Electronic Supplementary Information

Micro/Nano-Imprinted Substrates Grafted with a Thermoresponsive Polymer for Thermally Modulated Cell Separation

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Experimental Section

Materials

N-isopropylacrylamide (IPAAm) was kindly provided by KJ Chemicals (Tokyo, Japan). Styrene (St) and 4-vinylbenzyl chloride (VBC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The polymerization inhibitor was removed by passing the monomer solution through an inhibitor removal column (Sigma-Aldrich). Sodium hydroxide, formic acid, formaldehyde, tris(2-aminoethyl)amine (TREN), 2-propanol, tetrahydrofuran, methanol, acetone, toluene, CuCl, ethylenediamine-*N,N,N′,N′*-tetraacetic acid (EDTA), and 2,2'-azobisisobutyronitrile (AIBN) were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Ethyl 2-chloropropionate (ECP) was obtained from Tokyo Chemical Industry (Tokyo, Japan). Tris(2-*N,N*-dimethylaminoethyl)amine (Me_6TREN) was synthesized according to a previously reported method.[1] Glass coverslips (18 × 18 mm, 0.5-mm thick) were purchased from Matsunami Glass (Osaka, Japan). Phenylethyltrimethoxysilane (PETMS) was purchased from Gelest (Morrisville, PA, USA). The nano-imprinting mold (DTM-2-3) was obtained from Kyodo International (Kawasaki, Japan). Cell culture dishes were purchased from Corning (Corning, NY, USA). Red fluorescent protein-labeled human umbilical vein endothelial cells (RFP-HUVECs) and green-fluorescent protein normal human dermal fibroblasts (GFP-NHDFs) were purchased from Angio-Proteomie (Boston, MA, USA). The antifade reagent ProLong gold was obtained from ThermoFisher (Waltham, MA, USA). Other cells and cell culture media were purchased from Lonza (Basel, Switzerland).

Polymerization of P(St-*co*-VBC)

P(St-*co*-VBC) was prepared as the thermal nano-imprint lithography (NIL) substrate material. Bulk polymerization with an initiator was conducted. St (36.5 mL, 0.315 mol) and VBC (5 mL, 0.035 mol) were mixed in a 100-mL eggplant flask, and AIBN (164.2 mg, 1.0 mmol) was added to the monomer mixture solution. The monomer solutions were degassed by triplicate freeze-thaw cycles and sealed under reduced pressure with a stop-cock. Polymerization was performed at 70 °C for 20 h. The copolymer was purified by precipitation by dissolving in a small amount of toluene and dropping the solution into 2 L of methanol. The

copolymer was filtered and dried under vacuum for 2 h. The molecular weight of P(St-*co*-VBC) was determined using gel-permeation chromatography (GPC; GPC-8020: columns TSKgel SuperAW2500, SuperAW3000, and SuperAW4000, Tosho, Tokyo, Japan) with *N,N*-dimethylformamide containing 50 mM lithium chloride as the mobile phase, and was calibrated using polystyrene standards. The composition of each monomer in P(St-*co*-VBC) was determined by ^{1}H NMR spectroscopy, with deuterochloroform as the solvent.

Fabrication of micro/nano-convex or concave structures using NIL

The surfaces of glass coverslips were modified with hydrophobic phenethyl groups through sialinization reactions in order to increase the stability of the P(St-*co*-VBC) on the glass substrates. The glass coverslips were cleaned by oxygen plasma irradiation for 180 s (intensity: 400 W, oxygen pressure: 0.1 mmHg) in a plasma dry cleaner (PX-1000; March Plasma Systems, Concord, CA, USA). The cleaned glass coverslips were placed in a separator flask that was humidified at 60% for 2 h. The silane coupling reaction solution was prepared by dissolving PETMS (3.50 mL, 16.0 mmol) into toluene (340 mL). The solution was added to the flask and the silane coupling reaction was performed for 18 h at 25 °C under continuous stirring. After the reaction, the glass coverslips were rinsed with toluene and acetone, and then dried at 110 °C for 1 h.

P(St-*co*-VBC) was dissolved in toluene at a concentration of 10 wt%. Hydrophobized glass coverslips were set on a spin-coater (ACT-300D II, Active, Saitama, Japan), and the copolymer solution (360 μL) was dropped onto the glass. Spin coating was performed under the following conditions: the rotation rate was increased to 400 rpm and maintained for 5 s, and then increased to 3000 rpm and maintained for 30 s. The polymer-coated glass coverslips were dried overnight under reduced pressure at 45 °C.

