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Selective capture and noninvasive release of cells using a thermoresponsive polymer brush with affinity peptides

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Abstract

Tissue engineering and cell transplantation therapy have become promising therapies for intractable diseases. These approaches require cell separation technology without cell modification. Accordingly, in this study, we developed a novel cell separation method using a thermoresponsive block copolymer brush with an affinity peptide. A block copolymer brush with bottom poly(2-hydroxyethyl methacrylate [HEMA]-*co*-propargyl acrylate) and top poly(*N*-isopropylacrylamide-*co*-HEMA) segments was prepared through two steps of atom transfer radical polymerization. Then, cell affinity peptides were conjugated to the bottom segment of the copolymer brush through a click reaction. Using cRGD as a cell-affinity peptide, enhancement of cell adhesion with rapid adhesion on the copolymer brush was observed at 37°C, whereas the copolymer brush without cRGD did not exhibit cell adhesion. Temperature-modulated cell adhesion and detachment were performed with a relatively long upper segment because the affinity between peptides and cells was modulated by the swelling and shrinking of the upper thermoresponsive segment. Selective endothelial cell adhesion was performed at 37°C using GGGREDV as an affinity peptide. Smooth muscle cells and fibroblasts did not adhere to the copolymer brush. Adhered human umbilical vein endothelial cells (HUVECs) were successfully recovered by reducing the temperature to 20°C. Based on the properties of the copolymer brush, HUVECs could be purified using a mixture of cells simply by changing the temperature. These results demonstrated that the prepared copolymer brush with cell affinity peptides could be a useful cell separation tool because the cells could be separated with specificity and without cell modification using a simple procedure.

Keywords: Thermoresponsive polymer, Cell separation, Cell affinity peptide, Temperature-responsive chromatography

1. Introduction

In recent decades, regenerative therapies involving transplantation of cell suspensions or cellular tissues have become a promising approach for treating intractable diseases.[1-6] In these therapies, cell separation is an essential process for preparing cell suspensions and for the fabrication of cellular tissue. For example, when collecting cells from tissues in the living body, various cell types are mixed. Thus, target cells have to be purified from other types of cells before transplantation or cellular tissue fabrication. To date, various types of cell separation methods have been developed.[7-13] Among these approaches, cell separation methods via modification of cells with fluorescently labeled antibodies or antibodies conjugated to magnetic particles are widely used. However, these cell modification methods can alter the intrinsic properties of the cells, thereby reducing the therapeutic performance of the cells or the efficiency of cellular tissue fabrication. Thus, novel cell separation methods that do not modify the cells are needed.

Cell separation using poly(*N*-isopropylacrylamide) (PNIPAAm) is an example of a non-modifying cell separation method.[14-19] PNIPAAm has thermoresponsive properties attributed to hydration and dehydration across the phase transition temperature.[20] The polymer exhibits hydrophilic and hydrophobic alterations across the phase transition temperature. In addition, the polymer shows extension and shrinking below and above the phase transition temperature, respectively. The unique properties of PNIPAAm have been utilized for various types of biomedical applications,[18,19] such as thermally controlled drug and gene delivery systems,[21-23] biosensors and bio-imaging systems,[24-27] chromatographic separation,[28-30] and cell culture substrates for tissue engineering.[31-35] In cell separation applications, temperature-modulated cell adhesion and detachment have been carried out using PNIPAAm-modified substrates. At 37°C, PNIPAAm is hydrophobic owing to dehydration; thus, cells tend to adhere well to PNIPAAm-coated surfaces. In contrast, at reduced temperatures, such as 20°C, PNIPAAm becomes hydrophilic, and cells do not adhere well. Moreover, on PNIPAAm-modified substrates, cell adhesion and detachment behaviors

differ among cell types, leading to cell separation. For example, endothelial cells and myoblasts adhere to PNIPAAm-modified substrates at 37°C, but rapidly or slowly detach from the surface when the temperature is reduced to 20°C, respectively.[15] Thus, after the initial incubation period, endothelial cells can be recovered, and during the subsequent period, myoblasts can be recovered.[15]

Incorporation of ionic groups into PNIPAAm is an effective approach for increasing differences in cell adhesion behaviors because the cells have different electrostatic properties in the presence of different ionic groups, leading to variations in adhesion.[36-39] However, this approach is limited to the separation of cells with different electrostatic properties. Thus, cell specificity during adhesion should be increased through a different approach.

To increase the cell specificity, various types of ligands, such as antibodies and aptamers, have been investigated.[40-44] Among them, cell affinity peptides are strong candidates because they are stable and cost-effective compared with other types of biological ligands. Arg-Gly-Asp (RGD) and Arg-Glu-Asp-Val (REDV) peptides are widely used for enhancing cell adhesion to various types of biomaterials.[45-52] In particular, REDV peptide enhances endothelial cell adhesion through the recognition of integrin f$\alpha_4\beta_1$.[49, 50, 53] Thus, this peptide may be useful for cell separation applications with high specificity.

In the current study, we developed a thermoresponsive block copolymer brush with cell affinity peptides for temperature-modulated cell separation. Block copolymer brushes were prepared in two steps using surface-initiated activators regenerated by electron transfer atom transfer radical polymerization (ARGET-ATRP).[54, 55] Block copolymerization can introduce two types of segments in the polymer brush.[56, 57]: one is the bottom segment for peptide modification, and the other is the upper thermoresponsive segment for modulating affinity interaction between the peptide and the cells. To prepare the bottom segment for peptide conjugation, 2-hydroxyethyl methacrylate (HEMA) was used as the base monomer, because polyHEMA is commonly used as a hydrophilic and biocompatible polymer[58]

to prevent non-specific cell adhesion. Propargyl acrylate (PgA) was also used as a peptide conjugation site, because PgA has an alkyne group that can be used as the reaction group in the click reaction with copper-catalyzed azide-alkyne cycloaddition,[59, 60] for peptide modification to the polymer brush. To prepare the upper segment, p(NIPAAm-*co*-HEMA) was used. Although there are other types of thermoresponsive polymers, such as polyethylene glycol (PEG)-based thermoresponsive copolymers,[61-65] NIPAAm was used as a thermoresponsive segment because the NIPAAm copolymer exhibits a sharp hydrophobic and hydrophilic alteration leading to temperature-modulated cell adhesion and detachment. However, the hydrophobic property of PNIPAAm at 37°C would lead to non-specific cell adhesion. Thus, cell non-adhesive property was provided by the incorporation of HEMA to the PNIPAAm copolymer, and P(NIPAAm-*co*-HEMA) was used as an upper thermoresponsive segment. Using the prepared block copolymer brush, temperature-modulated cell separation was performed by simply changing the temperature.

