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Rate control of cell sheet recovery by incorporating hydrophilic pattern in thermoresponsive cell culture dish

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Abstract: Thready stripe-polyacrylamide (PAAm) pattern was fabricated on a thermoresponsive poly(*N*-isopropylacrylamide) (PIPAAm) surface, and their surface properties were characterized. A PIPAAm surface spin-coated with positive photoresist was irradiated through a 5 μm/5 μm or a 10 μm/10-μm black and white striped photomask, resulting in the radical polymerization of AAm on the photoirradiated area. After staining with Alexa488 bovine serum albumin, the stripe-patterned surface was clearly observed and the patterned surface was also observed by a phase contrast image of an atomic force microscope. NIH-3T3 (3T3) single cells were able to be cultured at 37°C on the patterned surfaces as well as on a PIPAAm surface without pattern, and the detachment of adhered cells was more rapidly from the patterned surface after reducing temperature. Furthermore, the rate of detachment of 3T3 confluent cell sheet on the patterned surface was accelerated, compared with on a conventional PIPAAm surface under the static condition. The rate control of cell sheet recovery should contribute the preservations of cell phenotype and biological functions of cell sheet for applying to clinical trials. © 2013 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 102A: 2849–2856, 2014.

Key Words: cell sheet, thermoresponsive surface, micropatterning, cell adhesion, cell detachment

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INTRODUCTION

Poly(*N*-isopropylacrylamide)s (PIPAAms) exhibit a lower critical solution temperature (LCST) in aqueous media in the vicinity of 32°C. Although they hydrate and form an expanded chain conformation in aqueous media below the LCST, they dehydrate and form a compact-aggregated structure above the LCST. Such a conformational change in response to temperature has been extensively used to modulate the physicochemical properties of polymeric surfaces.[1–4] At 37°C, PIPAAm-modified surface is slightly hydrophobic, allowing cells to adhere and proliferate under normal conditions. A decrease in temperature below 32°C, however, results in the hydration of polymer surface, leading to the spontaneous detachment of the cells as a single and uniform tissue-like cell sheet. As PIPAAm is covalently immobilized onto the culture surfaces, PIPAAm never shows fallout and stay on the surfaces even after cell detachment. These robust grafts give the noninvasive harvest of cultured cells as an intact layer cell sheet containing deposited ECM. The cell sheet can be collected simply by reducing culture temperature lower than 32°C for less than several hours without any enzymes such as trypsin. This technology allows us to transplant cell sheets to the host tissues without the use of biodegradable scaffolds and suturing. The direct transplantations have been initiated clinical application to corneal epithelia, periodontal ligament cells, myoblast cells, and esophageal epithelia.[5–8]

To achieve clinical use of cell sheet, the rate control of recovery of cell sheets is an important technology for maintaining the biological function and viability of recovered cell sheet. In addition, the rapid recovery also contributes the reduction of time necessary for the practical assembly of tissue structures. The clinical trials using cell sheets have recently started and the rate-control cell sheet detachment

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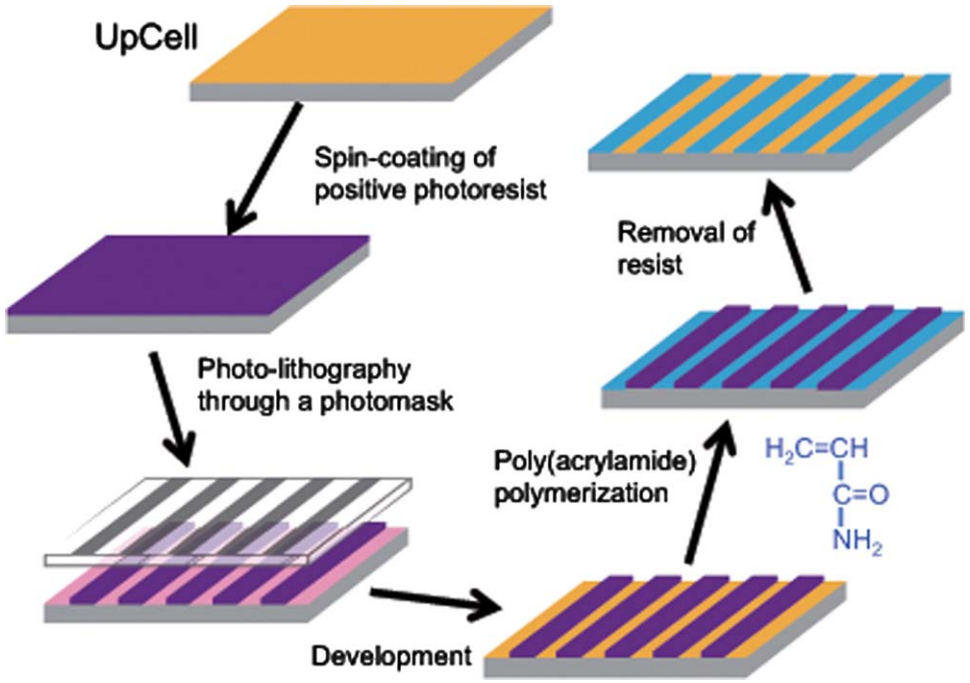