Micro/nano-structures were fabricated in the coated copolymer layer on the glass coverslip using a thermal NIL apparatus (Thermal mini, EHN-3250, Engineering System, Matsumoto, Japan) and a nano-imprinting mold with hole, pillar, and line patterns (DTM-2-3), respectively. The mold structures are shown in Fig. S1. The P(St-*co*-VBC)-coated glass coverslip was heated at 150 °C and the nano-imprinting mold was compressed against the coated P(St-*co*-VBC) layer at 1500 N for 120 s. The copolymer-coated glass coverslip was cooled to 50 °C, and the nano-imprinting mold was removed from the copolymer layer.

Thermoresponsive polymer brush grafting via ATRP

PIPAAm was grafted on the nano-imprinted P(St-*co*-VBC) layer surface through surface-initiated ATRP. IPAAm (226 mg, 2.0 mmol) was dissolved in 20 mL of methanol:water (80:20 v/v; 100 mM) in a 100-mL flask. The monomer solution was deoxygenated by argon gas bubbling for 1.5 h. CuCl (13.2 mg, 0.13 mmol) and $CuCl_2$ (1.75 mg, 0.013 mmol) were added to the solution under an argon atmosphere and the solution was stirred for 15 min. The glass coverslip with the nano-imprinted P(St-*co*-VBC) layer was placed in a 50-mL glass vessel, and the glass vessel and monomer solution in the flask were placed in the glove bag. Evacuation and argon gas purging of the glove bag were performed three times to remove the oxygen in the glove bag. Me_6TREN (33.8 mg, 0.150 mmol) was added to the monomer solution and mixed by shaking to generate the ATRP catalyst. The reaction solution was poured into the glass vessel containing the nano-imprinted substrate, and ECP (1.64 μL, 0.012 μmol) was immediately added to the reaction solution. ATRP was performed at 25 °C for 1 h with continuous shaking of the glass vessel. After the ATRP, the PIPAAm-modified nano-imprinted substrate was rinsed with the methanol:water (80:20 v/v) mixed solution and water, and dried under reduced pressure at 25 °C overnight. The reaction solution containing free PIPAAm was dialyzed against EDTA solution and water using a cellulose dialysis membrane (molecular weight cut-off, 1 kDa). The purified solution was lyophilized and PIPAAm was obtained.

Characterization of thermoresponsive micro/nano-imprinted substrates

The surface elemental composition of the prepared substrates was determined by X-ray photoelectron spectroscopy (XPS; K-Alpha, ThermoFisher) using a monochromatic Al $K\alpha_{1,2}$ source and a take-off angle of 90°.

The number-average molecular weight and polydispersity index of PIPAAm were determined using GPC (GPC-8020) and columns (SuperAW2500, SuperAW3000, and SuperAW4000; Tosho). *N,N*-dimethylformamide containing 50 mM lithium chloride was used as the mobile phase at a flow rate of 0.6 mL/min. The elution time was calibrated using poly(ethylene glycol) standards.

The surface morphologies of the thermoresponsive nano-imprinted substrates were examined using scanning electron microscopy (SEM; VE-9800, Keyence, Osaka, Japan) with Au sputter deposition.

The wettability of the PIPAAm-grafted nano-imprinted substrate was determined by static contact angle measurements. The prepared thermoresponsive nano-imprinted substrate was placed in the chamber of the contact angle meter (DSA 100S, Kruss, Hamburg, Germany). Dulbecco's modified Eagle medium (2 μL), used as the cell culture medium, was gently placed on the substrate via a syringe, and the contact angle of the droplet was measured at 20 °C or 37 °C using the circle-fitting method. Data are expressed as the mean of five measurements with the standard deviation.

Fibronectin adsorption on thermoresponsive NIL substrates

To investigate the protein adsorption properties of the prepared thermoresponsive nano-imprinted substrates, rhodamine-conjugated fibronectin was adsorbed on the substrates. A silicone rubber frame (2 × 2-cm square) was placed on the NIL substrates. Rhodamine-conjugated fibronectin was dissolved in phosphate-buffered saline (PBS) and a 4 μg/mL solution was prepared. Then, 1 mL of the fibronectin solution was dropped onto the NIL substrates inside the silicone frame. The solution on the NIL substrates was incubated at 37 °C for 24 h or at 20 °C for 4 h. After each incubation, the NIL substrates were rinsed with PBS and observed with fluorescent microscopy (ECLIPSE TE2000-U, Nikon, Japan). The observed image was analyzed by ImageJ (National Institutes of Health, Baltimore, MD, USA) and the fluorescence intensity attributed to the adsorbed rhodamine was determined.

