Micropatterning of the oligopeptides and cells

The cell-adhesive and cell-repulsive oligopeptides were patterned on the gold substrates by using microcontact printing.[20] Briefly, masters for preparing the PDMS mold were fabricated with SU-8 photoresist. The prepolymer solution composed of a silicone elastomer and a curing agent (10:1) was poured onto the molds and cured at 80°C for 30 min. The cured PDMS stamps were then peeled from the mold and cleaned with ethanol. To form the alternating patterns of cell-adhesive and cell-repulsive surfaces, the PDMS stamps were inked with a 50-μM solution of cell-adhesive oligopeptide and pressed gently onto the gold substrates for 5 min at room temperature. The stamps were peeled off, and the substrates were rinsed with ddH_2O and then immersed in a 50-μM solution of cell-repulsive oligopeptide overnight at 4°C. The substrates were removed from the solution and rinsed with ddH_2O, followed by 70% ethanol. Each substrate was then placed in a well of a six-well plate; fibroblasts were added (2.5×10^5 cells/mL, 2 mL/well), and the plates were incubated at 37°C. After 3 h of culture, the medium was replaced with a fresh medium. To quantify cell adhesion to the modified surfaces, cells were cultured for 3 h, 1 days, or 2 days; the modified substrates were removed and rinsed, and phase-contrast images were acquired as described above.

Transfer of cell micropatterns from gold substrates to collagen gels

The cell-adhesive and cell-repulsive oligopeptides were spatially patterned on gold substrates by microcontact printing as mentioned earlier. Modified substrates were cultured with GFP-HUVECs (2.5×10^5 cells/mL in 2 mL/well) in EBM-2 supplemented with SingleQuots growth supplement. After 3 h of culture, the culture medium was aspirated, and a type I collagen solution (2.4 mg/mL) was poured on the gold substrates and allowed to gel. A potential of −1.0 V was applied for 5 min, and the gel layer was then peeled from the substrate and placed into culture. Cells expressing GFP were observed at 3 h, 1 day, 2 days, and 3 days of culture on the collagen gels.

Data analysis

Data are expressed as means ± SD and were calculated from the results of at least three independent experiments. Numerical variables were statistically evaluated by the Dunnett's test for Figures 2 and 3B, by single-factor ANOVA for Figure 3C, and by Student's *t*-test for Figure 4D. A value of $p<0.05$ was considered significant.

Results and Discussion

Adsorption of proteins on oligopeptide-modified gold surfaces

The oligopeptides used in the present study were designed with alternating positively charged lysine (K) and negatively charged glutamic acid (E) residues (Fig. 1A, B), which contributed to the formation of a dense SAM through electrostatic forces.[21] The QCM measurements revealed that all three oligopeptides spontaneously adsorbed onto the gold surface. The adsorbed amounts of the cell-repulsive oligopeptide, CGGGKEKEKEK, and the cell-adhesive oligopeptide, CGGGKEKEKEKGRGDSP, were 140 ± 28 ng/cm^2 and 237 ± 49 ng/cm^2, respectively. There is little difference in the adsorbed molecular density between the oligopeptides when their molecular weights are taken into account (Fig. 2A). When the oligopeptides are aligned in the tetragonal configuration, these values are equivalent to an intermolecular distance of ~1.2 nm. This indicates the formation of a dense molecular layer, given that the intermolecular distance for alkanethiol SAMs is ~0.5 nm,[22] and an α-helix is ~1.2 nm in diameter.[23]

We next investigated whether nonspecific adsorption of proteins was blocked by the oligopeptide modification of the gold substrate. Two major glycoprotein components in the body, fibronectin and fibrinogen, were used for the experiments. Fibronectin, one of the extracellular matrix components, mediates cell adhesion through interactions with cell surface integrin receptors, and also serves as a bridge connecting other extracellular matrix components. Fibrinogen is a soluble plasma glycoprotein that is structurally similar to fibronectin and readily adsorbs onto culture surfaces to mediate cell adhesion.[24] Because both fibronectin and fibrinogen show avid nonspecific binding to various materials, a surface that can repel binding of these proteins may be useful for applications requiring controlled cell adhesion for cell micropatterning and electrochemical detachment. As shown in Figure 2B, the adsorption of both fibronectin and fibrinogen onto the gold surface was reduced by surface modification with the bridge-shaped and cell-repulsive oligopeptides; however, the zwitterionic oligopeptide modification was particularly effective in this regard. Thus, our

