro Stat StripXP2; NIPRO, Osaka-Shi, Japan) and lactate (Lactate Pro; ARKRAY, Inc., Edina, MN, USA) sensors.

To evaluate changes in a metabolic activity during the formation of thick cell sheets, fibroblasts in 2 mL culture medium were seeded on the substrate and conventional culture dishes at a density of  $2.0 \times 10^5$  cells/cm<sup>2</sup>. The culture medium was sampled every 24 h for 14 days of culture. The secretion of VEGF was quantified using an ELISA kit according to the manufacturer's instructions. All assays were conducted in duplicate and the concentrations were calculated using a standard curve obtained for recombinant VEGF.

#### 2.5. Fabrication of multilayered cell sheets

As depicted in Fig. 1c, a cell sheet was electrochemically detached. To alleviate shrinkage of a detached cell sheet and facilitate the subsequent stacking processes, a few drops of type I collagen solution (0.24% w/v) was poured onto the first cell sheet and gelled in a humidified incubator for 30 min at 37 °C. Subsequently, the cell sheet covered with a collagen gel layer was detached from the culture surface by applying the electrical potential. A silicone ring was used to harvest the detached cell sheet while passing PBS through the membrane substrate from the bottom. After harvesting the cell sheet, live/dead fluorescent staining was performed using FDA and EB. The detached cell sheet was then placed on another cell sheet and incubated for 30 min for attachment of the sheets to one another. The two-layered cell sheet was again detached from the

surface electrochemically and stacked onto another sheet to obtain three-layered cell sheets. By repeating these processes, a multi-layered cell sheet was fabricated. Cross-sections of the multilayered cell sheet were observed using H&E staining.

### 2.6. Transplantation of cell sheets in mice

Animal experiments were performed in accordance with the Guidelines for Animal Experimentation of Nagoya University School of Medicine. Four-week-old KSN/Slc nude mice, from the Chubu Kagaku Shizai Corporation (Nagoya, Japan), were anesthetized with isoflurane, and subcutaneous pockets were created on the dorsal surface at each side of the midline. Normal human neonatal skin fibroblasts (NB1RGB cells) were cultured on the modified substrates for 7 days to obtain thick cell sheets. Prior to transplantation, the cell sheets were labeled with PKH26 red fluorescent cell tracker and subsequently detached by applying an electrical potential. The cell sheets were lifted on a mesh support for handling, and then transplanted into dorsal subcutaneous pockets, followed by interrupted 5-0 nylon sutures.

After 1 and 2 months, the mice were anesthetized and skin samples, including the grafts, were harvested for H&E staining. For immunohistochemical staining, the samples were fixed with 4% paraformaldehyde, embedded in OTC compound and frozen sections were obtained on glass slides. The samples were immunostained with primary antibodies against human collagen and Alexa Fluor 488 conjugated secondary antibody, and then counterstained with DAPI. After washing the cells three times with PBS, the cells were examined using a fluorescence microscope (IX-71, Olympus).

## 3. Results and discussion

### 3.1. Detachment of single cells from oligopeptide-modified membrane substrates

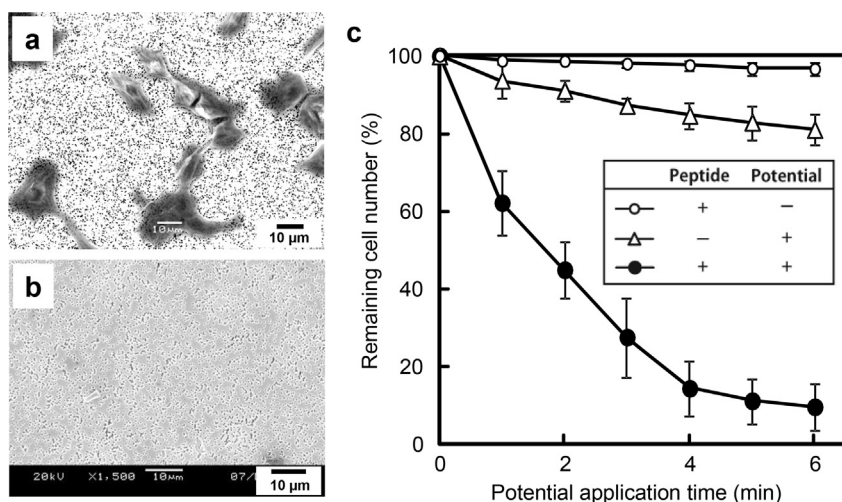
Fibroblasts attached and spread out on the gold-coated membrane substrates modified with the oligopeptide (Fig. 2a). Because a phase-contrast microscope could not be used to observe cells through the gold-coated membrane substrates, cell detachment was evaluated by images taken with a scanning electron