Cell adhesion and detachment behavior

Three types of cells, human umbilical vein endothelial cells (HUVECs),[2, 3] normal human dermal fibroblasts (NHDFs),[3-5] and human skeletal muscle myoblast cells (HSMMs)[6-8], were used for the investigation of cell adhesion and detachment behavior on the prepared substrates, since these cells are widely used for cardiovascular tissue engineering. HUVECs, NHDFs, and HSMMs were all cultured on conventional tissue culture polystyrene (TCPS) dishes (Falcon, 100-mm diameter, ThermoFisher Scientific, Waltham, MA, USA) using endothelial cell medium (EGM-2, Lonza), fibroblast cell medium (FGM, Lonza), and skeletal muscle myoblast cell medium (SkGM-2, Lonza), respectively. The cells were recovered from a conventional

TCPS dish by treatment with 0.1% trypsin containing 1.1 mM EDTA in PBS. Recovered cells were seeded on the prepared thermoresponsive NIL substrates at a density of 1.0×10^4 cells/cm^2. The cell-seeded substrates were incubated at 37 °C for 24 h in a humidified atmosphere of 5% CO_2 for 24 h, and then transferred to another incubator set at 20 °C. The cell morphology was observed at pre-determined intervals using a phase-contrast microscope (ECLIPSE TE2000-U, Nikon, Tokyo).

In observations of cell morphology in the co-culture condition, RFP-HUVECs and GFP-NHDFs were used for distinguishing between the cell types. The cell mixture suspension composed of RFP-HUVECs, GFP-NHDFs, and HSMMs was seeded on the prepared thermoresponsive NIL substrate at a density of 6.67×10^3 cells/cm^2 per cell type, for a total cell density of 2.0×10^4 cells/cm^2. SkGM-2 was used as the cell culture medium. The cell-seeded substrates were incubated at 37 °C for 24 h in a humidified atmosphere of 5% CO_2 for 24 h, and then transferred to another incubator set at 20 °C. The cell morphology was observed at pre-determined intervals using a fluorescence microscope (ECLIPSE TE2000-U).

To investigate the detailed cell adhesion behavior on the thermoresponsive NIL substrate, immunohistochemical staining of *p*-paxillin and actin fibers was performed. HUVECs, NHDFs, and HSMMs were seeded on the prepared NIL substrates with each cell culture medium and incubated for 37 °C for 24 h. Then, the cell culture medium was removed and replaced with pre-warmed PBS at 37 °C two times. Pre-warmed paraformaldehyde (4%, 37 °C) was added to the samples and the samples were incubated at 25 °C for 10 min. After the incubation, the paraformaldehyde was removed and the samples were rinsed with PBS two times. Triton (0.2%) was added to the sample and the sample was incubated at 25 °C for 10 min. After incubation with 0.2% Triton, the sample was rinsed with PBS two times. Then, 5% bovine serum albumin (BSA) was added to the samples, and the samples were incubated at 25 °C for 30 min and rinsed with 1% BSA two times. The samples were placed in the *p*-paxillin antibody solution prepared at a 50-times dilution with 1% BSA at 4 °C overnight. After the incubation, the samples were rinsed twice with 1% BSA solution. Then, the samples were incubated in an Alexa Fluor 488-conjugated goat anti-rabbit IgG(H+L) secondary antibody solution prepared at 500-times dilution with 1 % BSA at 25 °C for 30 min. The samples were rinsed with 1% BSA two times. Then, the samples were placed in 500 times-diluted Hoechst 33342 and 400 times-diluted Phalloidin 568 solutions prepared with 1% BSA and incubated at 25 °C for 30 min. After the incubation, the samples were rinsed with 1% BSA two times, followed by incubation with the antifade reagent

ProLong gold at 25 °C overnight. The stained samples were observed by a confocal laser-scanning microscopy (FluoView FV1200, Olympus, Tokyo).

In addition, field-emission (FE)-SEM was used for observing cell adhesion behaviors on the 2-μm hole pattern. Each cell type was incubated on the thermoresponsive nano-imprinted substrates at 37 °C for 24 h. Then, the samples were fixed with 4% paraformaldehyde which was warmed at 37 °C, and dehydrated using ethanol and tert-butyl alcohol, and freeze-dried. Au-sputtering was performed using a magnetron sputtering device (MSP-10, Vacuum Device, Ibaraki), and detailed cell adhesion behavior was observed using the FE-SEM apparatus (S-5500, Hitachi, Tokyo).