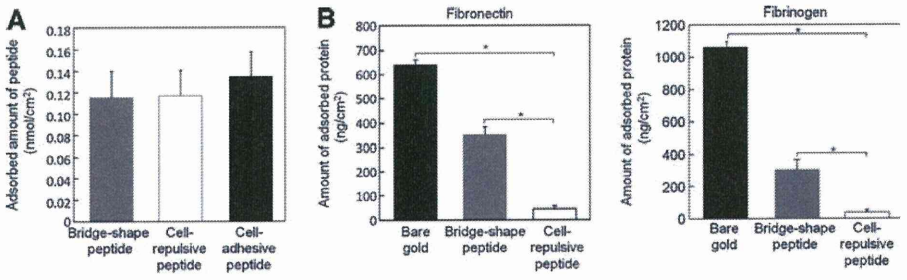

FIG. 2. Oligopeptide-modified gold surfaces and their nonfouling properties. **(A)** Spontaneous adsorption of cell-repulsive, cell-adhesive, and bridge-shaped oligopeptides. **(B)** Fibronectin and fibrinogen adsorption onto bare gold surfaces, or gold surfaces modified with the bridge-shaped or cell-repulsive oligopeptides. The error bars indicate SD calculated from three independent quartz crystal microbalance measurements. *$p<0.05$ compared to the bare gold and bridge-shaped oligopeptide surfaces.

results highlight the enhanced nonfouling properties of the oligopeptide modification when a zwitterionic molecule was used. This is consistent with previous reports in which peptides containing uniformly distributed negatively and positively charged residues were developed for ultra-low-fouling surface modifications.[21]

Contrary to our expectations, there was no difference in the amount of cell-repulsive and bridge-shaped oligopeptides adsorbed to the gold substrates (Fig. 2A). Nonetheless, there was a clear difference in the nonfouling properties of the two oligopeptides (Fig. 2B), which is likely due to the difference in the shapes adopted by the adsorbed molecules. The terminal cysteines of the bridge-shaped oligopeptides could form disulfide bonds with other molecules to create long molecular chains. This is far less likely to occur with the zwitterionic oligopeptide, because it contains only one cysteine, which favors formation of a densely packed single molecular layer.

Cell adhesion on oligopeptide-modified gold surfaces

To investigate the effects of the cell-repulsive and cell-adhesive oligopeptides on cell adhesion, the gold surfaces were modified with solutions containing varying molar ratios of the two zwitterionic molecules, ranging from 100% cell-adhesive oligopeptide to 100% cell-repulsive oligopeptide (Fig. 1D). After fibroblasts had been cultured on these surfaces for 3 h at 37°C, the substrates were washed, and images of the adherent cells were acquired. As expected, the number of adherent fibroblasts decreased in proportion to the decreasing ratio of the cell-adhesive to the cell-repulsive oligopeptide (Fig. 3A). Adherent cells exhibited a spread and extended morphology when incubated on surfaces coated with 100% and 1% cell-adhesive oligopeptide, but this was reduced on 0.1% surfaces, and almost all cells had a rounded morphology when present on surfaces modified with 0.01%, 0.0001%, and 0% cell-adhesive oligopeptide. Quantification of the images acquired after the 3-h incubation showed a small and insignificant difference ($p=0.10$) in the number of adherent cells on surfaces modified with 100% and 1% cell-adhesive oligopeptide (Fig. 3B). However, a further decrease in the oligopeptide ratio resulted in a dramatic reduction in the number of adherent cells. The modified surfaces also differed in their ability to support the growth of fibroblasts over 4 days of culture (Fig. 3C). Cells proliferated well on the surfaces with 100% and 1% cell-adhesive oligopeptide, with slightly better proliferation observed with 100% cell-adhesive oligopeptide. In contrast, cell proliferation was strongly limited on surfaces coated with <0.1% cell-adhesive oligopeptide. On day 2 of culture, the relative growth rate, calculated as the ratio of cells present compared to the initial cell number, was 2.9 and 3.0 for 100% and 1% surfaces, respectively, but was 1.7, 0.8, 1.5, and 0.9 for surfaces coated with 0.1%, 0.01%, 0.001%, and 0% cell-adhesive oligopeptide, respectively.