microscope after fixing the cells. Fibroblasts adhered on the membrane surface were readily detached by the application of  $-1.0$  V for 5 min at 1 day of culture (Fig. 2b). Cytocompatibility of the oligopeptide and the electrochemical detachment was confirmed using live/dead staining, chromosome abnormality test, and doubling time (Supplemental Fig. 1). To quantitatively estimate detachment, the number of cells withdrawn into a micropipette was counted using a hemocytometer every minute for 6 min of the potential application (Fig. 2c). When the substrate was modified with the oligopeptide and the electrical potential was applied,  $\sim 50\%$  and  $\sim 90\%$  of adhering cells were detached at 2 min and 5 min, respectively. In contrast, only a few cells were detached when no potential was applied. These results suggest that the electrical desorption of the oligopeptide is responsible for cell detachment. Approximately 20% cell detachment was observed even when the cells were directly attached to the gold surface and a potential was applied. This likely occurred because of desorption of nonspecifically adsorbed proteins from the bare gold substrate [26].

### 3.2. Cell sheet formation on the membrane substrate

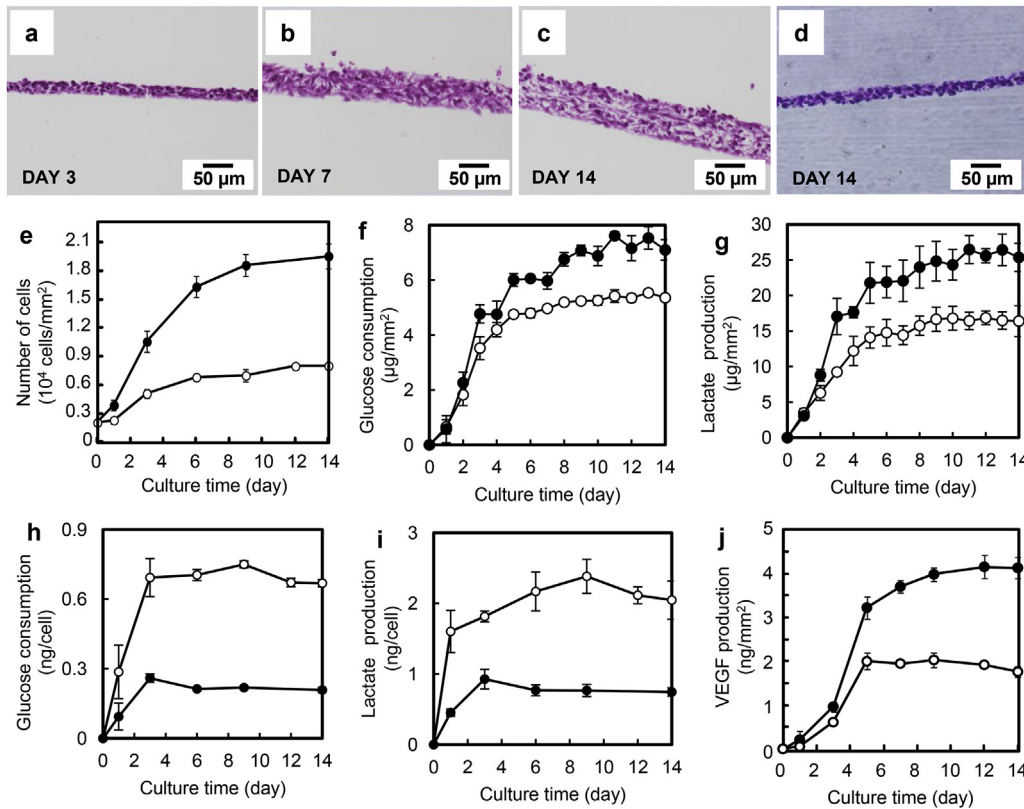
Fibroblasts were cultured on the oligopeptide-modified membrane substrate for 3, 7, and 14 days and their cross-sections were stained with H&E to estimate the changes in the thickness of a cell layer (Fig. 3a–d). Fig. 3a–d shows that the membrane substrate was removed during sectioning. As expected, the cells grew over time and formed a thicker cell layer on the membrane substrate. The thickness was  $58 \pm 4.6$   $\mu\text{m}$  at 14 days of culture (Fig. 3c), which was significantly greater than that on a conventional culture dish ( $20 \pm 3.6$   $\mu\text{m}$ , Fig. 3d). Changes in the number of cells were estimated using extracting DNA (Fig. 3e). The cell number increased rapidly on a membrane substrate by 6 days and nearly reached equilibrium after 10 days, whereas growth was stopped at 6 days on a conventional culture dish. This is likely related to the difference in oxygen and nutrient supply as in the membrane culture system, an improved supply from the upper and the lower side of the membrane substrate was expected.