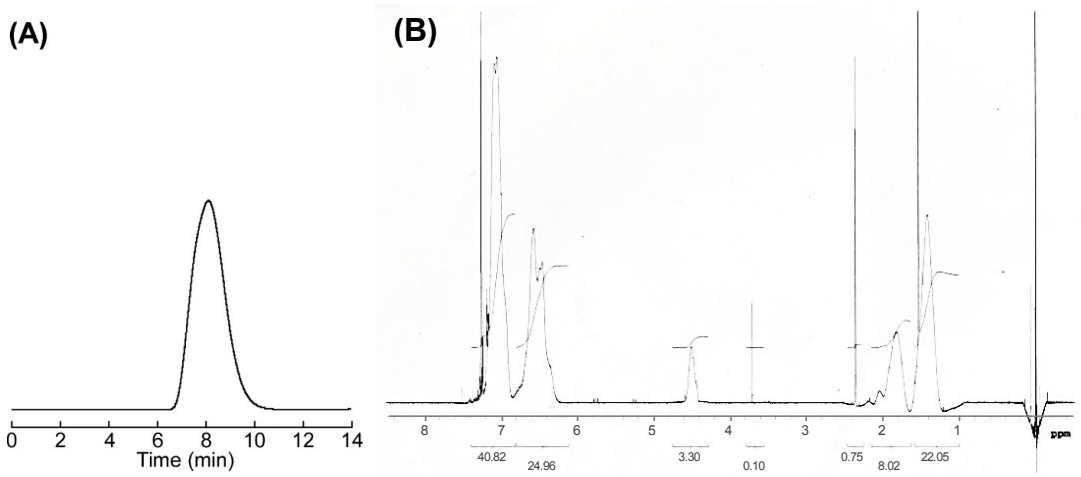

Figure S1. Gel-permeation chromatogram (A) and ^{1}H nuclear magnetic resonance spectrum (B) of synthesized P(St-*co*-VBC).

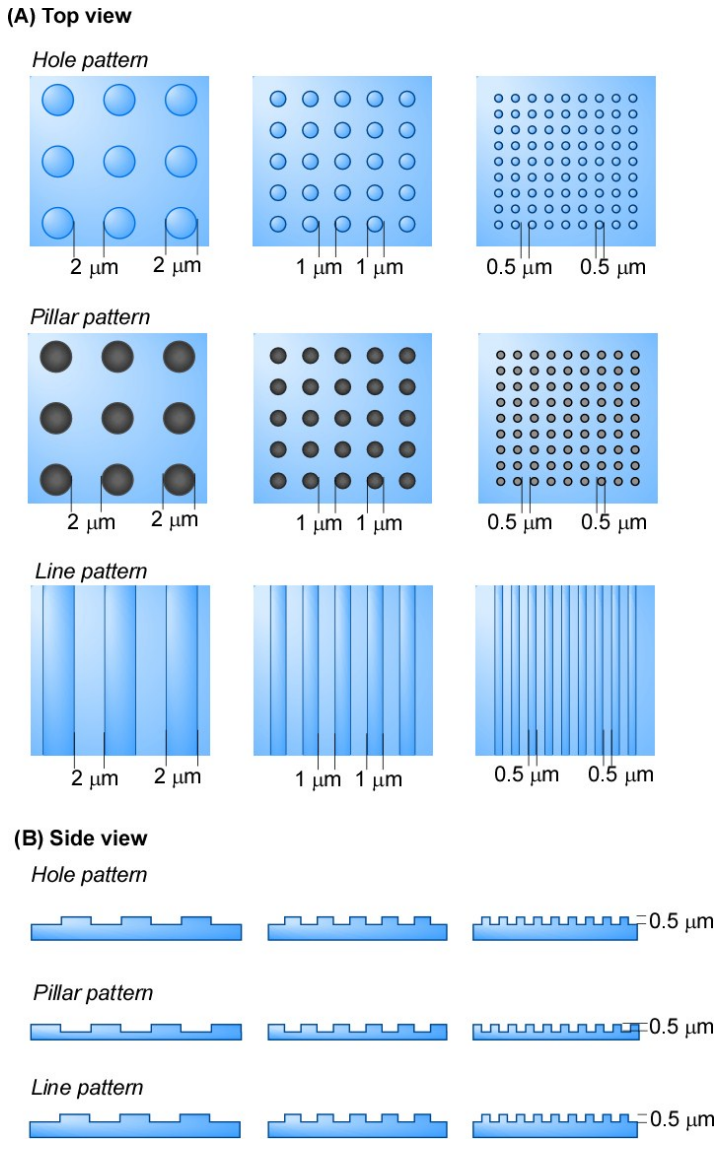

Figure S2. Structures of the NIL molds for preparing the thermoresponsive nano-imprinted substrates.

Table S1. Measurements of the thermoresponsive micro/nano-imprinted structures based on SEM images.