The minimum RGD density necessary to support cell adhesion has previously been examined for gold surfaces using mixed alkanethiol SAMs presenting RGD peptides and oligoethylene glycol. In those studies, the minimum RGD densities for cell adhesion and cell spreading were shown to

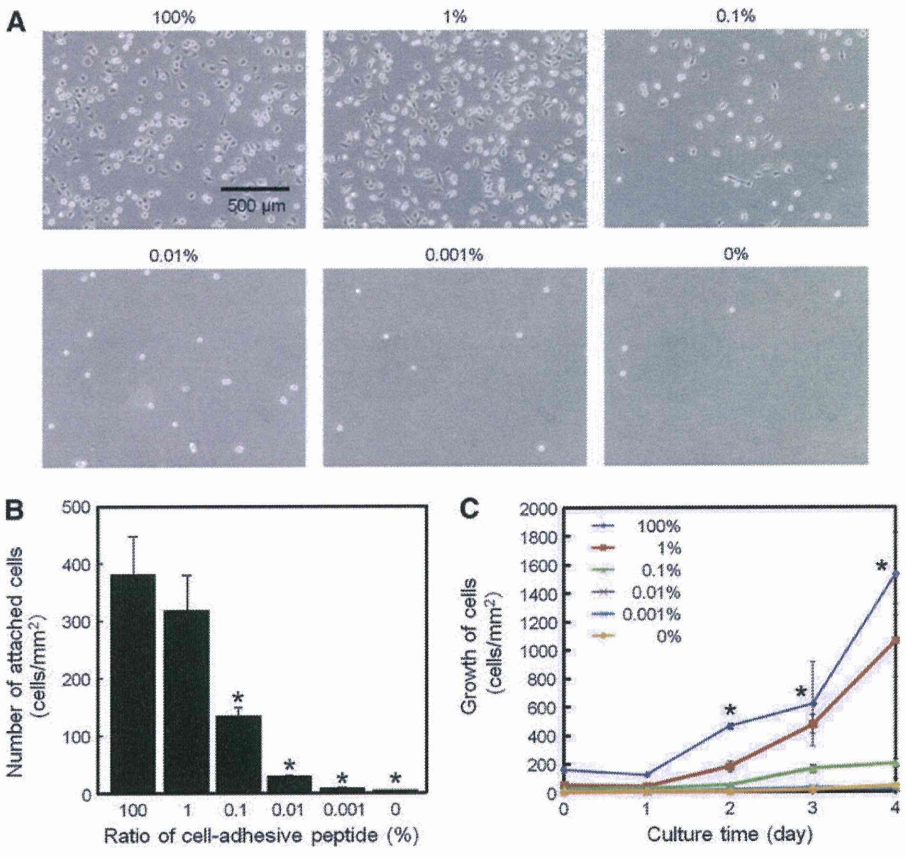

FIG. 3. Cell adhesion and growth on substrates prepared with different ratios of zwitterionic oligopeptides. **(A)** Phase-contrast images of cells after 3 h of culture. The percentage value above each panel indicates the molar ratio of the cell-adhesive oligopeptide to the cell-repulsive oligopeptide. **(B)** Quantitation of adherent cells obtained from image analyses. **(C)** Growth of adherent cells. The error bars indicate SD calculated from three independent experiments for each substrate. *$p<0.05$ compared to the 100% cell-adhesive oligopeptide **(B)**, or equal to or less than 0.1% cell-adhesive oligopeptide **(C)**. Color images available online at www.liebertpub.com/tea