FIGURE 1. Synthetic illustration of the procedure of polyacrylamide (PAAm) pattern on a thermoresponsive dish (UpCell). In the first step, a resist solution (purple) was spin-coated on the surface at 8,000 rpm for 50 s. In the second step, the coated surface was irradiated by a lamp through a photomask (5-, 10-, or 50-μm wide stripes), followed by the development in an alkali solution. In the third step, PAAm (blue) was fabricated on the patterned surface. In the last step, the residual resist (purple) was removed with an alkali solution. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

was required for the suitable transplantation of cell sheet to organs of patients depending on the skill of medical doctors, while cell–cell junctions of cell sheet were maintained.[6,7] Although cell sheet detachment can be controlled by pipetting or agitation, weak mechanical property of thin cell sheet is prone to be fragile because of weakened cell–cell junctions. Therefore, spontaneous recovery of cell sheet is strongly required. Our laboratory has developed various methods for rate-control cell sheet recovery by modifying the surfaces. PIPAAm surfaces including a few functional hydrophilic moieties in the side chain have been developed.[9–11] Adhered cells spontaneously detach themselves more rapidly from the surfaces by reducing temperature, because the hydrophilic moiety around hydrophobic side chain is important to accelerate the hydration on the PIPAAm surfaces below the LCST, followed by detaching cells. Another challenge for rate-control cell sheet recovery is attempted by using PIPAAm-modified microporous polyethylene terephthalate membrane, which has a high water permeability into the contact area between the cultured cell and the polystyrene surface. A PIPAAm-modified porous membrane substrate permits a rapid cell sheet manipulation by facilitating water movement to the interface between the cell sheets and membrane surfaces. Other challenge is to use PIPAAm-modified surfaces with PIPAAm comb-grafts in the side chain. A high temperature-sensitivity of comb-grafted PIPAAm molecules, which independently shrink and stretch from the PIPAAm main chain, triggers a high water supply on the surface by reducing temperature, followed by the rapid cell sheet recovery.

From these studies describe above, change in the hydration of thermoresponsive surfaces is suggested to be a dominant factor for accomplishing the rate control of cell sheet detachment. Micropatterned fabrication based on surface chemistry exhibits the possibility of improving the chemical, physical, and structural properties of materials interface.[12–17] From the previous studies of cell culture on micropatterned surfaces, cell characters, such as cell differentiation and cellular death, can be modulated.[18–27] The microstripe-patterned surfaces with various lengths, which consisted cell adhesive and cell nonadhesive areas, were fabricated for controlling cell adhesion.[28,29] When the pattern width was smaller than 750 nm, many cells detached themselves from the surface, and when the width was 250 nm, cells could not attach to the surface, indicating that the focal adhesion of cells is strongly limited by the pattern.[28] In addition, NIH/3T3 cells are able to adhere, spread, proliferate, and reach confluency on the microstripe-patterned surface with 3-μm pitches.[29] According to the knowledge, a thermoresponsive polymeric dish with thready striped hydrophilic polymeric pattern, which was smaller in size than a cell and was larger than the length of focal adhesion, was fabricated and characterized in this study. Since the size of pattern was much smaller than that of cell, the thermoresponsive area in the patterned surface was assumed to keep its cell adhesion and proliferation abilities at 37°C, like a conventional thermoresponsive surface without pattern. Then, other hydrophilic area in the surface was speculated to trigger a possible rapid cell and cell sheet detachment after reducing temperature. In addition, the effect of