Patterns	Diameter or pitch in the mold (μm) [a)]	Distance in the mold (μm) [b)]	Diameter or pitch in the imprinted layer (μm) [c)]	Distance in the imprinted layer (μm) [d)]
Hole (2 μm)	2.00	2.00	2.12 ± 0.02	1.75 ± 0.02
Hole (1 μm)	1.00	1.00	1.09 ± 0.03	0.86 ± 0.05
Hole (0.5 μm)	0.50	0.50	0.56 ± 0.02	0.42 ± 0.03
Pillar (2 μm)	2.00	2.00	2.14 ± 0.03	1.72 ± 0.01
Pillar (1 μm)	1.00	1.00	1.14 ± 0.02	0.82 ± 0.04
Pillar (0.5 μm)	0.50	0.50	0.62 ± 0.03	0.40 ± 0.01
Line (2 μm)	2.00	2.00	2.05 ± 0.01	1.90 ± 0.01
Line (1 μm)	1.00	1.00	1.10 ± 0.03	0.88 ± 0.01
Line (0.5 μm)	0.50	0.50	0.55 ± 0.01	0.44 ± 0.01

a) Diameter of the pillar and hole patterns and pitch of the line pattern in the NIL mold. b) Distance between each pillar, hole, and line patterns in the NIL mold. c) Diameter of the pillar and hole patterns in the thermoresponsive micro/nano-imprinted substrates. d) Distance between each pillar, hole, and line pattern in the thermoresponsive micro/nano-imprinted substrates.

Table S2. Elemental analyses of the thermoresponsive micro/nano-imprinted substrates determined by XPS at a take-off angle of 90°.

Code	Atom (%)					N/C ratio
	C	N	O	Cl	Si	
Unmodified glass substrate	11.9	0.45	61.2	0.56	25.9	0.038
P(St-*co*-VBC)-coated glass	93.9	0.16	4.08	1.02	0.88	0.002
Flat [a)]	91.7	1.17	4.96	0.84	1.29	0.013
Hole (2 μm)	93.0	1.61	3.72	0.80	0.83	0.017
Hole (1 μm)	90.8	1.60	4.94	0.92	1.77	0.018
Hole (0.5 μm)	93.2	1.69	3.58	1.01	0.51	0.018
Pillar (2 μm)	92.6	1.59	4.21	0.56	1.05	0.017
Pillar (1 μm)	89.4	1.55	6.12	0.71	2.18	0.017
Pillar (0.5 μm)	91.0	2.13	4.96	0.84	1.08	0.023
Line (2 μm)	93.9	1.70	3.33	0.43	0.66	0.018
Line (1 μm)	91.4	1.63	4.85	0.57	1.56	0.018
Line (0.5 μm)	90.7	2.11	5.14	0.79	1.25	0.023

a) "Flat" denotes the PIPAAm-modified flat P(St-*co*-VBC)-coated glass.

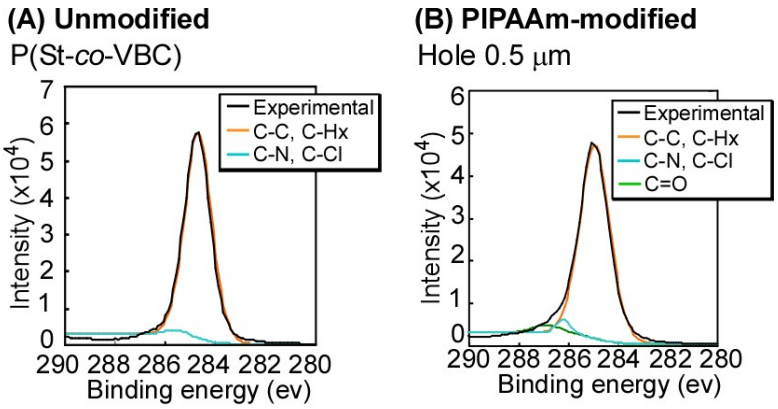

Figure S3. XPS deconvolution of the C1s peaks of the (A) unmodified P(St-*co*-VBC) layer and (B) PIPAAm-modified P(St-*co*-VBC) layer (0.5-μm hole). In the spectrum of the PIPAAm-modified P(St-*co*-VBC) layer, an additional peak was observed at 288 eV, corresponding to the C=O bond of PIPAAm, whereas no such peak was observed in the spectrum of the P(St-*co*-VBC) layer.

Table S3. Surface wettability of the thermoresponsive micro/nano-imprinted substrates determined by contact angle measurements in the sessile drop method.