be 0.001% and 0.1%, respectively, expressed as the ratio of RGD alkanethiol to oligoethylene glycol alkanethiol.[25,26] The RGD molar density was calculated based on the assumption that the relative molar ratio of the alkanethiols is the same in solution and on the gold surface; although this assumption may not be completely accurate, the relative trends are likely to be similar.[27,28] We also assumed that the RGD alkanethiols and oligoethylene glycol alkanethiols formed well-packed SAMs[28] with a molecular density of 0.93 nmol/cm^2.[22] Based on those assumptions, the minimum RGD density necessary to support cell adhesion and cell spreading was calculated to be 9.3 and 930 fmol/cm^2, respectively. In the present study, we estimated the density of surface-adsorbed zwitterionic oligopeptide to be 140 ng/cm^2, and noted that the transition between supportive and nonsupportive substrates was observed in surfaces coated with 0.1% ratio of cell-adhesive to cell-repulsive oligopeptides. Therefore, if we again assume that the ratio of oligopeptides in solution is proportional to that on the gold surface, then we calculate the RGD density required to support fibroblast adhesion at 140 fmol/cm^2. This calculation is an estimate and does not consider three-dimensional architecture and dynamics. Nevertheless, this estimate does suggest that the results observed here with oligopeptides are consistent with the previous reports using alkanethiol SAMs.

Detachment of cells after electrochemical desorption of oligopeptides

It is well documented that molecules adsorbed onto gold surfaces via gold–thiolate bonds can be reductively desorbed by applying a negative electrical potential.[22] In our previous studies, cells adhering on gold surfaces modified with either alkanethiol SAM or the bridge-shaped oligopeptides were