To examine the respiration activity of the cells, the consumption of glucose and the production of lactate were evaluated. Glucose consumption and lactate production rapidly increased both on the



**Fig. 2.** Single cell detachment from oligopeptide-modified membrane substrates. (a) SEM image of fibroblasts adhering on the substrate at 1 day of culture. (b) SEM image of the membrane surface after applying  $-1.0$  V for 5 min and replacement with fresh culture medium, indicating cells were detached and withdrawn from the surface. (c) Changes in the cell number on the membrane substrate. After potential application,  $\sim 90\%$  of cells were detached within 5 min along with desorption of oligopeptide (closed circle). Only few cells detached from the surface without potential application (open circle) or in the absence of the oligopeptide (i.e., cells were directly attached to a gold surface (open triangle)). The error bars indicate SD calculated from 3 independent experiments.





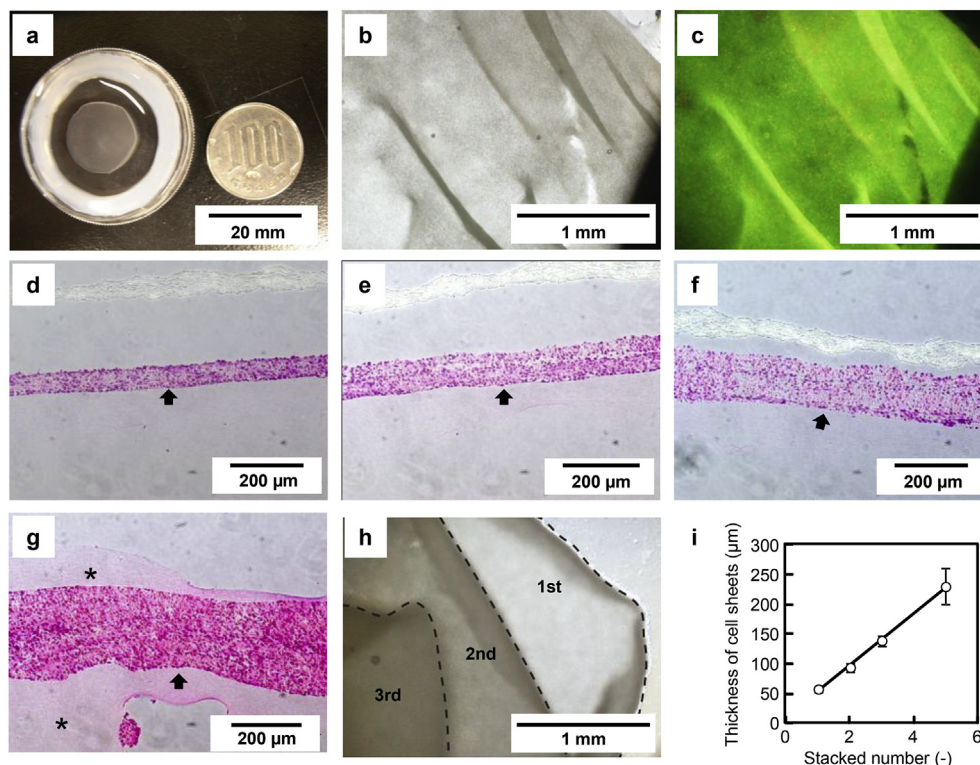
**Fig. 3.** Formation of fibroblast sheets on oligopeptide-modified membrane substrates, and their respiration activity and VEGF production. (a–d) H&E staining of cross-section of cell sheets. Cells were cultured for 3 days (a), 7 days (b), and 14 days (c) on the membrane substrates and for 14 days on a conventional culture dish (d). (e) Changes in the number of cells. (f–i) Respiration activity. The amounts of consumed glucose (f, h) and produced lactate (g, i), which were normalized by the culture surface areas (f, g) and by the number of cells at each time point (h, i). (j) VEGF secretion. Cells were cultured on the membrane substrate (closed circle) or a culture dish (open circle). The error bars indicate SD calculated from 3 independent experiments.