Code	Contact angle [a]			
	37 °C		20 °C	
	degree	cosθ	degree	cosθ
P(St-*co*-VBC)-coated glass	82.0 ± 2.0	0.139 ± 0.034	88.0 ± 1.3	0.035 ± 0.020
Flat	83.1 ± 1.7	0.120 ± 0.030	80.2 ± 1.8	0.170 ± 0.031
Hole (2 μm)	94.8 ± 1.9	–0.083 ± 0.032	90.3 ± 2.6	-0.005 ± 0.046
Hole (1 μm)	94.5 ± 1.8	–0.078 ± 0.032	89.5 ± 3.5	0.008 ± 0.062
Hole (0.5 μm)	93.8 ± 3.4	–0.067 ± 0.059	89.4 ± 3.6	0.010 ± 0.062
Pillar (2 μm)	89.7 ± 1.1	0.005 ± 0.019	85.5 ± 1.2	0.078 ± 0.021
Pillar (1 μm)	89.8 ± 2.9	0.004 ± 0.051	86.5 ± 2.6	0.061 ± 0.045
Pillar (0.5 μm)	88.9 ± 1.4	0.019 ± 0.024	85.9 ± 1.6	0.072 ± 0.027
Line-parallel (2 μm) [b]	81.6 ± 7.5	0.146 ± 0.129	86.3 ± 3.0	0.064 ± 0.052
Line-Parallel (1 μm) [b]	79.8 ± 7.2	0.175 ± 0.121	86.7 ± 3.1	0.057 ± 0.053
Line-Parallel (0.5 μm) [b]	76.1 ± 4.4	0.240 ± 0.073	82.5 ± 2.6	0.130 ± 0.045
Line-orthogonal (2 μm) [c]	77.6 ± 1.3	0.215 ± 0.022	86.7 ± 3.8	0.057 ± 0.065
Line-orthogonal (1 μm) [c]	79.8 ± 3.6	0.177 ± 0.062	83.8 ± 3.7	0.107 ± 0.065
Line-orthogonal (0.5 μm) [c]	78.2 ± 1.7	0.204 ± 0.029	81.4 ± 3.0	0.150 ± 0.051

a) Mean ± SD, n = 5. b) Droplets were observed in parallel to the line pattern. c) Droplets were observed orthogonal to the line pattern.

Table S4. Fluorescent intensities of adsorbed rhodamine-conjugated fibronectin on the nano-imprinted substrates [a)].

Code	Brightness of adsorbed rhodamine-conjugated fibronectin			
	PIPAAm-modified		Unmodified	
	37 °C	20 °C	37 °C	20 °C
Tissue culture polystyrene (TCPS)			69.2	82.2
Flat [b)]	66.6	17.9	80.2	82.6
Hole (2 μm)	53.3	21.9	94.3	70.4
Hole (1 μm)	60.1	39.5	88.3	72.3
Hole (0.5 μm)	65.2	44.6	97.7	91.8
Pillar (2 μm)	53.0	22.9	89.6	82.5
Pillar (1 μm)	58.3	35.6	93.2	40.7
Pillar (0.5 μm)	66.5	35.9	98.1	95.8
Line (2 μm)	52.0	22.0	86.9	82.2
Line (1 μm)	54.6	27.0	88.6	46.3
Line (0.5 μm)	58.8	39.8	96.4	86.1

a) Quantitatively analyzed by ImageJ. b) “Flat” denotes the PIPAAm-modified flat P(St-*co*-VBC)-coated glass.