2. Materials and Methods

2.1 Materials

N-isopropylacrylamide (NIPAAm) was obtained from KJ Chemicals (Tokyo, Japan) and recrystallized from *n*-hexane. 2-Hydroxyethyl methacrylate (HEMA) was obtained from Tokyo Chemical Industries (Tokyo, Japan) and purified using distillation before use. Propargyl acrylate (PgA) was synthesized from propargyl alcohol and acroyl chloride. The detailed synthesis is described in Supporting Information S1. Toluene, 2-propanol, dimethyl sulfoxide (DMSO), copper (II) chloride ($CuCl_2$), ascorbic acid, α-chloro-*p*-xylene, tris(2-aminoethyl)amine (TREN), and copper(II) sulfate pentahydrate were obtained from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). Tris[(2-dimethylamino)ethyl]amine (Me_6TREN) was synthesized from TREN.[66] ([Chloromethyl]phenylethyl) trimethoxysilane (CPTMS) was purchased from Gelest (Morrisville, PA, USA). Tris([1-benzyl-1H-1,2,3-triazol-4-yl]methyl)amine (TBTA) was obtained from Tokyo Chemical Industries. Glass cover slips were purchased from Matsunami Glass Industry (Osaka, Japan).

Cell affinity peptides with azide groups, i.e., cyclic RGD-N_3, K(N_3)-REDV, and K(N_3)-GGGREDV, were synthesized through solid-phase peptide synthesis using a peptide synthesis apparatus (Initiator$^+$ SP Wave, Biotage, Uppsala). The detailed peptide synthesis is described in the Supporting Information S2. Normal human umbilical vein endothelial cells (HUVECs), human aortic smooth muscle cells (AoSMCs), normal human dermal fibroblasts (NHDFs), and cell culture media for AoSMCs and NHDFs were purchased from Lonza (Basel, Switzerland). Endothelial cell growth medium was purchased from Promocell (Heidelberg, Germany). Dulbecco's modified Eagle's medium (DMEM) and cell staining reagents were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Fetal bovine serum (FBS) was obtained from Biosera (Nuaille, France).

2.2 Preparation of thermoresponsive block copolymer brushes with cell-affinity peptides

Thermoresponsive block copolymer brushes with affinity peptides were prepared via two steps using surface-initiated ARGET-ATRP[54, 55] and subsequent click reaction with copper-catalyzed azide-alkyne cycloaddition,[59, 60] for peptide modification to the polymer brush (Fig. 1A). Glass plates (24 × 50 mm, thickness: 0.2 mm) were placed in a glass plate holder, and the glass surfaces were cleaned and activated using a plasma modifier (PM100; Yamato, Japan). Then, the glass plates were set into a 500-mL separation flask. The flask was humidified at 60% relative humidity for 2 h. ATRP initiator solution was prepared by dissolving 3.504 mL CPTMS in 300 mL toluene. The prepared solution was poured into glass plates in a separable flask. The silane coupling reaction for CPTMS modification to glass surfaces was performed at 25°C for 16 h. After the reaction, the glass plates were rinsed with toluene and acetone and placed in a drying oven at 110°C for 3 h.

The first ATRP was performed to prepare the P(HEMA-*co*-PgA) brush as a peptide modification segment. PgA has an alkine group for the click reaction, and HEMA was used as a hydrophilic monomer for prevention of cell adhesion induced by hydrophobic interactions between the polymer brush and cells. During polymerization, the PgA feed molar ratio was set at 10 mol% and 20 mol% to prevent polymerization involving the alkine group. In the preparation of copolymer consisting of PgA 10 mol% and HEMA 90 mol%, PgA (413 mg, 3.75 mmol) and HEMA (4.39 g, 33.8 mmol) were dissolved in the mixed solvent of 2-propanol (280 mL) and water (15 mL) in a 500-mL separable flask. Then, $CuCl_2$ (8.10 mg, 0.06 mmol), Me_6TREN (138 mg, 0.6 mmol), and α-chloro-*p*-xylene (26.3 μL, 0.2 mmol) were dissolved in the solution. The solution was deoxygenated by bubbling argon gas for 30 min. Then, the prepared ATRP initiator-modified glass plates containing a glass holder were immersed in the solution. The ascorbic acid solution, prepared by dissolving ascorbic acid (106 mg, 0.6 mmol) in pure water (5 mL), was added to the ATRP reaction to start the ATRP. Polymerization was performed at 25°C for 1 h. After

polymerization, the glass plates were rinsed with acetone and dried in vacuo. The ATRP reaction solution of the first ATRP was dialyzed using a cellulose dialysis membrane (molecular weight cut off: 1000 Da) against water to purify the synthesized polymer from α-chloro-*p*-xylene for 5 days. After dialysis, the polymer was obtained by lyophilization. The prepared first segments were designated as "P1" and "P2" and were prepared using PgA compositions of 10 mol% and 20 mol%, respectively.

The second ATRP was performed to prepare the P(PNIPAAm-*co*-HEMA) brush as a thermoresponsive segment for the modulation of cell adhesion and detachment by changing the affinity of cells and peptides. The ATRP procedure was similar to the first step of ATRP. In the second ATRP, the feed monomer compositions of NIPAAm and HEMA were 90 mol% and 10 mol%, respectively. The total monomer concentrations were set at 500 and 1000 mM to modulate the length of the thermoresponsive segment. In the preparation of 500 mM monomer concentration solution, NIPAAm (15.3 g, 135 mmol) and HEMA (1.95 g, 15.0 mmol) were dissolved in the mixed solvent of 2-propanol (280 mL) and water (15 mL) in a 500-mL separable flask. Then, $CuCl_2$ (8.10 mg, 0.06 mmol), Me_6TREN (138 mg, 0.6 mmol), and α-chloro-*p*-xylene (26.3 μL, 0.2 mmol) were dissolved in the solution. The solution was deoxygenated by bubbling argon gas for 30 min. Then, the prepared ATRP initiator-modified glass containing the glass holder was immersed in the solution. The ascorbic acid solution, prepared by dissolving ascorbic acid (106 mg, 0.6 mmol) in pure water (5 mL), was added to the ATRP solution to initiate it. Polymerization was performed at 25°C for 20 h. After polymerization, glass plates were rinsed with acetone and dried in vacuo. The synthesized polymer from α-chloro-*p*-xylene in the ATRP reaction was obtained using dialysis and lyophilization following the same procedure as the first ATRP. The thermoresponsive segments were designated as "T500" and "T1000" based on the monomer concentration. The block copolymer brushes were denoted using the names of each segment (e.g., "P1T500" and "P1T1000").

Cell affinity peptides were modified to block copolymer brushes via copper-catalyzed azide-alkyne cycloaddition, a type of click reaction.[59, 60] $CuSO_4$ aqueous solution (5 mM, 10 mL) and TBTA DMSO solution (10 mM, 11 mL) were mixed to form the Cu-TBTA complex, and the mixed solution was deoxygenated by bubbling argon for 30 min. Peptide and sodium ascorbate were added to the Cu-TBTA complex solution and diluted using pure water to reach final concentrations of 0.25 mM $CuSO_4$, 0.5 mM TBTA, 100 μM peptide, and 5 mM sodium ascorbate. The prepared block copolymer brush-modified glass plates were cut into 24 × 25 mm pieces and placed in a 35-mm polystyrene petri dish. The prepared solution was added to the copolymer brush in the dish. The reaction proceeded at 25°C for 18 h with continuous shaking. After the reaction, the block copolymer brush-modified glass plates were rinsed three times with water and DMSO (50%:50%, v/v) and subsequently rinsed with pure water. The prepared copolymer brush-modified plates were sterilized using 70% ethanol.