membrane substrate and in the culture dish at the beginning of culture, and over the culture period, these rates were significantly greater on the membrane substrate than on the culture dish (Fig. 3f and g). However, these rates normalized to the number of cells at each culture time (Fig. 3e) were more than double on a culture dish than on the membrane substrate during the culture period (Fig. 3h and i). These results indicate that the membrane substrate provides a more aerobic environment even when the cell number was 2.7-fold greater than that on a culture dish at 14 days of culture. These results are consistent with those of a previous report in which enhanced oxygen supply through a silicone membrane substrate improved the respiration activity and led to the formation of thicker cell sheets (~50 μm in thickness at 15 days) [27]. Fibroblasts secrete various growth factors, including VEGF, which play an important role in wound healing and the regeneration of necrotic tissues [28]. Changes in the time course of VEGF secretion were correlated with those in cell number; VEGF secretion increased rapidly at the beginning of culture and was later saturated on both on the membrane substrate and the culture dish (Fig. 3j). Although VEGF production is known to accelerate under low oxygen concentration conditions [29], there was no significant difference in the VEGF secretion rate per cell between the membrane substrate ( $2.1 \pm 0.13 \times 10^{-4} \text{ ng cell}^{-1} \text{ day}^{-1}$  at 14 days) and a culture dish ( $2.2 \pm 0.16 \times 10^{-4} \text{ ng cell}^{-1} \text{ day}^{-1}$  at 14 days). This may be attributed to other factors influencing VEGF production, such as positive effects by cell–cell interactions in the cell sheet and negative effects by changes in pH in the culture dish.

### 3.3. Detachment of cell sheets

We examined whether a cell sheet formed on the oligopeptide-modified membrane substrate could be detached electrochemically. Fibroblasts were grown for 14 days on the substrate and  $-1.0 \text{ V vs. Ag/AgCl}$  was applied for 7 min. The detached cell sheet was then harvested along with the flow from the bottom through the membrane substrate using a silicone ring (Fig. 1c). The detached cell sheet immediately shrank to ~65% of its original projected area, which was typically ~20 mm in diameter (Fig. 4a). Live/dead fluorescent staining revealed that all cells in the harvested cell sheet were viable (Fig. 4b and c).

The detachment of cell sheets may depend on the geometry of membrane substrates such as pore size. In a preliminary study, we examined another membrane substrate composed of pores that were 3.0 μm in diameter, which are much larger than those of the substrate used in this study (0.4 μm). Both substrates were composed of poly(ethylene terephthalate) and the same procedure was conducted to detach the cell sheet, but no detachment was observed for the 3.0 μm-pore-substrate even when the potential application time was extended to 10 min. This is probably because fibroblasts migrated into the pores during cell sheet formation. Thus, the pore of a membrane should be sufficiently small to prevent intrusion of cells into the pore. In general, cells cannot migrate into a pore less than 1 μm [30]; thus, a membrane substrate with pores 0.4 μm in diameter was used in this study.



**Fig. 4.** Stacking of cell sheets. (a) Representative appearance of a fibroblast sheet electrochemically detached from the membrane substrate at 14 days of culture. (b, c) Live/dead staining of a detached cell sheet. Phase contrast (b) and fluorescent (c) images. Green indicates live cells and red indicates dead cells. (d–g) H&E staining of cross sections of stacked fibroblast sheets. Single (d), double (e), triple (f), and quintuple (g) layered sheets. The asterisks and arrows indicate collagen gels and cell sheets, respectively. (h) Macroscopic image of peripheral of a three-layered fibroblast sheet. (i) Dependence of the thickness of cell sheets on the repeating number of stacking. The error bars indicate SD calculated from 3 independent experiments.