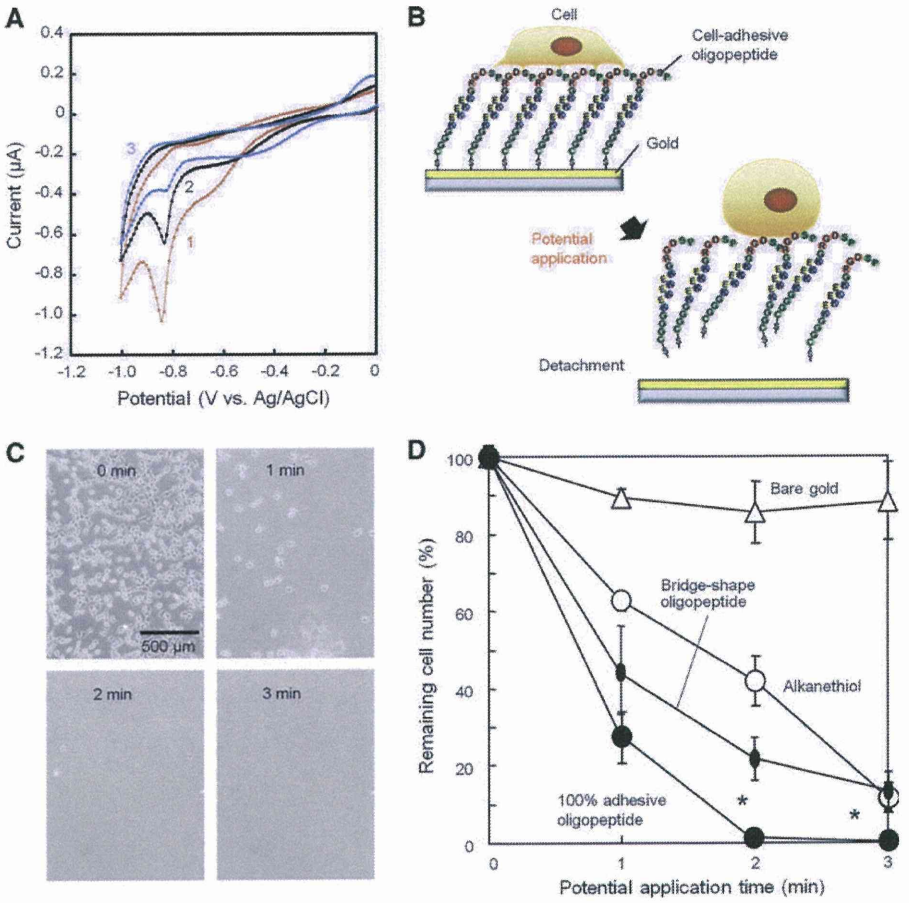

FIG. 4. Cell detachment after electrochemical desorption of the zwitterionic oligopeptides. **(A)** Cyclic voltammogram obtained during the reductive desorption of the oligopeptide; 1, 2, and 3 are scan numbers. Cyclic voltammograms were recorded at a scanning rate of 20 mV/s with respect to an Ag/AgCl reference electrode. The working electrode area was 8.0 mm^2. **(B)** Schematic diagram of the electrochemical detachment of cells along with desorption of the oligopeptide. **(C)** Phase-contrast micrographs indicating fibroblasts on the gold surface modified with the cell-adhesive oligopeptide were readily detached within 2 min. **(D)** Percentage fibroblasts remaining bound to substrate after negative potential application. Cells were enumerated by image analysis of surfaces modified with the cell-adhesive oligopeptide, bridge-shaped oligopeptide, 10-carboxy-1-decanethiol, or bare gold surface. Potential application was −1.0 V with respect to an Ag/AgCl reference. The error bars indicate SD calculated from three independent experiments for each plot. *$p < 0.05$ compared to the bridge-shaped oligopeptide. Color images available online at www.liebertpub.com/tea

detached by applying −1.0 V with respect to an Ag/AgCl reference electrode.[13,15] Here, we characterized the zwitterionic oligopeptide desorption process by cyclic voltammetry analysis, which showed that the peak potential for the reductive desorption of the oligopeptide appeared at −0.85 V in the first scan (Fig. 4A). In the second and third scans, the apparent peak at −0.85 V decreased, demonstrating that the oligopeptide was readily desorbed by the potential scanning. These measurements were performed in a strong alkaline KOH solution, whereas the cell detachment experiments were performed in PBS. The difference between the solutions could result in a potential shift of <100 mV.[29] In addition, the electrical resistance of the cells themselves may cause a drop in the potential. Considering the unpredictability of such contributions, we used a potential of −1.0 V for the detachment of cells in the subsequent experiments.

To examine detachment of cells adhering on a gold surface via the cell-adhesive oligopeptide (Fig. 4B), we acquired phase-contrast images after application of −1.0 V for 1, 2, or 3 min (Fig. 4C), and quantified the adherent cells. Although relatively few cells detached from the bare gold surface over the 3-min period, almost all cells detached within 2 min from the surfaces modified with the cell-adhesive zwitterionic oligopeptide (Fig. 4D). Detachment from the zwitterionic peptide-modified surface was much more rapid than from either the alkanethiol or bridge-shaped oligopeptide-modified surfaces. Notably, 100% of cells detached with the zwitterionic oligopeptide, whereas ~10% of cells did not detach with the bridge-shaped oligopeptide, even after 7-min potential application.[13] To our knowledge, this is the first description of an oligopeptide-based electrochemical cell detachment platform that allows complete cell retrieval in minutes.

The cell-adhesive oligopeptide contains the cell-anchoring sequence, GRGDSP, which forms a β-turn structure[30] and could create a bulkier peptide than the cell-repulsive oligopeptide, as illustrated in Figure 1D. We had expected that when the cell-adhesive and cell-repulsive oligopeptides are mixed at a certain ratio, a denser SAM may form that would accelerate cell detachment. To this end, we prepared solutions containing mixtures of the cell-adhesive and cell-repulsive