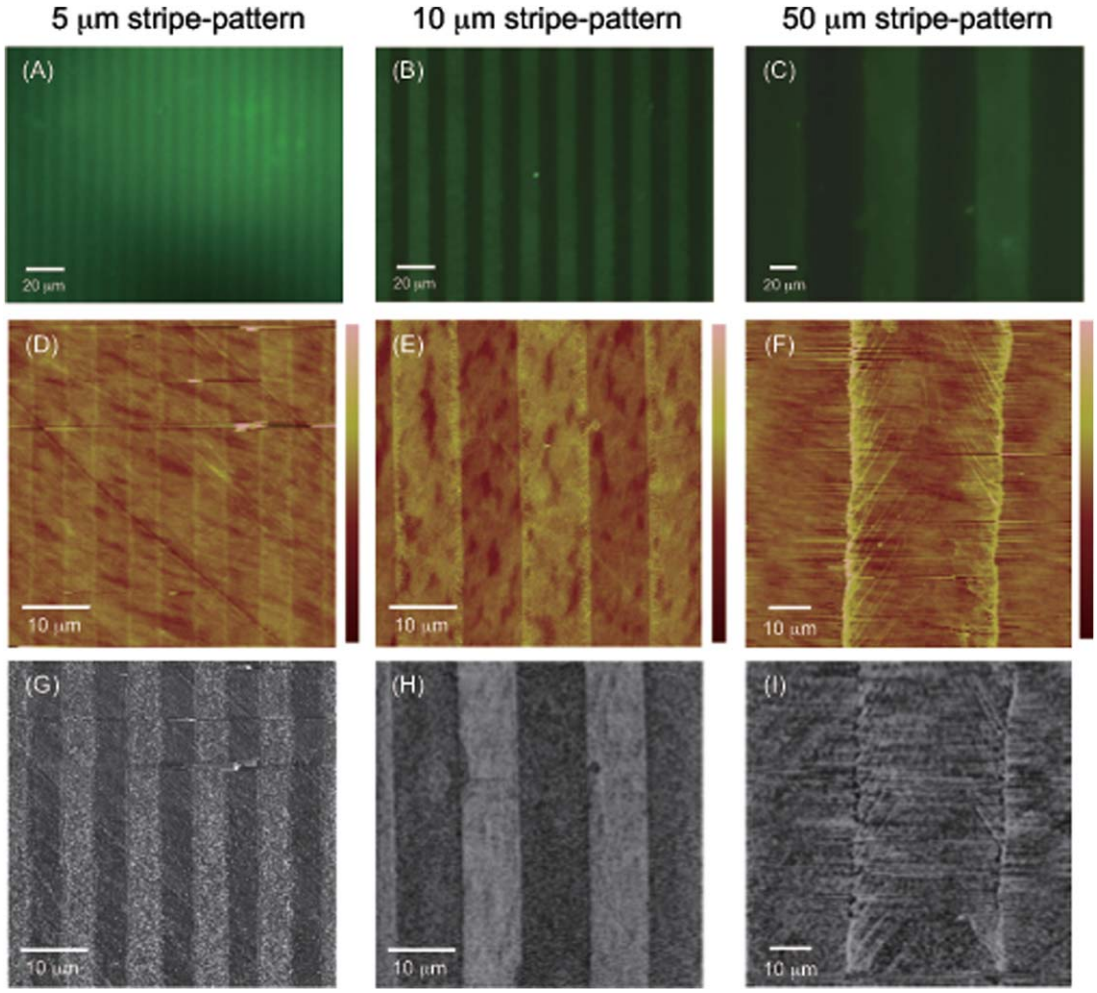

FIGURE 2. Characterization of patterned thermoresponsive surfaces (UpCell). Fluorescence image of the Alexa488 conjugated bovine serum albumin (Alexa488-BSA) absorbed micropatterned surface, which included 5-μm wide stripes of poly(N-isopropylacrylamide) (PIPAAm) alternating with 5-μm wide stripes of poly(acrylamide) (PAAm)-on-UpCell (A), 10-μm wide stripes of PIPAAm alternating with 10-μm wide stripes of PAAm-on-UpCell (B), and 50-μm wide stripes of PIPAAm alternating with 50-μm wide stripes of PAAm-on-UpCell (C) show that green fluorescence selectively on the PIPAAm surface. Scale bar: 20 μm. Atomic force microscope images of patterned UpCell at an ambient condition. Images of (D–F) are the topographic images of 5-, 10-, and 50-μm wide stripe patterns. Images (G–I) are the phase contrast images of 5-, 10-, and 50-μm wide stripe patterns. Scale bar: 10 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

stripe-pattern size was also discussed for controlling the rate of cell sheet recovery.

MATERIALS AND METHODS

Materials

Tissue culture polystyrene dishes (35–3002) were obtained from Becton Dickinson Labware (Franklin Lakes, NJ). NIH-3T3 cells (3T3s) were purchased from Health Science Research Resources Bank (Osaka) and were used after 15–20 passages for cell culture studies. Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA solution, and Mg^{2+}- and Ca^{2+}-free Dulbecco's phosphate-buffered saline (PBS) were from Sigma-Aldrich (St. Louis, MO). Streptomycin and penicillin were purchased from Gibco BRL (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Japan Bioserum (Hiroshima). Water used in this study was purified by a water purification system, Milli-Q A10 (Millipore, Billerica, MA), unless otherwise mentioned.