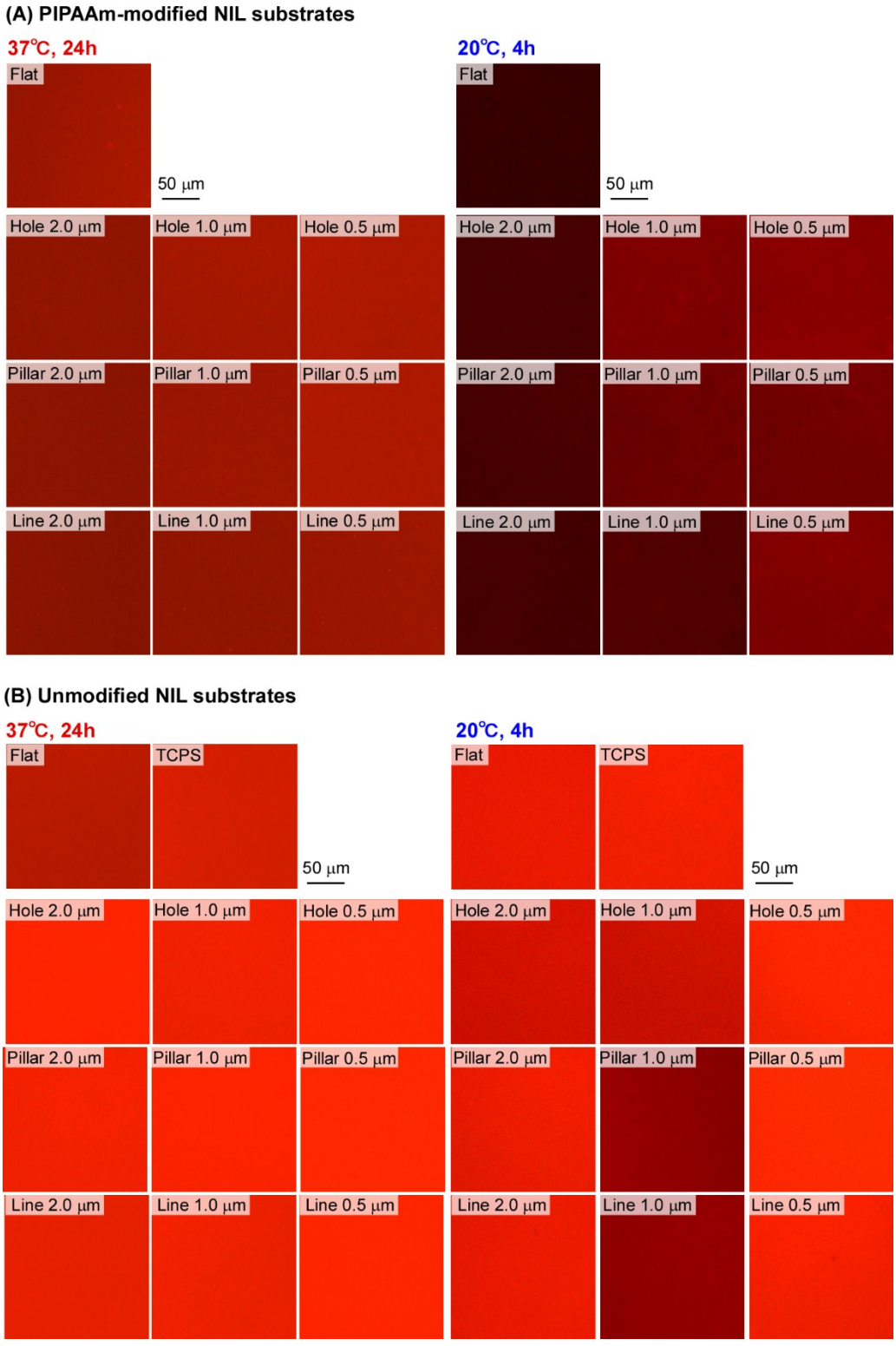

Figure S4. Fluorescent microscopic images of adsorbed rhodamine-conjugated fibronectin on the prepared NIL substrates for investigating protein adsorption properties. (A) PIPAAm-modified thermoresponsive substrates, (B) unmodified NIL substrates. “Flat” indicates the non-imprinted region of the prepared thermoresponsive NIL substrates.