### 3.4. Stacking of cell sheets

We were concerned that the oligopeptides used for electrochemical detachment may remain on the surface of the detached cell sheet and hinder the cell sheet from attaching to another cell sheet during stacking. RGD peptides supplemented in culture medium have been used to prevent cell adhesion to a culture dish [31]. In this study, however, the detached cell sheet was readily attached onto another cell sheet within 30 min of incubation. This rapid adhesion may be attributed to the extracellular matrix (ECM) secreted by fibroblasts and which accumulated between a cell sheet and the substrate. Because the ECM is typically composed of huge molecules, the influence of the oligopeptide on the adhesion of cell sheets should be negligible. This ECM layer was detected in cell sheets formed using thermo-sensitive polymer-grafted substrate and was found to be responsible for the attachment of a cell sheet onto another cell sheet [32]. In the system using the thermo-sensitive polymer-grafted substrate, ~30 min were required to detach the cell sheet [15]. In our electrochemical system, only <7 min was required for detachment, and the cells were not exposed to non-physiological temperatures, which may be beneficial when repeating the processes to obtain a multilayered cell sheet. In this study, the 7-min detachment of a cell sheet and 30-min attachment to another cell sheet were repeated 2–5 times to fabricate multilayered cell sheets. The cross-sectional images (H&E) of multilayered cell sheets are shown in Fig. 4d–g. Because of the improved oxygen supply as described above, fibroblasts formed a thick cell sheet on the membrane substrate (Fig. 4d), making it easy to handle each cell sheet (Fig. 4h), repeat the stacking processes, and fabricate a thick cell sheet. By simply repeating the processes 5

times, the thickness of a stacked cell sheet reached more than 200  $\mu\text{m}$  (Fig. 4i).

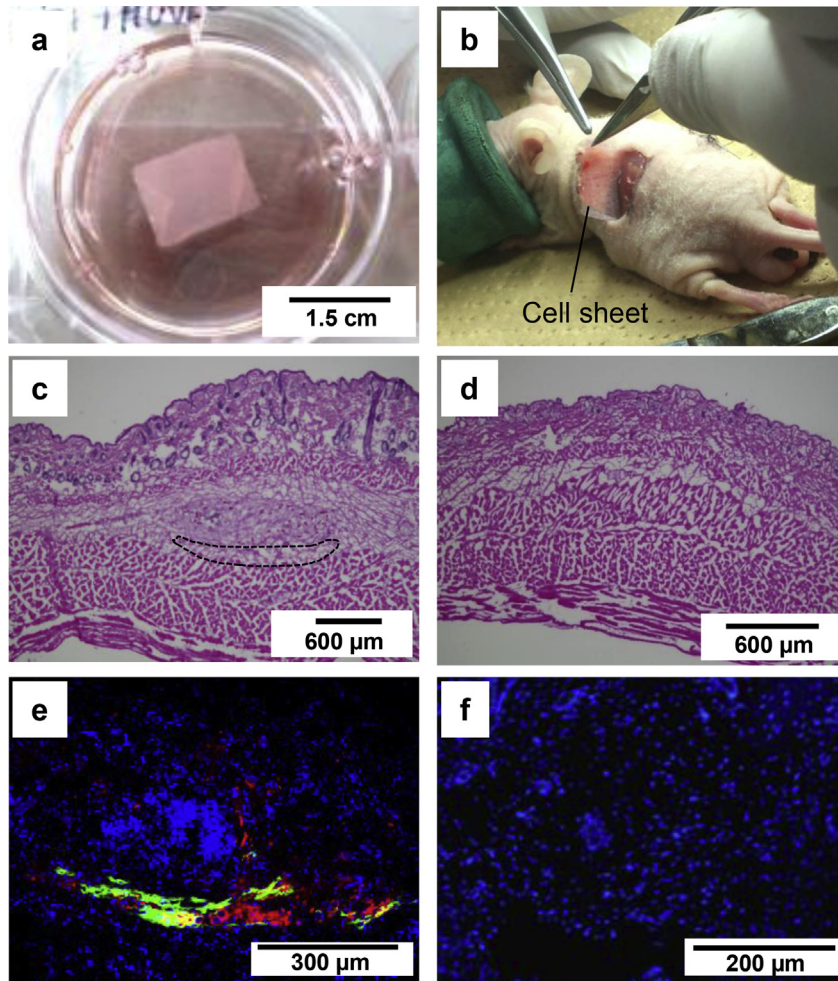
As the processes were repeated, thicker cell sheets were fabricated, but necrotic cell death occurred at the center of the cell sheet where the thickness was too large. The maximum thickness of a cell sheet,  $W_{max}$  [cm], defined here as the thickness containing a nonzero oxygen concentration at the center of cell sheet, can be estimated roughly from the mass balance of oxygen diffusion and consumption based on the one-dimensional Fick's law [27,33],