Preparation of thermoresponsive culture surfaces introduced polyacrylamide-micropattern

Thermoresponsive surface with a thready stripe-PAAm pattern was prepared by a similar method reported in a previous study (Fig. 1).[30] *g*-Line positive photoresist (TBR-001, Tokyo Ohka Kogyo, Kanagawa) was spin-coated at 8,000 rpm for 50 s on thermoresponsive culture dishes (UpCell®) (CellSeed, Tokyo), and the dishes were dried at 60°C for 2 h for evaporating organic solvents.[31] Then, photoirradiation was performed by a visible light using a lamp (250 W) for 3.5 s through a quartz mask (Dai Nippon Printing, Tokyo, Japan) (size: 18 × 18 mm, pattern size: 5-, 10-, and 50-μm black/white repeated pattern), followed by the development using a developer solvent (2.38% tertamethylammmonium

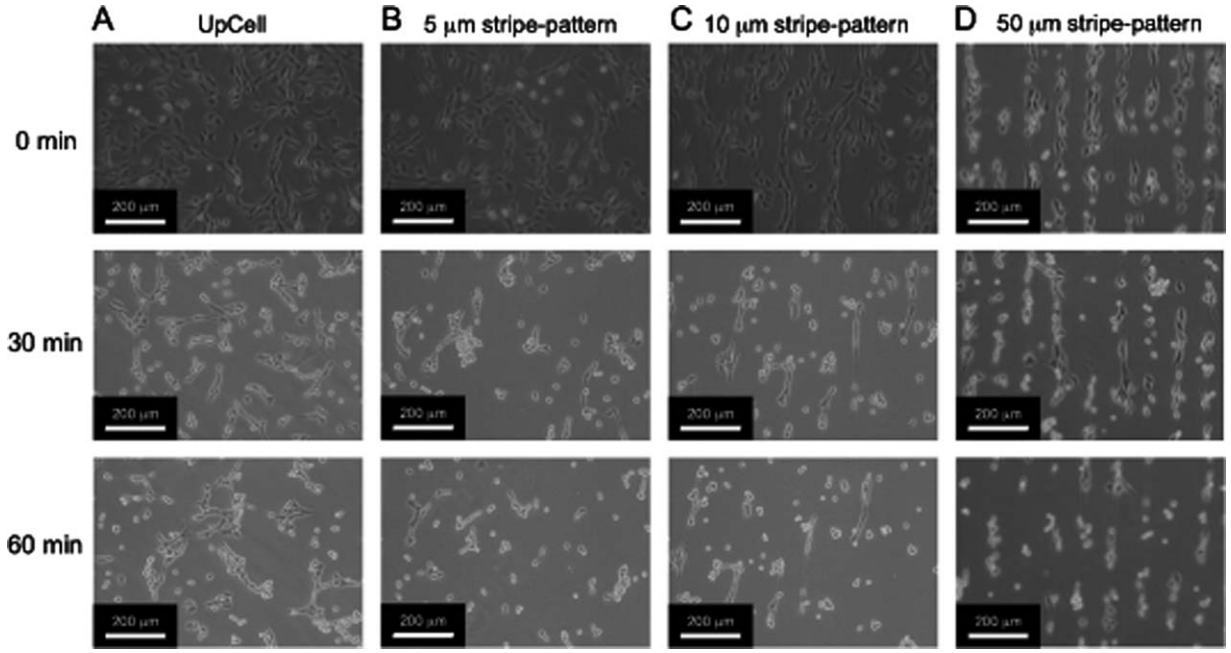

FIGURE 3. Microscopic photographs of (A) adhering cells on the control thermoresponsive dish (UpCell), (B) 5-μm wide stripe-poly(acrylamide) (PAAm) pattern on UpCell, (C) 10-μm wide stripe-PAAm pattern on UpCell, and (D) 50-μm wide stripe-PAAm pattern on UpCell after 0 min (37°C incubation at 24 h), 30 min, and 60 min at 20°C incubation.

hydroxide solution) (NMD-3) (Tokyo Ohka Kogyo) for 150 s. A total of 3.5 mL of 20 wt % acrylamide (AAm) (Wako Pure Chemical Industries, Osaka) aqueous solution containing 5 wt % 7,7-dimethyl-2,3-dioxobicyclo-[2,2,1]heptane-1-carboxylic acid (CQ-COOH) (Tokyo Chemical Industries, Tokyo) as an initiator was added on the photoresist-patterned culture dish, followed by radical polymerization on the developed position for 2 h by a blue light emitting diode (LED) (wave length: 450–470 nm) with an LED light source system for laboratory use (MIL-C1000T, MIL-U200, and MILB18(A), SANYO Electric, Osaka, Japan) After the reaction, the dish was thoroughly washed with warm water and was dried in oven at 50°C. Then, the surfaces were immersed in the developer solvent again to remove the residual photoresist for exposing the thermoresponsive area. Finally, the surfaces were washed with ultra pure water and dried to obtain micropatterned thermoresponsive culture surfaces.