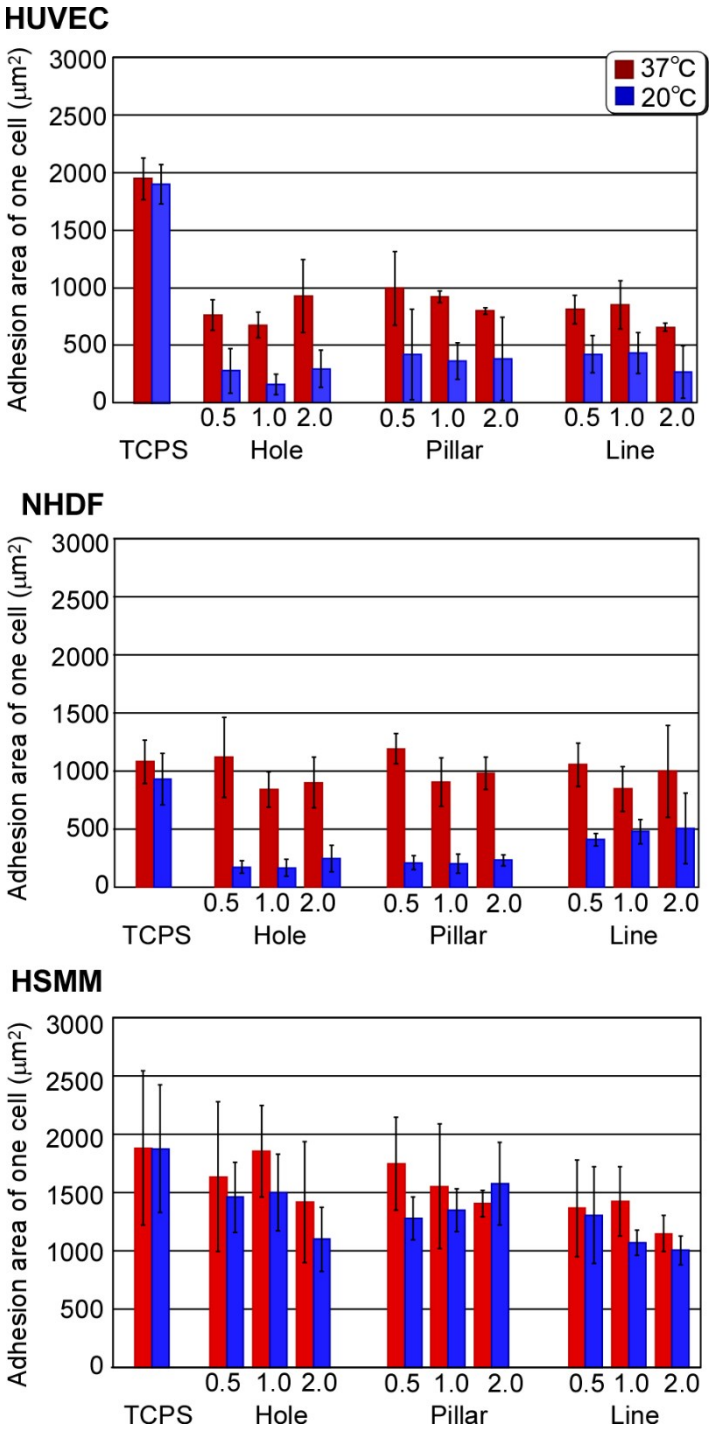

Figure S5. The areas of adhered cell on each pattern for (A) HUVECs, (B) NHDFs, and (C) HSMMs. The data were obtained from analysis of the microscopic images of the adhered cells on the prepared NIL substrates. Mean ± SD, n = 3.

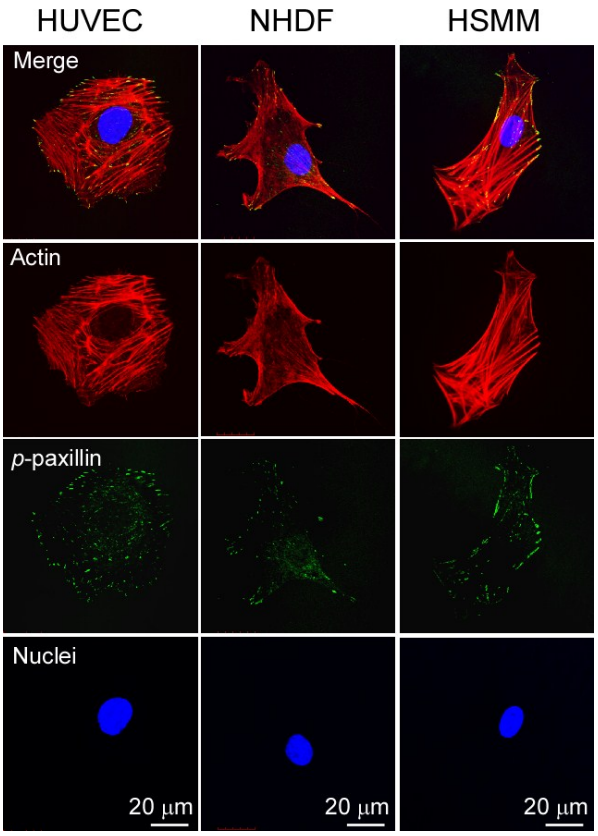

Figure S6. Observations of HUVECs, NHDFs, and HSMMs adhering on a conventional tissue culture polystyrene dish by immunofluorescent staining using Phalloidin 568 (actin fiber, red), Alexa Fluor 488 (*p*-paxillin, green), and Hoechst 33342 (nuclei, blue).

References

1. M. Ciampolini and N. Nardi, *Inorg. Chem.*, 1966, **5**, 41-44.
2. T. Sasagawa, T. Shimizu, S. Sekiya, Y. Haraguchi, M. Yamato, Y. Sawa and T. Okano, *Biomaterials*, 2010, **31**, 1646-1654.
3. N. Asakawa, T. Shimizu, Y. Tsuda, S. Sekiya, T. Sasagawa, M. Yamato, F. Fukai and T. Okano, *Biomaterials*, 2010, **31**, 3903-3909.
4. Y. Tsuda, T. Shimizu, M. Yamato, A. Kikuchi, T. Sasagawa, S. Sekiya, J. Kobayashi, G. Chen and T. Okano, *Biomaterials*, 2007, **28**, 4939-4946.
5. H. Takahashi, M. Nakayama, K. Itoga, M. Yamato and T. Okano, *Biomacromolecules*, 2011, **12**, 1414-1418.
6. H. Kondoh, Y. Sawa, S. Miyagawa, S. Sakakida-Kitagawa, I. A. Memon, N. Kawaguchi, N. Matsuura, T. Shimizu, T. Okano and H. Matsuda, *Cardiovasc. Res.*, 2006, **69**, 466-475.
7. H. Hata, G. Matsumiya, S. Miyagawa, H. Kondoh, N. Kawaguchi, N. Matsuura, T. Shimizu, T. Okano, H. Matsuda and Y. Sawa, *J. Thorac. Cardiovasc. Surg.*, 2006, **132**, 918-924.
8. Y. Sawa, S. Miyagawa, T. Sakaguchi, T. Fujita, A. Matsuyama, A. Saito, T. Shimizu and T. Okano, *Surg. Today*, 2012, **42**, 181-184.