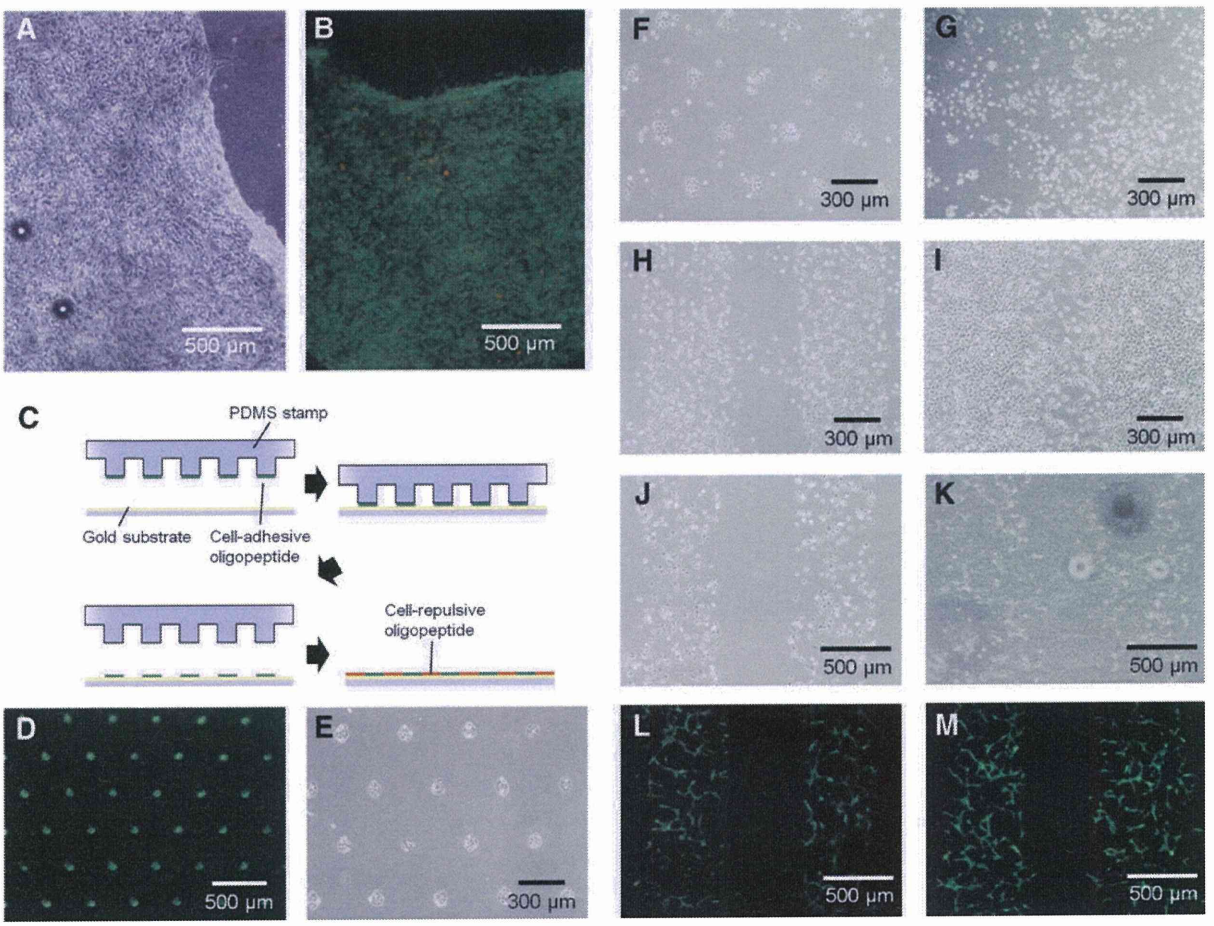

FIG. 5. Electrochemical transfer of cell sheets and micropatterned cells to collagen gels. **(A)** Detached human bone marrow-derived mesenchymal stem cell (hBMSC) sheet. **(B)** Live/dead fluorescent staining of the detached hBMSC sheets: green indicates live cells, and red indicates dead cells. **(C)** Procedure for micropatterning of the two oligopeptides. **(D)** Stamped fluorescein patterning. Fibroblasts adherent on the micropatterned surface at 3 h **(E)**, 1 day **(F)**, and 2 days **(G)** of culture and on striped pattern at 1 day **(H)** and 2 days **(I)** of culture. **(J)** Human umbilical vein endothelial cells constitutively expressing green fluorescent protein (GFP-HUVECs) on striped micropatterns at 3 h of culture. GFP-HUVECs transferred to collagen gels and cultured for 1 **(K)**, 2 **(L)**, and 3 days **(M)**. Color images available online at www.liebertpub.com/tea

oligopeptides at ratios of 10:90 and 1:99. However, cells detached from both the compositions within 2 min, similar to the kinetics observed on using 100% cell-adhesive oligopeptide. Optimization of the oligopeptide sequence, such as by varying the lengths of the alternating KE and GGG spacer, might provide a well-defined structure and closely packed monolayer.[31] In future investigations, we will focus on the design of SAM with shorter cell detachment times.