Characterization of thermoresponsive culture surfaces with polyacrylamide-pattern

For the characterization of thermoresponsive culture dish with a stripe-PAAm pattern, the patterned surface was stained by a fluorescent-protein. Alexa488 bovine serum albumin (BSA) (1:200 diluted) (Invitrogen, Carlsbad, CA) was dissolved in 10% PBS at a concentration of 10 μg/mL. Two milliliters of the protein solution was spreaded to the patterned surface, and the surface was incubated at 37°C for 2 h. The patterned surface was then rinsed with PBS and examined by a fluorescence microscope (Eclipse TE2000, Nikon, Tokyo). Obtained images were processed with software (AxioVision 4.6; Carl Zeiss, Jena, Germany).

The depth of the patterned surface was measured by the height mode of an X-Y scanning tapping mode atomic force microscopy (AFM) (NanoScope® V Multimode) (Veeco, Santa Barbara, CA) using a n-doped Si tip (MPP-21100-10) (Veeco) at room temperature. Root-mean-square (RMS) values of the flattened images were further obtained using software.

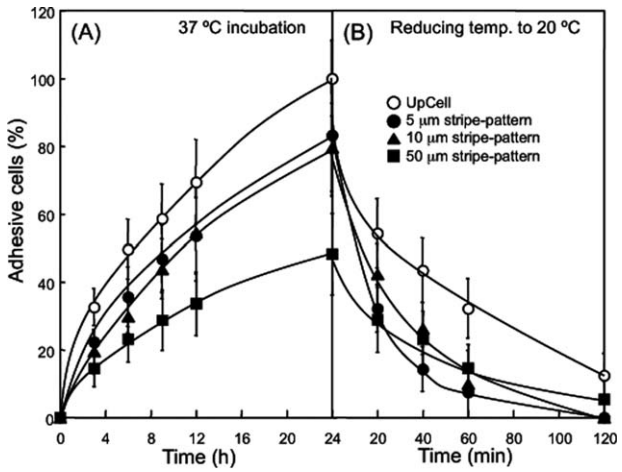

FIGURE 4. Time course of cell adhesion and detachment on the control thermoresponsive dish (UpCell) (the open circle), 5-μm wide stripe-poly(acrylamide) (PAAm) pattern on UpCell (the closed circle), 10-μm wide stripe-PAAm pattern on UpCell (the closed triangle), and 50-μm wide stripe-PAAm pattern on UpCell (the closed square) by temperature change. NIH 3T3 cells (3T3s) were incubated (A) at 37°C for 24 h and then (B) at 20°C for 120 min. Adhering cells were counted by using a microscope and averaged from four separate experiments. The number of adhering cells was shown as percentage of which 100% was the number on the UpCell at 24 h for each experiment.

TABLE I. Detachment of Single Cells and Cell Sheet on the Stripe-Patterned Thermoresponsive Surface

Code	Rate Constant of Single Cell Detachment[a]	Average Recovery Time of Cell Sheet ($n = 15$) (min)
UpCell	0.028	13.7 ± 8.7[b]
5-μm stripe pattern on UpCell	0.048	21.3 ± 12.3[b]
10-μm stripe pattern on UpCell	0.036	64.7 ± 14.7[b]
50-μm stripe pattern on UpCell	0.029	Not fabricated[c]

[a]Rate constant expresses the ability of 3T3 detachment from stripe-patterned surface by reducing temperature.

[b]Significant differences ($p < 0.05$) between UpCell and 5 μm stripe pattern and between UpCell and 10 μm stripe pattern determined by Mann–Whitney *U* test.

[c]Not fabricated means 3T3 cell sheets were hardly cultured on the surface.