To characterize the polymer brush, the molecular weights of the obtained polymers in the reaction solution were measured using gel permeation chromatography (GPC). The polymer was dissolved in DMF containing 50 mM LiCl at a concentration of 5 mg/mL. The prepared sample was analyzed using a GPC system (HLC-8020GPC; Tosoh, Tokyo, Japan) equipped with two serially connected TSK-GEL α-M columns. DMF containing 50 mM LiCl was used as the mobile phase at a flow rate of 1.0 mL/min. The elution capacity of the polymer was detected using an equipped RI detector. The calibration curve for the estimation of the molecular weight was obtained using polyethylene glycol standards.

The phase transition behavior of the prepared thermoresponsive copolymers, i.e., T500 and T1000, was observed by evaluating temperature-dependent changes in the optical transmittance of the polymer solution. The copolymer was dissolved in water, phosphate-buffered saline, and DMEM containing 10% FBS at a concentration of 5 mg/mL. The transmittance of the copolymer solution was observed with the increasing temperature using an ultraviolet-visible spectrometer (V-630; JASCO, Tokyo, Japan). The

solution temperature in the cuvette was controlled and increased by 0.1°C using equipped temperature control device (ETC-717; JASCO and CTU-100, JASCO). The transmittance of the solution was monitored at 20–35°C. The temperature at which the solution transmittance reached 50% was defined as the lower critical solution temperature. In addition, the phase transition temperature of the copolymer was observed using differential scanning calorimetry (DSC-60 Plus, Shimadzu, Kyoto). P(NIPAAm-*co*-HEMA) was dissolved in water and PBS at 30% (w/v). The sample was heated at a rate of 2.5°C/min, and microcalorimetric endotherms of the copolymer were obtained.

The polymer modification on the glass plate was confirmed via surface elemental analysis of initiator and polymer-modified substrates using an X-ray photoelectron spectroscopy (XPS) (K-Alpha, Thermo Fisher, Waltham, MA, USA).

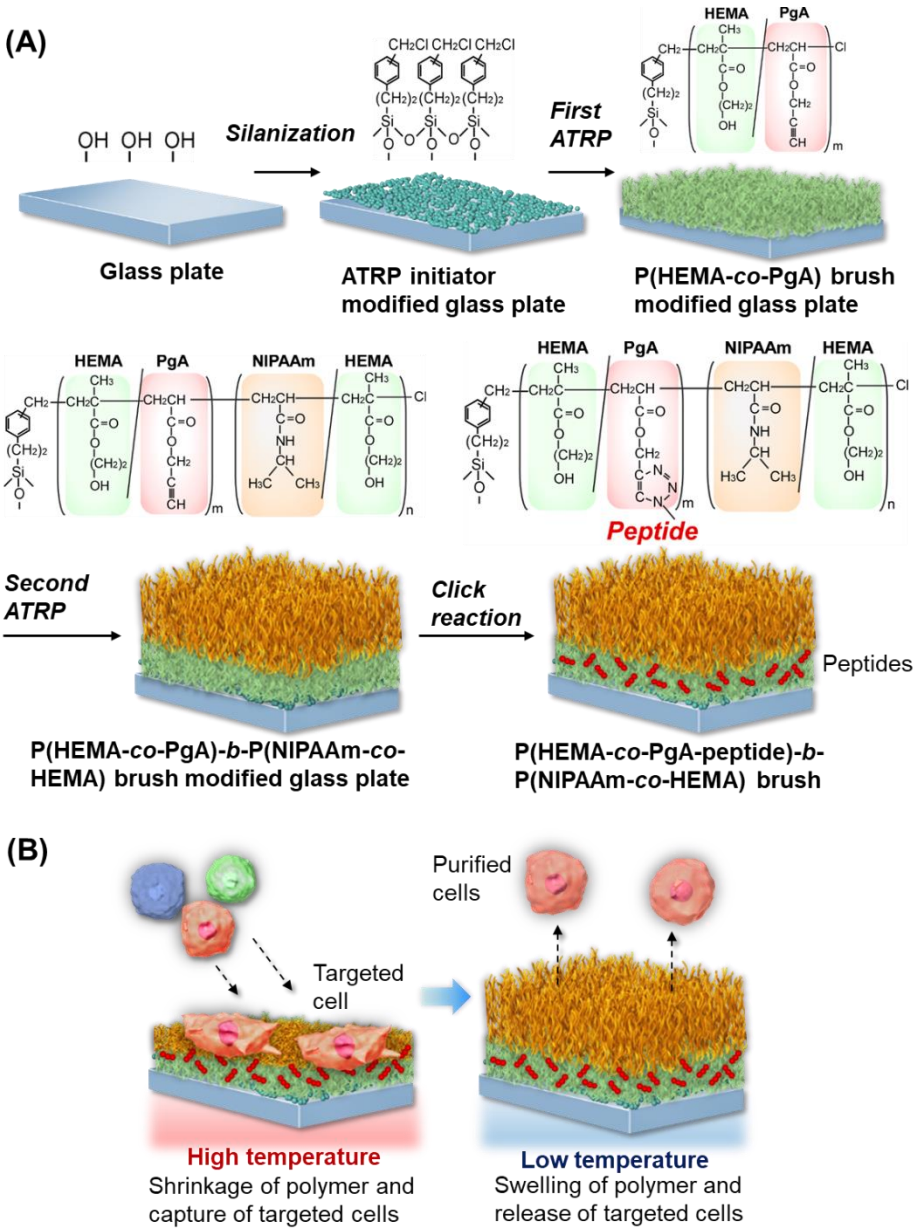

Fig. 1 Schematics showing (A) the preparation of thermoresponsive polymer brush with cell affinity peptide and (B) the temperature-modulated cell separation processes.

2.3 Temperature-modulated cell adhesion and detachment using a copolymer brush with peptides

In this study, we used three types of cells, i.e., HUVECs, AoSMCs, and NHDFs, to evaluate cell adhesion and detachment with peptide-conjugated copolymer brushes. These three types of cells are commonly utilized in vascular tissue engineering.[3, 67-70] Thus, manipulating cells with simple temperature changes may be useful for these applications. Cells were cultured in the appropriate cell culture medium for each cell type (Table S1).

To evaluate the cell adhesion and detachment behaviors of thermoresponsive copolymer brushes with cell affinity peptides, copolymer brush-modified glass plates were placed into a 35-mm polystyrene petri dish. The copolymer brush-modified glass in the dish was sterilized using 70% ethanol and then rinsed with PBS. The cell suspension was prepared using DMEM containing 10% FBS and seeded onto the modified glass in the dishes at a density of 1×10^5 cells/dish. The cell density of 1×10^5 cells/dish was set to prevent cell-cell contact on the glass because the adhesion of each cell prevents cell recovery. The dishes were incubated at 37°C for 90 min. Microscopic images of adhered cells were obtained every 30 min using a microscope (CKX53; Olympus, Tokyo, Japan) with an equipped camera (EOS X8i; Canon, Tokyo, Japan). After incubation at 37°C for 90 min, the copolymer brush-modified glass in the dishes was rinsed with DMEM without FBS three times. Next, DMEM containing 10% FBS and maintained at 20°C was added to the dishes, and the dishes were incubated at 20°C for 90 min. Micrographs were obtained every 30 min during incubation. The cell adhesion ratio was calculated as the ratio of the number of adhered cells to that of the seeded cells.