$$D \frac{d^2 C}{dx^2} = OCR \cdot d \quad (1)$$

where  $x$  [cm] is the position along the thickness of the cell sheet and is zero at the center of cell sheet,  $C$  is concentration of oxygen [ $\text{mol}/\text{cm}^3$ ] and is a function at  $x$ ,  $D$  is diffusion coefficient of oxygen [ $\text{cm}^2/\text{s}$ ],  $OCR$  is oxygen consumption rate [ $\text{mol}/\text{cell}/\text{s}$ ], and  $d$  is the density of cells [ $\text{cells}/\text{cm}^3$ ]. Assuming that the system is in a steady state under controlled diffusion and  $D$ ,  $OCR$  and  $d$  are constant, Eq. (1) can be integrated as follows with the following boundary conditions: when  $x = 0$  and  $C = 0$ , when  $x = \pm W_{max}/2$ ,  $C = C_s$ , where  $C_s$  [ $\text{mol}/\text{cm}^3$ ] is the oxygen concentration at the surface of a cell sheet,

$$W_{max} = 2 \sqrt{\frac{2 \cdot D \cdot C_s}{OCR \cdot d}} \quad (2)$$

$W_{max}$  was estimated to be ~840  $\mu\text{m}$  when  $D$  was  $2 \times 10^{-5} \text{ cm}^2/\text{s}$  [34],  $C_s$  was  $2.1 \times 10^{-7} \text{ mol}/\text{cm}^3$  [35],  $OCR$  was  $1.4 \times 10^{-17}$  [35], and  $d$  was  $3.4 \times 10^8 \text{ cells}/\text{cm}^3$ , where  $d$  was calculated from the thickness of the cell sheet (Fig. 3c) and the number of cells (Fig. 3e) at 14



**Fig. 5.** Transplantation of fibroblast sheets in nude mice. (a) Fibroblast sheet after electrochemical detachment for transplantation. (b) Dorsal subcutaneous pocket of a nude mouse. (c, d) H&E staining of skin samples. The samples were obtained after 2 months of transplantation from mice with (c) or without (d) the transplant of a cell sheet. The area enclosed with dotted line in (c) indicates transplanted cell sheet. (e, f) Immunohistochemical staining of skin samples. The samples were obtained after 2 months of transplantation from mice with (e) or without (f) the transplant of a cell sheet. Red (PKH26) indicates previously labeled fibroblasts, blue (DAPI) indicates nucleus, and green (Alexa Fluor 488) indicates human collagen.

days of culture. In this calculation, we assumed that  $C_5$  was the saturated oxygen concentration in a 21%  $O_2$  atmosphere, and rotation or perfusion culture might satisfy this assumption.  $W_{max}$  should be lower than the above value in this study because cell sheets were cultured under static conditions. To fabricate a thicker cell sheet over  $W_{max}$ , it is necessary to engineer vascular networks and perfuse culture medium in a cell sheet [25,36]. Repeated transplantation of cell sheets has been used to fabricate a vascularized cell sheet *in vivo* and the thickness of cell sheet reached ~1 mm without a necrotic layer [37]. In this study, a cell suspension solution of GFP-HUVECs ( $1.25 \times 10^5$  cells/mL) and normal human neonatal skin fibroblasts ( $3.25 \times 10^5$  cells/mL) in 2 mL of culture medium were seeded in the membrane substrate. Over 7 days of culture, vascular networks were formed in the cell sheet (Supplemental Fig. 2) and branching of luminal structures was integrated as the vasculatures matured over time. Our future studies will involve transplantation of this prevascularized cell sheet into an animal model and evaluation of whether the vasculatures were anastomosed to *in vivo* vasculatures.

### 3.5. Transplantation of detached cell sheet in mice

To examine the *in vivo* survival and function of cell sheets, human neonatal skin fibroblasts were grown to form a cell sheet on the membrane substrate for 7 days and electrochemically detached

and transplanted into the subcutaneous pockets created on the dorsal surface of nude mice (Fig. 5a and b). One and 2 months after transplantation, no significant inflammatory responses and tumorigenic transformation were observed at the transplanted sites (Fig. 5c and d, 2 months). Accumulation of human collagen was observed near the PKH26-stained cell sheet (Fig. 5e), but not in non-transplanted mice (Fig. 5f). These results indicate that our electrochemical approach can be used to readily detach and transplant cell sheets.