Cell culture and cell sheet recovery on the thermoresponsive patterned surfaces

3T3s were cultured in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 microg/mL streptomycin. 3T3s were recovered from tissue culture poly(styrene) dishes by treatment with 0.05% trypsin and 0.05% ethylenediaminetetraacetic acid (EDTA) in PBS and were routinely cultured passage at 1/2 confluency. Then, 3T3s were seeded onto treated patterned surfaces. The seeded density of 3T3s was fixed at 1.0×10^4 cells/cm^2 for single cells experiment. Adhesion of cultured cells on the patterned surfaces was monitored by a phase contrast microscope (Eclipse TE2000, Nikon, Tokyo). Then, for investigating a temperature effect on cell detachment from the patterned surfaces, the patterned surfaces were incubated at 20°C at 5% CO_2 for 2 h, and the number of adhering cells was counted for another 2 h for evaluating single cell detachment. For the experiments of cell sheet harvest and recovery, cells were incubated at a density of 1.0×10^5 cells/cm^2 inside an viscoelastic poly(dimethyl siloxane) ring (18 mm in diameter) as a wall at 37°C for 48 h, then the patterned surfaces were incubated at 20°C at 5% CO_2, and the detachment of cell sheets were visually monitored by a video camera (Handycam, Sony, Tokyo). For confirming the cell–cell junctions after confluently cultured cells, adhesion of cultured cells on the patterned surfaces was monitored at 37°C after 24 h incubation by a phase contrast microscope. In addition, the recovered 3T3 cell sheets were fixed at 37°C with prewarmed 4% paraformaldehyde in PBS for 20 min and routinely processed into 10-μm-thick paraffin-embedded sections. Hematoxylin and eosin stained sections were prepared by conventional methods and examined by an optical microscope.

RESULTS AND DISCUSSION

Surface characterization of thermoresponsive patterned surfaces

Fluorescent images on the patterned surfaces after stained with Alexa488 BSA were shown in Fig. 2(A–C). Although Alexa488 BSA can attach on hydrophobic PIPAAm surfaces at 37°C, the fluorescent BSA was repelled on the hydrophilic PAAm-on-UpCell at the same temperature. Figure 2(A–C) clearly indicates that PAAm was chemically modified to a thermoresponsive surface (UpCell) as the designed pattern. Figure 2 also shows the stripe-patterned PAAm on UpCell evaluated by topographic and phase-contrast image measured by the tapping Mode AFM. The boundary of the patterned surface was observed from the topography image and the immobilized PAAm layer was confirmed to be very thin by the cross-sectional profiles of the images (data not shown) [Fig. 2(D–F)]. A clear contrast was also observed from phase-contrast images, indicating that the component of bright area (the brighter area in the topographic image) of PAAm was

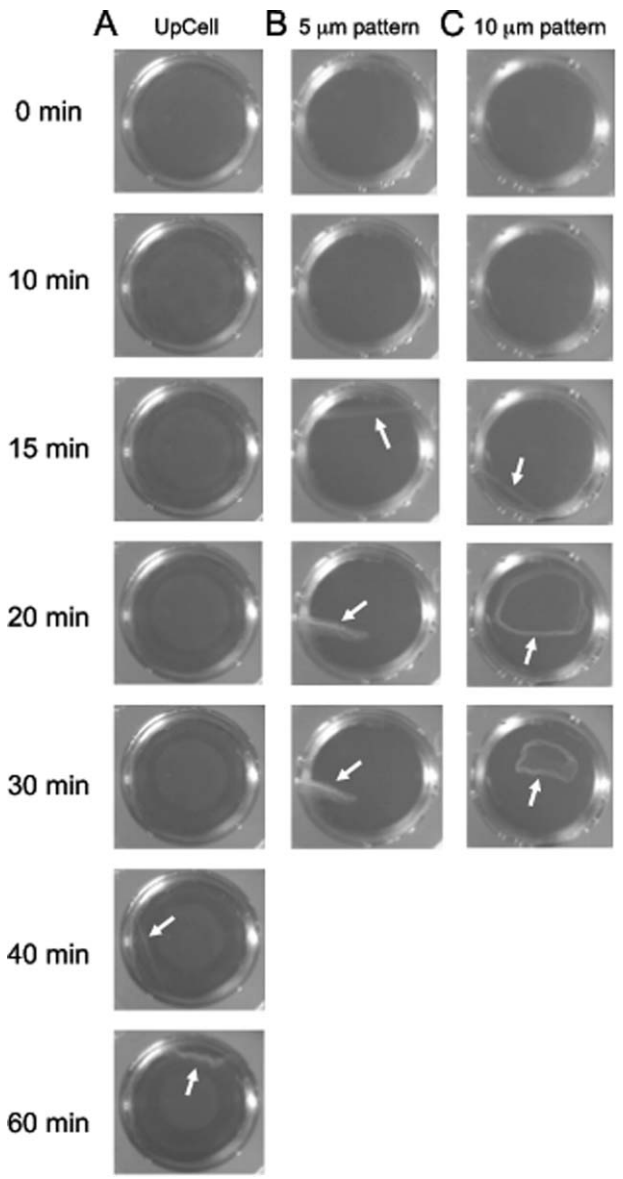

FIGURE 5. Macroscopic photographs of cell sheet detachment from (A) the controlled thermoresponsive dish (UpCell), (B) 5-μm wide stripe-poly(acrylamide) (PAAm) pattern on UpCell, and (C) 10-μm wide stripe-PAAm pattern on UpCell. Cell sheet spontaneously detached itself by reducing temperature from 37 to 20°C. The white arrows indicate the edge of spontaneously recovered cell sheet.