In the cell separation experiment, HUVECs, AoSMCs, and NHDFs were stained red, blue, and green, respectively, using cell staining reagents. The cells were mixed at a ratio of 1:1:1 and seeded into the copolymer brush-modified glass within the dishes at a density of 1×10^5 cells/dish. The dishes were incubated at 37°C for 90 min. Microscopic images of adhered cells every 30 min were obtained using a fluorescent microscope (BZ-X810; Keyence, Osaka, Japan). After incubation at 37°C for 90 min, the

copolymer brush-modified glass in the dish was rinsed with DMEM without FBS three times. Then, DMEM maintained at 20°C, was added to the dishes, and the dishes were incubated at 20°C for 90 min. Micrographs were obtained every 30 min during incubation. The cell adhesion ratio was calculated as the ratio of the number of adhered cells to that of the seeded cells. The cell adhesion ratio of recovered cells for each cell type after incubation at 20°C was estimated by subtracting the cell adhesion ratio at 20°C from that at 37°C. Next, the cell composition of recovered cells was calculated by dividing the recovered cell ratio by the sum of the recovered cell ratios for the three types of cells.

3. Results and discussion

3.1 Characterization of copolymer brushes

The prepared copolymer brushes were characterized via GPC, and the phase transition profiles of the copolymers were observed (Table 1 and Fig. 2). The GPC results indicated that the molecular weights of the first segment of the block copolymers, P1 and P2, were 9400 and 8400, respectively. The molecular weight was relatively high compared with that of prepared PNIPAAm copolymers in previous research using a similar ATRP procedure.[37] This is probably because of the relatively high reactivity of acrylate and methacrylate monomers compared with that of the acrylamide monomer. These copolymers exhibited a small polydispersity index, indicating that polymerization was controlled by the ATRP procedure in the current study. The molecular weights of the second temperature-responsive segments, T500 and T1000, were 14000 and 18100, respectively. The molecular weight increased as the monomer concentration in the ATRP procedure increased because polymerization was enhanced.[71] A relatively low polydispersity index was observed in the copolymers of the second segment, indicating that the second ATRP was also controlled in the ATRP procedure, despite the relatively higher molecular weight and copolymerization of other types of monomers, such as acrylamide and methacrylate.

The phase transition behaviors of the thermoresponsive copolymer P(NIPAAm-*co*-HEMA) segments were investigated by observing changes in the transmittance of the copolymer (Fig. 2). The phase transition temperature of the thermoresponsive copolymer in pure water was approximately 29°C, which was relatively low compared with that of the homopolymer of PNIPAAm at 32°C. This is because of the increased hydrophobicity attributed to the hydrogen bonding between the hydroxyl group of HEMA and amide groups of NIPAAm, as previous reports suggested.[72, 73]

The phase transition temperature of the copolymer decreased in PBS and DMEM containing 10% FBS because of the salting effect of the sodium chloride contained in PBS and DMEM.[74] There were no large differences in phase transition temperatures between PBS and DMEM, although DMEM containing 10% FBS and large amounts of proteins were present in the copolymer solution. A previous report indicated that human serum albumin in polymer solution does not affect phase transition behaviors.[75] In the current study, proteins in FBS did not affect the phase transition behaviors of the copolymers.

We also measured the phase transition temperature of the P(NIPAAm-*co*-HEMA) copolymer using DSC (Fig. S1). A similar phase transition temperature of the copolymer was observed using the transmittance change of the copolymer solution.

These results indicated that the phase transition of the copolymer was modulated by changing the temperature from 37 to 20°C, which are commonly used temperatures for cell adhesion and detachment, respectively. Further, in the copolymer brush, the peptide was conjugated to the P(HEMA-*co*-PgA) segment, different from the thermoresponsive segment, so that the phase transition temperature would not change after peptide modification.

Surface elemental analysis of the prepared initiator and polymer-modified glass was performed using XPS (Table S2). P1 and P1-T1000 exhibited higher carbon composition than initiator modified substrates. This was because the carbon atom attributed to P(HEMA-*co*-PgA) and P(HEMA-*co*-PgA)-*b*-P(NIPAAm-

co-HEMA) was detected using XPS measurement. Furthermore, the silicon composition decreased after copolymer modification because the grafted copolymer prevented the access of X-rays to the basal glass. Thus, the silicon composition attributed to the base glass substrate decreased after polymerization. After peptide modification, the carbon and nitrogen composition of the peptide-modified copolymer brush increased slightly compared with that before modification because the carbon and nitrogen atoms attributed to peptide and azide group were detected by XPS.

Table 1 Characterization of copolymer brush-prepared surface-initiated ATRP

Code	Copolymer type	Monomer conc. (mM)	Molar ratio (mol%)			M_n [a]	M_w [a]	M_w/M_n [a]	LCST (°C) [b]		
			PgA	NIPAAm	HEMA				In water	In PBS	In DMEM
P1	Poly(HEMA-*co*-PgA)	125	10	-	90	9400	11900	1.27			
P2		125	20	-	80	8400	10700	1.28			
T500	Poly(NIPAAm-*co*-HEMA)	500	-	90	10	14000	19600	1.40	29.1	26.6	26.1
T1000		1000	-	90	10	18100	25000	1.38	29.6	27.1	27.1

a) Determined via GPC using DMF with 50 mM LiCl as mobile phase. b) Determined as temperature at which the copolymer solution showed 50% transmittance

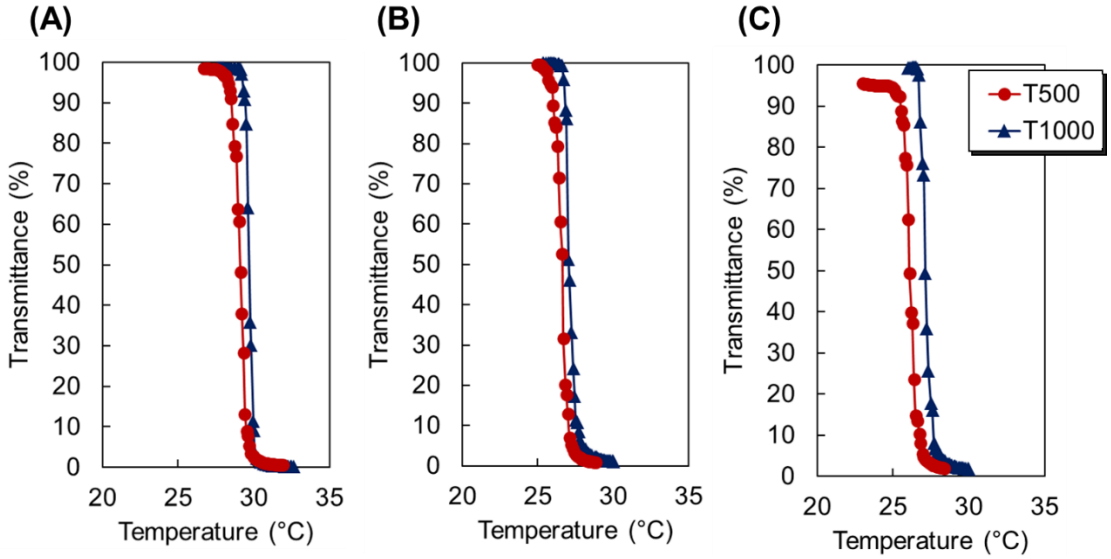

Fig. 2 Phase transition behavior of the thermoresponsive copolymers P(NIPAAm-*co*-HEMA) in water (A), PBS (B), and DMEM containing 10% FBS (C).