## 4. Conclusion

In this study, a gold-coated membrane substrate modified with oligopeptides was utilized for cell sheet engineering. Cells grew and formed a thick cell sheet, typically 50  $\mu\text{m}$  in thickness, on the substrates because of the improved supply of oxygen and nutrients. The cell sheets were rapidly detached along with desorption of oligopeptides by the application of a negative potential. Detached cell sheets were stacked with one another to form a multilayered cell sheet. Electrochemically detached cell sheets were transplanted into subcutaneous pockets and remained near the transplanted sites for at least 2 months without causing significant inflammatory responses. This approach is promising for preparing and surgically transplanting thick cell sheets for regenerative medicine and tissue engineering applications.



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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.reth.2015.12.003>.

## References

- [1] Langer R, Vacanti JP. Tissue engineering. *Science* 1993;260:920–6.
- [2] Atala A. Engineering organs. *Curr Opin Biotechnol* 2009;20:575–92.
- [3] Vacanti CA, Langer R, Schloo B, Vacanti JP. Synthetic-polymers seeded with chondrocytes provide a template for new cartilage formation. *Plast Reconstr Surg* 1991;88:753–9.
- [4] Atala A, Bauer SB, Soker S, Yoo JJ, Retik AB. Tissue-engineered autologous bladders for patients needing cystoplasty. *Lancet* 2006;367:1241–6.
- [5] Park H, Cannizzaro C, Vunjak-Novakovic G, Langer R, Vacanti CA, Farokhzad OC. Nanofabrication and microfabrication of functional materials for tissue engineering. *Tissue Eng* 2007;13:1867–77.
- [6] Ronneberger B, Kao WJ, Anderson JM, Kissel T. In vivo biocompatibility study of ABA triblock copolymers consisting of poly(L-lactic-co-glycolic acid) A blocks attached to central poly(oxyethylene) B blocks. *J Biomed Mater Res* 1996;30:31–40.
- [7] Sung HJ, Meredith C, Johnson C, Galis ZS. The effect of scaffold degradation rate on three-dimensional cell growth and angiogenesis. *Biomaterials* 2004;25:5735–42.
- [8] Ohashi K, Yokoyama T, Yamato M, Kuge H, Kanehiro H, Tsutsumi M, et al. Engineering functional two- and three-dimensional liver systems in vivo using hepatic tissue sheets. *Nat Med* 2007;13:880–5.
- [9] Fukuda J, Khademhosseini A, Yeo Y, Yang XY, Yeh J, Eng G, et al. Micromolding of photocrosslinkable chitosan hydrogel for spheroid microarray and co-cultures. *Biomaterials* 2006;27:5259–67.
- [10] Kachouie NN, Du YA, Bae H, Khabiry M, Ahari AF, Zamanian B, et al. Directed assembly of cell-laden hydrogels for engineering functional tissues. *Organogenesis* 2010;6:234–44.
- [11] Nishida K, Yamato M, Hayashida Y, Watanabe K, Maeda N, Watanabe H, et al. Functional bioengineered corneal epithelial sheet grafts from corneal stem cells expanded ex vivo on a temperature-responsive cell culture surface. *Transplantation* 2004;77:379–85.
- [12] Nishida K, Yamato M, Hayashida Y, Watanabe K, Yamamoto K, Adachi E, et al. Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. *N Engl J Med* 2004;351:1187–96.
- [13] Sawa Y, Miyagawa S, Sakaguchi T, Fujita T, Matsuyama A, Saito A, et al. Tissue engineered myoblast sheets improved cardiac function sufficiently to discontinue LVAS in a patient with DCM: report of a case. *Surg Today* 2012;42:181–4.
- [14] Kwon OH, Kikuchi A, Yamato M, Okano T. Accelerated cell sheet recovery by co-grafting of PEG with PIPAAm onto porous cell culture membranes. *Biomaterials* 2003;24:1223–32.
- [15] Kwon OH, Kikuchi A, Yamato M, Sakurai Y, Okano T. Rapid cell sheet detachment from poly (N-isopropylacrylamide)-grafted porous cell culture membranes. *J Biomed Mater Res* 2000;50:82–9.
- [16] Cole MA, Voelcker NH, Thissen H, Griesser HJ. Stimuli-responsive interfaces and systems for the control of protein–surface and cell–surface interactions. *Biomaterials* 2009;30:1827–50.