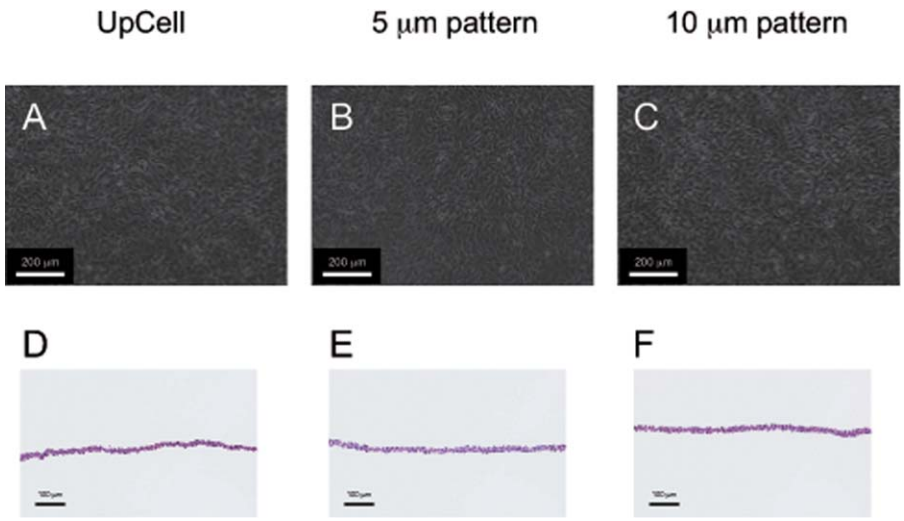

FIGURE 6. Microscopic photographs of confluent cells on (A) the controlled thermoresponsive dish (UpCell), (B) 5-μm wide stripe-poly(acrylamide) (PAAm) pattern on UpCell, and (C) 10-μm wide stripe-PAAm pattern on UpCell. Histological analyses of recovered cell sheets stained by hematoxylin–eosin (HE) from (D) the UpCell, (E) 5-μm wide stripe-PAAm pattern on UpCell, and (F) 10-μm wide stripe-PAAm pattern on UpCell. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

different from that of dark area (the darker area in the topographic image) of PAAm-on-UpCell [Fig. 2(G–I)]. From these results, PAAm molecules on UpCell were confirmed to be covalently grafted as a thready stripe pattern.

Single cells detachment on thermoresponsive patterned surfaces

The adhesion and detachment of 3T3 cells on the surfaces after reducing temperature was shown in Figure 3. The ratio of adhesion of 3T3 on the patterned surface was found to slightly reduce on that on UpCell after 24-h incubation at 37°C [Figs. 3(A–C) and 4(A)]. On the other hand, the ratio of adhesion of 3T3 on 50-μm wide stripe-patterned surface was ∼50%, because the size of pattern was bigger than that of cells and 3T3s hardly adhered on the PAAm-on-UpCell [Figs. 3(D) and 4(A)]. Although no cell detachment was observed on TCPS after reducing temperature to 20°C (data not shown), the cells were able to detach themselves on the controlled UpCell and the patterned surfaces, indicating that the patterned surfaces kept the function of the thermoresponsibility as well as the UpCell [Figs. 3 and 4(B)]. Importantly, less than 60 min for cell detaching on the patterned surface was required, while it seemed to require more than 60 min for detaching themselves on UpCell. For obtaining the rate constant of cell detachment, the single Langmuir models were performed using Eq. (1) as follows.[32]