3.2 Temperature-modulated cell adhesion and detachment using a copolymer brush

Cell affinity peptides were immobilized to the prepared copolymer brush through a click reaction, and cell adhesion and detachment on the polymer brush were observed.

First, the cyclic RGD peptide (cRGD) was used as a cell affinity peptide because it has been widely used in biomedical studies.[45, 47, 48, 76, 77] Especially, cRGD peptide was utilized because it exhibits affinity for vascular cells near the tumor.[76, 77] To investigate the effect of cell affinity peptides on cell adhesion to the copolymer brush, cell adhesion of the prepared copolymer brushes with or without cRGD was observed at 37°C using HUVECs as model cells (Fig. 3A). Tissue culture polystyrene (TCPS) dishes were used as controls for the evaluation of cell adhesion. P1T500-cRGD, the copolymer brush harboring the cRGD peptide, exhibited excellent cell adhesion properties with a short incubation time of 90 min, whereas cells did not adhere to P1T500, which harbored the copolymer brushes without the cRGD peptide. The results indicated that cell adhesion was related to the affinity for cRGD peptide at the bottom segment of the copolymer brush. In addition, the three types of cells showed very low adhesion to the P1T500 copolymer brushes, even at 37°C (Fig. 3B). The results indicated that the prepared thermoresponsive block copolymer brushes had non-cell adhesive properties at 37°C. In contrast, a similar thermoresponsive copolymer brush was found to show cell adhesion properties in a previous report.[15] The difference is attributed to the relatively long copolymer of the thermoresponsive segment. Previous reports have indicated that longer PNIPAAm segments prevent cell adhesion in the copolymer brushes owing to the increased hydrophilic properties of the long PNIPAAm.[15, 38, 78] The lengths of the thermoresponsive segments in the copolymer brushes were relatively long, conferring the copolymer brushes with non-adhesive properties.

The cell adhesion rate on P1T500-cRGD was quite high compared with that on thermoresponsive polymer brushes reported in previous studies.[15, 16] P1T500-cRGD reached the maximum cell adhesion

ratio within 90 min, whereas thermoresponsive polymer brushes reach the maximum cell adhesion ratio within 24 h.[15] These findings indicated that the cell separation process could be shortened with cell adhesion peptides. Previous investigation of cell separation using PNIPAAm brushes reported that approximately 24 h is required for cell adhesion.[15] In contrast, using copolymer brushes with cell affinity peptides, cell adhesion was achieved within 90 min. Thus, the prepared polymer brushes with cell affinity peptides dramatically reduced the time for cell adhesion, leading to a shorter temperature-modulated cell separation procedure.

The cell adhesion ratio on copolymer brushes after incubation at 37°C for 90 min was also investigated using AoSMCs and NHDFs, and the adhesion ratios of HUVECs, AoSMCs, and NHDFs were compared (Fig. 3B). Adhesion of all three types of cells was enhanced using copolymer brushes with the cRGD peptide, although the adhesion properties of each cell were slightly different. The results suggested that copolymer brushes with affinity peptides could be used for various types of cells.

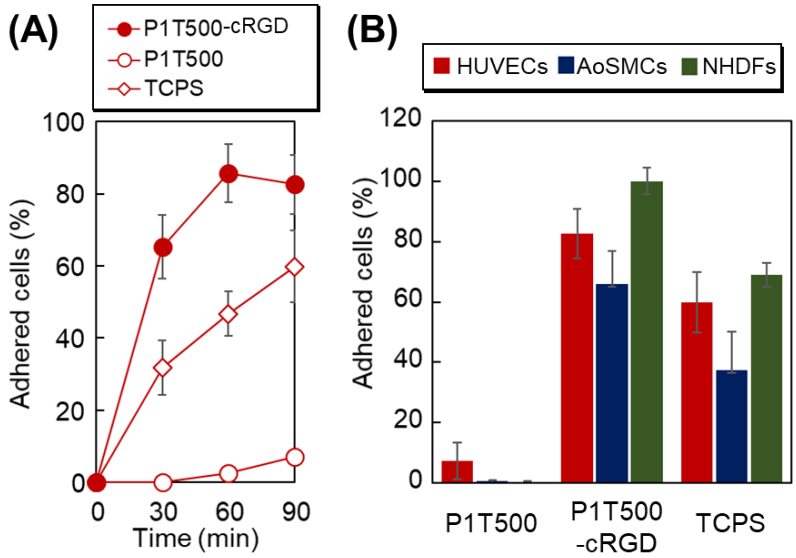

Fig. 3 Effects of cRGD on the enhancement of cell adhesion. (A) HUVEC adhesion profiles on polymer brushes with and without cRGD and TCPS as control at 37°C. (B) Cell adhesion ratios for HUVECs, AoSMCs, and NHDFs after incubation at 37°C for 90 min.

Because the cell affinity peptide cRGD in the bottom segment of the copolymer brush showed an affinity for cells, temperature-dependent cell adhesion and detachment were investigated using P1T500-cRGD and P1T1000-cRGD (Fig. 4). Three types of cells, i.e., HUVECs, AoSMCs, and NHDFs, adhered to the short copolymer brush P1T500-cRGD at 37°C after incubation for 90 min (Fig. 4A). However, these three cell lines did not detach at 20°C after 90 min of incubation. In contrast, the three cell lines adhered to the long copolymer brush P1T1000-cRGD during incubation at 37°C for 90 min and detached after incubation at 20°C for 90 min (Fig. 4B). The difference could be attributed to the length of the upper thermoresponsive segment in the copolymer brush. For P1T1000-cRGD, the thermoresponsive copolymer segment successfully modulated the affinity interaction between cells and affinity peptides in response to temperature changes. At 37°C, the thermoresponsive copolymer segment shrunk, and cells interacted with cell affinity peptides in the bottom segment of the copolymer brush, leading to cell adhesion on the copolymer brush. Notably, at 20°C, the thermoresponsive copolymer segment extended and reduced the affinity between cells and peptides in the bottom segment, leading to detachment of cells from the copolymer brush. However, in the case of P1T500-cRGD, the length of the thermoresponsive copolymer segment was insufficient in the extended state to prevent the affinity between the cells and peptide, leading to non-detachment of cells. These results indicated that cell adhesion and detachment on copolymer brushes with the cRGD peptide could be controlled based on changes in the length of the thermoresponsive upper segment.