Electrochemical detachment of cell sheets

Cell sheet engineering is a promising approach in the field of regenerative medicine.[32] We therefore examined whether zwitterionic oligopeptide substrates might be useful for engineering cell sheets. To do this, we examined adhesion of hBMSCs, which have great potential as a cell source for tissue regeneration in humans.[33] hBMSCs were cultured on gold surfaces modified with the cell-adhesive oligopeptide for 3 days until the cells had formed confluent monolayers. The cell sheets were then coated with a collagen gel layer, and −1.0 V was applied for 5 min, after which the detached cell sheet was peeled off with the gel layer (Fig. 5A). The cell sheets were stained with fluorescent probes to discriminate live (FDA, green) and dead (EB, red) cells, which showed that almost all the cells in the harvested cell sheets were viable (Fig. 5B). This electrochemical cell detachment process is very rapid compared to other approaches, for example, thermoresponsive surfaces, which typically require 40–60 min for cell detachment.[8,34] This feature will be particularly beneficial to maintain cell viability when cell sheets are stacked during fabrication of multilayered cell sheets for transplantation.

Micropatterning and transfer of cells from the gold surface to collagen gels

We used micropatterning to create gold substrates modified with spatially controlled patterns of cell-adhesive and cell-repulsive oligopeptides (Fig. 5C). Cell-adhesive islands were first prepared with microcontact printing of the cell-adhesive oligopeptide, and the remaining regions were then modified with the cell-repulsive oligopeptide (Fig. 5C). Figure 5D shows a stamped pattern visualized with fluorescein. Fibroblasts seeded on the patterned surfaces selectively adhered to the islands containing the cell-adhesive oligopeptide (Fig. 5E). After 1 day of culture, most of the cells were confined to these adhesive regions, and only a few cells had migrated into the region containing the cell-repulsive oligopeptide (Fig. 5F). However, after 2 days of culture, a significant number of cells had randomly migrated over the entire surface, and the patterns virtually disappeared (Fig. 5G). This phenomenon was independent of the pattern geometry, and was also observed when the oligopeptides were applied in a striped pattern (Fig. 5H, I). These results suggest that the cell-repulsive oligopeptide inhibited the initial cell adhesive events, but did not block cell migration over a sustained period. We therefore examined the long-term stability of the bound cell-repulsive oligopeptide. The surface modified with the cell-repulsive oligopeptide was exposed to the culture medium containing 10% FBS for 24 h, and the amount of adsorbed protein was then quantified using QCM. We found that the surface coated with the cell-repulsive oligopeptide effectively prevents adsorption of proteins even after 24 h, suggesting that the oligopeptide remained attached on the surface (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea). However, we cannot exclude the possibility that there may be some oligopeptide SAM instability or contamination with other molecules, especially in the presence of cells, which may induce oligopeptide degradation or overlay of adhesive proteins on the SAM. Further investigation using real-time QCM or atomic-force microscopy will be necessary to clarify this.

We next determined whether cell micropatterns can be transferred to a hydrogel. As previously, micropatterned oligopeptides were applied to the gold surface; GFP-HUVECs were allowed to adhere; and a collagen layer was overlaid. A potential of −1.0 V was applied for 5 min, and the gel layer with detached cells was removed. The striped micropattern of adherent cells (Fig. 5J) was transferred to the hydrogel (Fig. 5K). The transferred HUVECs maintained their activity of network assembly as is typically observed in conventional collagen gel culture (Fig. 5L, M).[35] Collectively, these results demonstrate the noninvasive electrochemical transfer of cells from modified substrates to collagen gels. This process has the potential for rapid fabrication of complex tissues in a spatially controlled manner for tissue-engineering applications.

Conclusions

Custom-designed zwitterionic oligopeptides spontaneously formed SAMs on gold surfaces via gold–thiolate bonds and intermolecular electrostatic forces. The SAMs of cell-repulsive oligopeptides significantly decreased nonspecific protein adsorption and cell adhesion, whereas cell attachment and growth were supported by SAMs formed with >1% cell-adhesive oligopeptide. Adherent cells were completely detached from these surfaces within 2 min by applying a negative electrical potential. Furthermore, the difference in the ability of the two oligopeptide SAMs to support cell adhesion allowed fabrication and transfer of cell micropatterns to a hydrogel.

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Disclosure Statement

No competing financial interests exist.

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