$$C_d = \alpha - \beta e^{-\gamma t} \quad (1)$$

where C_d is the amount of adhered cell, α is the initial amount of adhered cells at 37°C for 24 h, β is the constant, γ is the rate constant, and t is time. In our study, 2D cell sheet having cell adhesive proteins at a basal side, such as ECM, was spontaneously detached after reducing temperature, meaning that cell sheet detachment should be promoted by the spontaneous detachment of proteins. Since one of the most useful models for evaluating a protein desorption is a exponential model, a Langmuir model for calculating the rate of detachment of cell sheet was used in this study.[33–35] The raw data for the detachment of 3T3s were able to be fit to Eq. (1), and the rate constants on UpCell, 5-, 10-, and 50-μm wide stripe-patterned surface were found to be ∼0.028, 0.048, 0.036, and 0.029, respectively (Table I). Generally, a high and low rate constants indicate a rapid and slow cell detachment, respectively. Although the detachment on 5- and 10-μm wide stripe-patterned surface were confirmed to be rapid, the detachment on controlled UpCell was slow. The grafts of hydrophilic thready PAAms were suggested to slightly change the interfacial hydration on surfaces, resulting in accelerating the single cell recovery on the surface. Considering the pattern size, the sizes of the 5- and 10-μm wide stripe pattern were smaller than that of a cell, and the lengths of pattern were much larger than that of focal adhesion of a cell, meaning that 3T3 were able to adhere on the 5- and 10-μm wide stripe-patterned surface in this study.[28,36,37] These results confirmed that the recovery of 3T3 cell sheet on the patterned surface was more rapid than that on the unpatterned UpCell.

Cell sheet detachment on thermoresponsive patterned surfaces

3T3 cells were seeded on thermoresponsive patterned surface at a density of 1.0×10^5 cells/cm^2 and 3T3 cell sheet was able to fabricated on the thermoresponsive patterned surface (5- and 10-μm wide stripe patterns) as well as the conventional thermoresponsive surface (UpCell) after 24-h incubation. Confluently cultured cell sheet, like single cells, was also readily detached itself from thermoresponsive surfaces. These sheets were facilely detached themselves from the surfaces, maintaining their cell–cell junctions by

lowering the culture temperature to 20°C. Although 64.7 min incubation at 20°C was required to detach nearly all adhering cells from the controlled UpCell after reducing temperature [Fig. 5(A)], 13.7 min required for recovering a cell sheet from the 5-μm wide stripe-patterned surface [Fig. 5(B)], indicating that the thready hydrophilic pattern accelerated the rapid cell sheet recovery. Furthermore, 21.3 min required for recovering cell sheet from 10-μm wide stripe-patterned surface [Fig. 5(C)]. Considering the effect of PAAm pattern toward the recovery of cell sheet, highly hydrophilic PAAm grafts were suggested to enhance water diffusion to the interface between the patterned surface and attached cell sheet. Detailed information of cell sheet recovery is shown in movie files (Supporting Information).

The average recovery time of 15 cell sheets was ~13.7 min on 5-μm wide stripe-patterned surface, and the average of recovery time was 64.7 min on the controlled UpCell. Furthermore, the average of recovery time of 15 cell sheets was ~21.3 min on 10-μm wide stripe-patterned surface, indicating that the recovery rate was possibly controlled by the size of pattern. From the statistical analyses of the rate of cell sheet recovery was performed using Mann–Whitney U test, significant differences ($p < 0.05$) were showed between the recovery times on 5-μm wide stripe patterned and the control thermoresponsive surfaces, 10-μm wide stripe patterned and the control UpCell, and 5- and 10-μm wide stripe-patterned surfaces. Considering the difference of the recovery rate on 5- and 10-μm wide stripe-patterned surfaces, more narrow thready pattern was assumed to trigger more rapid detachment of cell sheet, because the detachment of single cells was more rapid on the 5-μm wide stripe-patterned surface in Figure 4(B). Figure 6(A–C) shows the microscopic photographs of cultured 3T3s on thermoresponsive patterned surfaces after 24 h at 37°C. Cells were found to be confluently cultured on the surfaces and the shape of adhered cells was hardly corresponded to the patterned figure. The histological analyses of cell sheets also revealed that the cultured cell sheets maintained the cell–cell junctions, indicating that 3T3s bridged across the thready hydrophilic stripe patterns [Fig. 6(D–F)]. Since the size of pattern was much smaller that that of the cell, cell adhesion was hardly inhibited on the patterned surface as the similar results of single cell adhesion in Figures 3 and 4.

CONCLUSION

In this study, thready striped and ultrathin hydrophilic polyacrylamide (PAAm) pattern on thermoresponsive surfaces was fabricated and characterized. The striped patterned surface was clearly observed by a fluorescence stain and a phase contrast image of an atomic force microscope. NIH-3T3 (3T3) single cells adhered on the patterned surface at 37°C as well as on a conventional thermoresponsive surface and adhered 3T3s detached themselves more rapidly on the patterned surface than on the thermoresponsive surface after reducing temperature. Furthermore, the rate of detachment of 3T3 confluent cells sheet on the patterned surface was much faster than that on the conventional thermoresponsive surface. This acceleration of cell sheet detachment should contribute not only to maintaining cell phenotype and biological functions of cell sheet but also to supporting clinical trials.

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