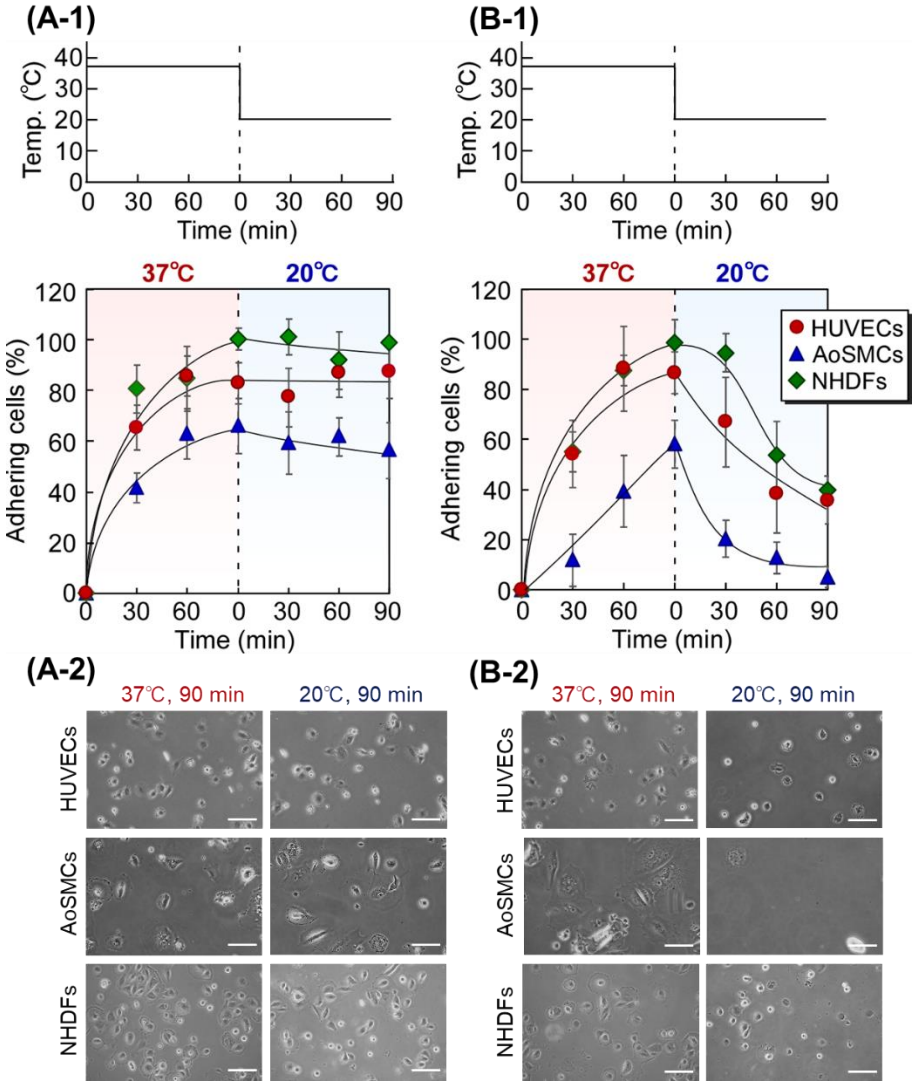

Fig. 4 Cell adhesion and detachment profiles on thermoresponsive copolymer brushes with cRGD. (A) P1T500-cRGD and (B) P1T1000-cRGD. (-1) Cell adhesion profile during incubation at 37°C for 90 min and subsequent incubation at 20°C for 90 min, with the time-dependent temperature profile. (-2) Cell morphology on copolymer brushes. Scale bar: 100 μm.

Using the peptide-conjugated copolymer brushes, cell-selective adhesion and detachment were investigated for use in cell separation applications by changing the peptide from cRGD to REDV because

REDV peptide has specific affinity for endothelial cells, whereas cRGD has an affinity for a variety of cells. A block copolymer brush conjugated with REDV peptide (P1T500-REDV) was prepared, and the adhesion of HUVECs was observed (Fig. S2). However, HUVECs did not adhere to the copolymer brushes during a 90-min incubation at 37°C, potentially because the affinity between HUVECs and REDV was insufficient. In previous reports, cell affinity peptides were conjugated to the base materials through spacers, such as glycine residues or polyethylene glycol chains, thereby increasing the accessibility of peptides to cells.[47, 49, 79] Thus, REDV with three glycines as a spacer (GGGREDV) was used as an affinity peptide, and copolymer brushes with the peptide (P1T500-GGGREDV and P1T1000-GGGREDV) were prepared. Three types of cells (i.e., HUVECs, AoSMCs, and NHDFs) were seeded on the prepared brushes and cell adhesion and detachment were observed (Fig. 5). Only HUVECs adhered to both P1T500-GGGREDV and P1T1000-GGGREDV following incubation at 37°C, whereas AoSMCs and NHDFs did not adhere to the brushes. The results indicated that the GGGREDV peptide functioned as a cell affinity peptide by adding GGG to the peptide as a spacer. In addition, the conjugated GGGREDV in the bottom segment of the copolymer brush recognized HUVECs, leading to selective adhesion of HUVECs. However, in P1T500-GGGREDV, most of the HUVECs did not detach during incubation at 20°C because the affinity between peptide and cells could not be modulated by the thermoresponsive upper segment in the brush; this result could be attributed to the insufficient length of the upper segment, similar to the case of cell adhesion and detachment using P1T500-cRGD. In contrast, P1T1000-GGGREDV adhered to HUVECs at 37°C and successfully detached at 20°C. P1T1000-GGGREDV had a sufficiently long thermoresponsive copolymer segment for modulating the interaction between cells and peptides. Thus, these results indicated that the thermoresponsive copolymer with a GGGREDV peptide could selectively capture HUVECs, whereas other types of cells did not. Moreover, the captured HUVECs could be successfully released by reducing the temperature. To verify the effect of GGGREDV peptide on cell

adhesion, we observed HUVEC adhesion on the prepared copolymer brush using the GGGRDEV peptide, synthesized by changing the peptide sequence (Fig. S3). HUVEC adhesion was suppressed on GGGRDEV peptide modified copolymer brush compared to that on GGGREDV modified copolymer brush. This suggested that the adhesion of HUVECs was induced by the affinity between the HUVECs and the conjugated peptides in the copolymer brush.