- [17] Nandivada H, Ross AM, Lahann J. Stimuli-responsive monolayers for biotechnology. *Prog Polym Sci* 2010;35:141–54.
- [18] Wildt B, Wirtz D, Searson PC. Programmed subcellular release for studying the dynamics of cell detachment. *Nat Methods* 2009;6:211–3.
- [19] Chan EW, Park S, Yousaf MN. An electroactive catalytic dynamic substrate that immobilizes and releases patterned ligands, proteins, and cells. *Angewandte Chemie Int Ed* 2008;47:6267–71.
- [20] Yeo WS, Mrksich M. Electroactive self-assembled monolayers that permit orthogonal control over the adhesion of cells to patterned substrates. *Langmuir* 2006;22:10816–20.
- [21] Guillaume-Gentil O, Akiyama Y, Schuler M, Tang C, Textor M, Yamato M, et al. Polyelectrolyte coatings with a potential for electronic control and cell sheet engineering. *Adv Mater* 2008;20:560–5.
- [22] Inaba R, Khademhosseini A, Suzuki H, Fukuda J. Electrochemical desorption of self-assembled monolayers for engineering cellular tissues. *Biomaterials* 2009;30:3573–9.
- [23] Seto Y, Inaba R, Okuyama T, Sassa F, Suzuki H, Fukuda J. Engineering of capillary-like structures in tissue constructs by electrochemical detachment of cells. *Biomaterials* 2010;31:2209–15.
- [24] Mochizuki N, Kakegawa T, Osaki T, Sadr N, Kachouie NN, Suzuki H, et al. Tissue engineering based on electrochemical desorption of an RGD-containing oligopeptide. *J Tissue Eng Regen Med* 2011;7:236–43.
- [25] Lovett M, Lee K, Edwards A, Kaplan DL. Vascularization strategies for tissue engineering. *Tissue Eng Part B Rev* 2009;15:353–70.
- [26] Mallon CT, Chaumont CD, Moran N, Keyes TE, Forster RJ. Electrochemical desorption of fibrinogen from gold. *Langmuir* 2009;26:293–8.
- [27] Evenou F, Fujii T, Sakai Y. Spontaneous formation of highly functional three-dimensional multilayer from human hepatoma Hep G2 cells cultured on an oxygen-permeable polydimethylsiloxane membrane. *Tissue Eng Part C Methods* 2010;16:311–8.
- [28] Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996;86:353–64.
- [29] Thangarajah H, Yao DC, Chang EI, Shi YB, Jazayeri L, Vial IN, et al. The molecular basis for impaired hypoxia-induced VEGF expression in diabetic tissues. *Proc Natl Acad Sci U S A* 2009;106:13505–10.
- [30] Wolf K, Te Lindert M, Krause M, Alexander S, Te Riet J, Willis AL, et al. Physical limits of cell migration: control by ECM space and nuclear deformation and tuning by proteolysis and traction force. *J Cell Biol* 2013;201:1069–84.
- [31] Pierschbacher MD, Ruoslahti E. Influence of stereochemistry of the sequence Arg-Gly-Asp-Xaa on binding specificity in cell adhesion. *J Biol Chem* 1987;262:17294–8.
- [32] Yamato M, Akiyama Y, Kobayashi J, Yang J, Kikuchi A, Okano T. Temperature-responsive cell culture surfaces for regenerative medicine with cell sheet engineering. *Prog Polym Sci* 2007;32:1123–33.
- [33] Sumaru K, Sugiura S, Kanamori T. Optimal design of cell culture chip on the basis of oxygen and glucose supply to cultivated cells in the chip. *Biochem Eng J* 2007;36:304–9.
- [34] Powers MJ, Domansky K, Kaazempur-Mofrad MR, Kalezi A, Capitano A, Upadhyaya A, et al. A microfabricated array bioreactor for perfused 3D liver culture. *Biotechnol Bioeng* 2002;78:257–69.
- [35] Yanagawa F, Kaji H, Jang YH, Bae H, Yanan D, Fukuda J, et al. Directed assembly of cell-laden microgels for building porous three-dimensional tissue constructs. *J Biomed Mater Res A* 2011;97:93–102.
- [36] Sadr N, Zhu M, Osaki T, Kakegawa T, Yang Y, Moretti M, et al. SAM-based cell transfer to photopatterned hydrogels for microengineering vascular-like structures. *Biomaterials* 2011;32:7479–90.
- [37] Shimizu T, Sekine H, Yang J, Itoi Y, Yamato M, Kikuchi A, et al. Polysurgery of cell sheet grafts overcomes diffusion limits to produce thick, vascularized myocardial tissues. *FASEB J* 2006;20:708–10.