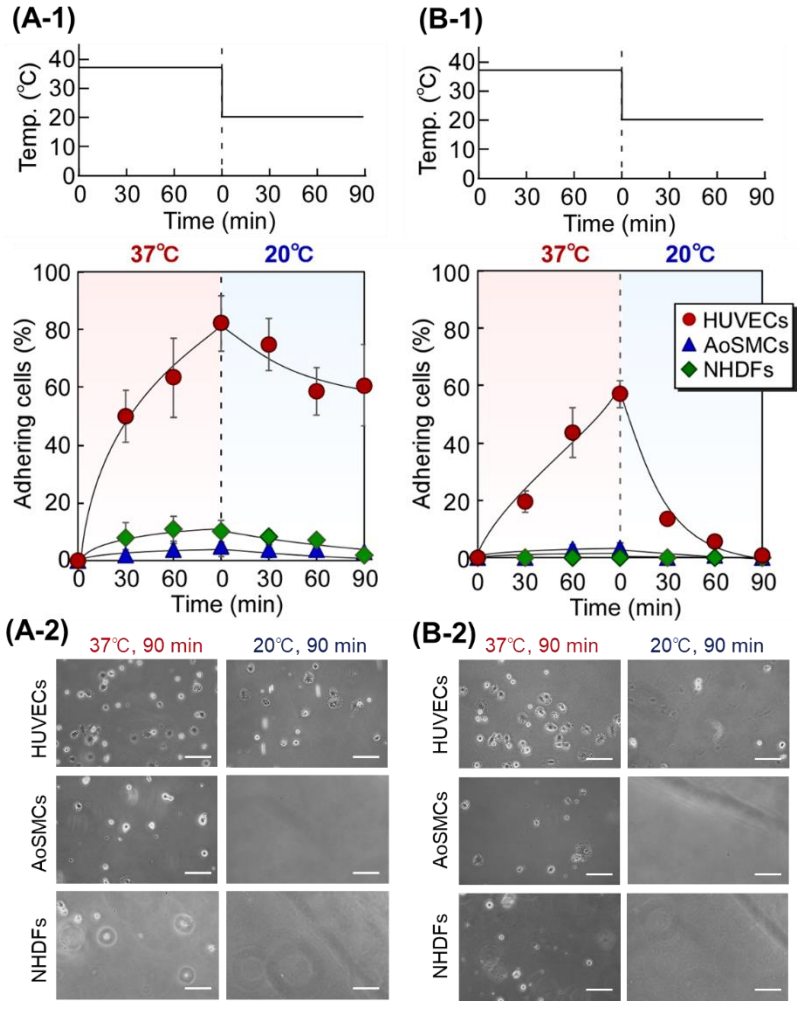

Fig. 5 Cell adhesion and detachment profiles on thermoresponsive copolymer brushes with GGGREDV. (A) P1T500-GGGREDV and (B) P1T1000-GGGREDV. (-1) Cell adhesion profiles during incubation at 37°C for 90 min and subsequent incubation at 20°C for 90 min, with time-dependent temperature profile. (-2) Cell morphology on copolymer brushes. Scale bar: 100 μm.

To increase the cell affinity of the peptide in the copolymer brushes, the peptide binding site in the bottom segment was increased from 10 mol% ("P1") to 20 mol% ("P2"). A block copolymer brush with GGGREDV was prepared using P1 and P2 segments, and HUVEC adhesion behaviors were observed (Fig. S4). There were no major differences in cell adhesion between P1 and P2 copolymers, indicating that cell adhesion could not be enhanced by increasing peptide conjugation sites in the bottom segment. In addition, the required peptide density in the copolymer brush depended on the seeded cell density. The result indicated that the amount of peptide in the P1T1000 copolymer brush was sufficient for the seeded cells at a density of 1×10^5 cells/dish.

Additionally, the effects of FBS concentration on cell adhesion were investigated (Fig. S5) because previous reports indicated that the cell affinity to the peptide is achieved in serum-free conditions.[45, 46] The non-adhesive properties of P1T1000 without the conjugated peptide were investigated at various concentrations of FBS using NHDFs (Fig. S5). However, NHDFs adhered to the P1T1000 copolymer brush under serum-free conditions (0% FBS). As the concentration of FBS in the culture medium increased, NHDF adhesion was suppressed, with complete suppression observed at 10% FBS. In a previous study, the initial adhesion of fibroblasts on the cell culture dish was induced using secreted fibronectin under serum-free conditions.[80] In the present study, NHDFs similarly adhered to the copolymer brush. With increasing serum concentrations, the albumin in the serum prevented non-specific adhesion of NHDFs onto the copolymer brush. Thus, NHDF adhesion decreased with increasing serum concentrations. The results indicated that 10% FBS in cell culture medium was essential for suppressing the non-specific adhesion of cells on the block copolymer brushes.

To investigate the availability of the developed GGGREDV-conjugated polymer brush for cell separation, cell separation experiments were performed using a mixture of HUVECs, AoSMCs, and NHDFs. HUVECs, AoSMCs, and NHDFs were stained red, blue, and green, respectively, using different

cell staining reagents to identify each cell type and were mixed at a ratio of 1:1:1. The prepared cell suspension was seeded on P1T500-GGGREDV and P1T1000-GGGREDV, and cell adhesion and detachment were observed after incubation at 37°C for 90 min and subsequent incubation at 20°C for 90 min (Fig. 6). HUVEC adhesion on these copolymer brushes at 37°C was slightly reduced compared with individually seeded HUVECs, as shown in Fig. 5, probably because the cell staining reagent reduced HUVEC activity. Using P1T500-GGGREDV, HUVECs selectively adhered at 37°C. However, the HUVECs did not detach at 20°C, potentially because the affinity between the peptide and the cells could not be modulated by the thermoresponsive upper segment in the brush. This result could be attributed to the insufficient length of the upper segment. On the contrary, using the P1T1000-GGGREDV copolymer brush, HUVECs could selectively adhere at 37°C and detach at 20°C. The results indicated that HUVECs could be obtained from a mixture of cells using the prepared peptide-conjugated copolymer brushes simply by changing the temperature (Fig. 6C). The cell composition of recovered cells after incubation at 20°C was estimated, and HUVECs were recovered at approximately 90% purity.

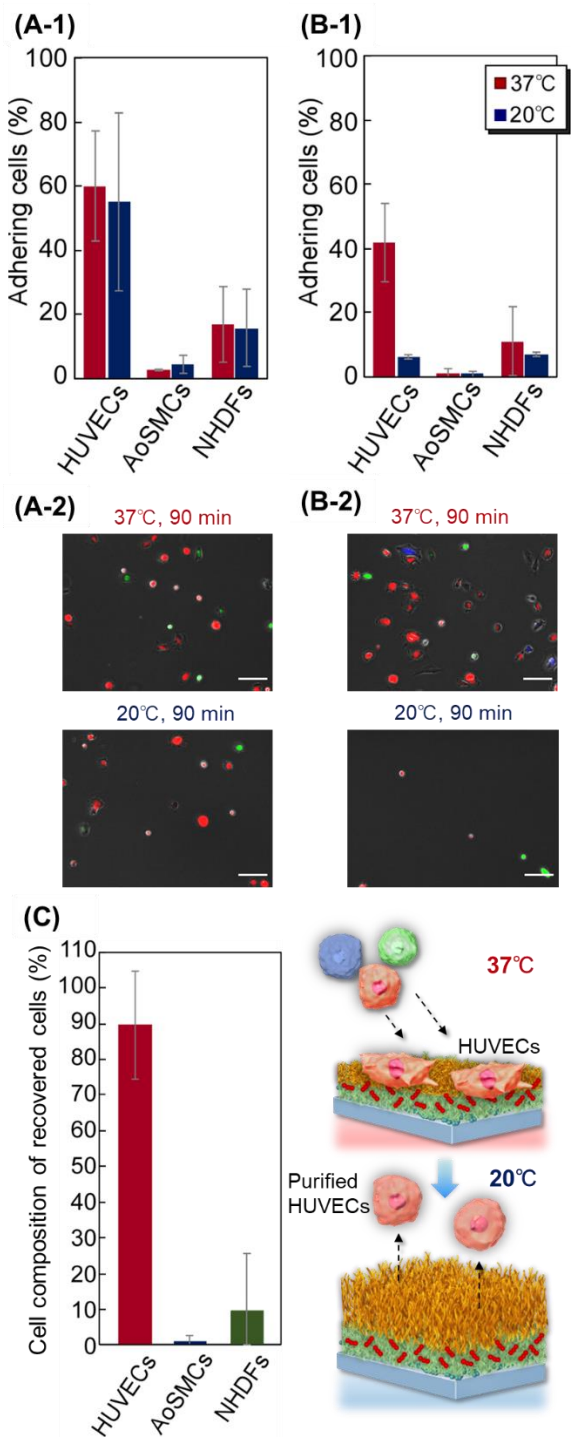

Fig. 6 Cell adhesion and detachment on thermoresponsive copolymer brushes with GGGREDV in mixtures of HUVECs, AoSMCs, and NHDFs. (A) P1T500-GGGREDV and (B) P1T1000-GGGREDV. (-1) Cell adhesion ratio after incubation at 37°C for 90 min and subsequent incubation at 20°C for 90 min. (-2) Cell morphology on copolymer brushes in coculture conditions. HUVECs: red, AoSMCs: blue, and NHDFs: green. Scale bar: 100 μm. (C) Estimated cell composition of recovered cells after incubation at 37°C for 90 min and subsequent incubation at 20°C for 90 min.

To investigate the change in cell activity during the temperature-modulated cell separation process, HUVEC viability before seeding and after recovery with P1T1000-GGGREDV was evaluated using trypan blue assays (Fig. S6). There were no significant differences in cell viability before seeding and after recovery ($p > 0.05$). Thus, the results indicated that cell separation methods could separate cells while maintaining cell viability.

These results demonstrated that the thermoresponsive block copolymer brushes harboring a cell affinity peptide could separate specific types of cells without modification. Thus, these copolymer brushes might be powerful tools for the purification of cells in tissue engineering and cell therapy applications.

4. Conclusions

We developed a thermoresponsive block copolymer brush with cell affinity peptides for temperature-modulated cell separation with high selectivity. A block copolymer brush consisting of a bottom peptide conjugating segment and an upper thermoresponsive segment was prepared via two steps of ATRP and subsequent conjugation of cell affinity peptides to the bottom segment of the block copolymer brush. The affinity between peptides on the copolymer brush and cells was investigated using cRGD as a cell affinity peptide. The cRGD-conjugated copolymer brush exhibited enhanced cell adhesion with a rapid adhesion rate. In contrast, cells barely adhered to the copolymer brush in the absence of cRGD because of the affinity between the peptide and cells. Temperature-modulated cell adhesion and detachment were achieved with a relatively long thermoresponsive segment in the copolymer brush owing to the extension and shrinkage of the thermoresponsive segment, leading to the modulation of affinity between cells and peptides. Selective HUVEC adhesion was also achieved using GGGREDV peptide at 37°C, whereas AoSMCs and NHDFs did not adhere to the copolymer brush. The adhered HUVECs was successfully recovered by reducing the temperature from 37 to 20°C with a longer thermoresponsive copolymer

segment. Using this property, HUVEC purification from a mixture of cells was performed simply by changing the temperature. These results demonstrated that thermoresponsive block copolymer brushes harboring the cell affinity peptide could be a useful cell separation tool for tissue engineering and cell therapy applications because cells could be separated simply by changing the temperature without modifying the cell surface.

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Conflict of interests

There are no conflicts of declare.

Electronic supplementary information

Supplementary data can be found in the online version.

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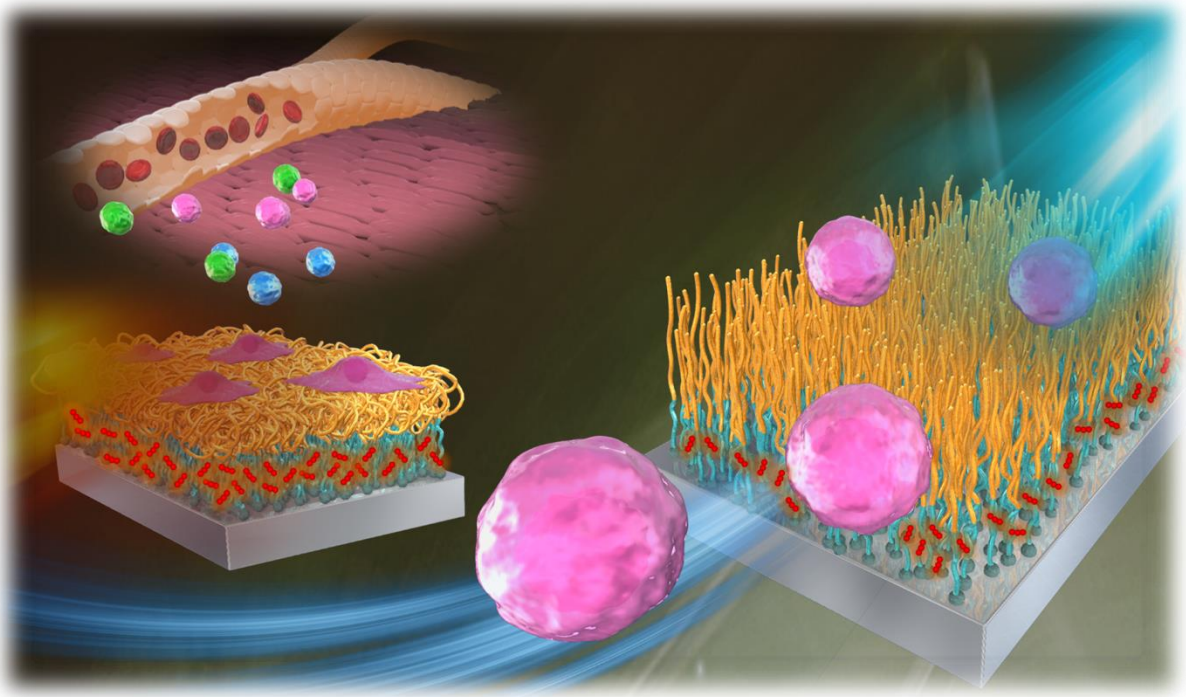
Graphical Abstract



Thermoresponsive block copolymer brush with cell affinity peptides was prepared via two steps of ATRP and subsequent click reaction. The prepared polymer brush can capture target cells with high selectivity at 37 °C and non-invasively release cells at 20 °C using polymer brush property.