Journal of Materials Chemistry B

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Journal:	*Journal of Materials Chemistry B*
Manuscript ID	TB-ART-04-2020-000972.R1
Article Type:	Paper
Date Submitted by the Author:	04-Jun-2020
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Enhanced mechanical properties and cell separation with thermal control of PIPAAm-brushed polymer-blend microfibers

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Abstract

We developed thermoresponsive microfibers with improved mechanical properties and enhanced temperature modulated-cell separation. Microfiber substrates were electrospun using poly(4-vinylbenzyl chloride) (PVBC)–poly(*n*-butyl methacrylate) (PBMA) blend materials in different ratios. Although diameters were similar to that of the PVBC homofiber, polymer-blend microfibers exhibited excellent mechanical properties of non-brittle softness, owing to PBMA with low T_g. These polymer-blend microfibers enabled preparation of thin, dense mats that were superior in the experimental handling of cell separation. Poly(*N*-isopropylacrylamide) (PIPAAm) brushes were grafted via surface-initiated atom transfer radical polymerization from the initiation sites of PVBC in the polymer-blend microfiber substrates. The microfiber in the 25:75 ratio of PVBC:PBMA had a reasonable amount of the initiation site and superior mechanical properties. The PIPAAm-brushed microfibers of the 25:75 blend substrate were capable of temperature-modulation, both in wettability and cell separation. Among normal human dermal fibroblasts (NHDFs), human umbilical vein endothelial cells (HUVECs), and human skeletal muscle myoblasts (HSMMs), HUVEC cells showed significantly poor adhesion on fibers at 37 °C; they were separated from adhered NHDF and HSMM cells in the initial step. Reducing the temperature to 20 °C remarkably detached NHDF cells, allowing their separation from HSMM cells. Compared with the PIPAAm-brushed PVBC homopolymer microfiber, these cell-separating functions were enhanced in the thermoresponsive PBMA-rich polymer-blend microfiber, probably ascribed to the property of PBMA and the moderate density of PIPAAm-brush. Thus, the developed microfibers could be useful for temperature-modulated cell separation systems.

Keywords: Electrospinning, Thermoresponsive polymer, Cell separation, Polymer-blend substrate, mechanical property

1. Introduction

For several decades, tissue engineering and regenerative medicine have been utilized for the development of new therapies for intractable diseases.[1-8] In these therapies, cell suspension or engineered cellular tissues are transplanted in patients. Effective cell separation techniques are required to achieve safe transplantations, using highly purified cells of the appropriate type. Various types of cell separation technologies have been hitherto developed.[9-16] Fluorescent activated cell sorting or magnetic activated cell sorting are widely used owing to their accurate cell-separation performance. However, these cell separation techniques require the modification of cell surfaces with fluorescent dyes or magnetic particles. Label-free cell separation has the advantages of easy operation, low cost, and versatile applications for the collected cells. Microfluidics devices can be used for label-free cell separation.[15, 16] However, separation of large amounts of cells at once remains difficult. Thus, technology enabling large-scale separation is urgently required.

Poly(*N*-isopropylacrylamide)—PIPAAm—is known to exhibit a unique thermoresponsive property; it reversibly becomes hydrophobic/hydrophilic responding to thermal stimuli across its lower critical solution temperature (LCST) of 32 °C.[17] Because changes in polymer chain morphologies are accompanied by changes in bio-affinities, PIPAAm is used in various biomedical applications,[18, 19] including drug and gene delivery systems,[20-22] biosensors, diagnostic devices,[23-25] temperature-responsive chromatography,[26-29] and cell culture substrates for fabrication of cell sheets.[30-33] We applied PIPAAm to the process of intact cell separation.[34-39] PIPAAm or PIPAAm copolymer-modified glass plates were used as substrates.[34-37] In principle, cells were shown to adhere to substrates at 37 °C under which PIPAAm-modified surfaces were hydrophobic, whereas after reducing the temperature to 20 °C, PIPAAm became hydrophilic and the adhered cells were observed to be detached from the substrates. Cell adhesion and detachment behaviors varied depending on cell type, leading to temperature-modulated cell separation without modification of cell surfaces. However, the PIPAAm-modified glass plates used in our study had a small surface area, which made the scale of cell

separation also small. To overcome this issue, we have developed thermoresponsive microfibers to be used as cell separation material with large surface area.[39] These thermoresponsive microfibers were prepared by electrospinning of poly(4-vinylbenzyl chloride)—PVBC, a derivative of polystyrene—and subsequent PIPAAm grafting from a methyl chloride group of PVBC via surface-initiated atom transfer radical polymerization (ATRP).[39] However, the PVBC microfibers were observed to be fragile, restricting its handling and practical utility as a cell separation tool. In addition, cell adhesion on the PIPAAm-grafted PVBC microfibers was not satisfactorily high; it was approximately 40–60 % at 37 °C.[39] Thus, simultaneous enhancement of mechanical properties of the material and cell adhesion should be crucial.

In the present study, we aimed to prepare thermoresponsive microfibers with excellent mechanical properties and cell adhesion as a new cell separation tool. Base microfiber substrates were electrospun using polymer-blends[40, 41] of PVBC and poly(*n*-butyl methacrylate)—PBMA, because PBMA is known to be softer and more flexible than PVBC. Moreover, in the previous study, BMA enhanced cell adhesion properties when copolymerized with IPAAm and grafted as a polymer brush.[35] In this study, a PBMA homopolymer was used, and a polymer mixture containing more than half PBMA was used as a substrate for PIPAAm grafting. It is interesting how this different molecular environment affected cell adhesion, detachment properties, and cell separation functionality. Furthermore, BMA copolymers have been used for drug delivery and tissue engineering applications.[42, 43] Hence, our blend polymer substrate should be non-cytotoxic. Thermoresponsive PIPAAm was grafted on the PVBC-PBMA microfiber surfaces via surface-initiated ATRP. Accordingly, we examined the cell adhesion and detachment behaviors on the prepared microfibers and the feasibility of cell separation, using cells for vascular and muscular tissue engineering.

2. Materials and methods

2.1 Materials

N-isopropylacrylamide (IPAAm) was purchased from KJ Chemicals (Tokyo, Japan) and recrystallized before use. 4-Vinylbenzyl chloride (VBC), poly(*n*-butyl methacrylate) (PBMA), and ethyl 2-chloropropinate (ECP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, toluene, dichloromethane, tris(2-aminoethyl)amine (TREN), copper(I) chloride (CuCl), copper(II) chloride ($CuCl_2$), and 2,2′-azobis(isobutyronitrile) (AIBN) were purchased from Fujifilm Wako Pure Chemicals (Osaka, Japan). Tris[(2-dimethylamino)ethyl]amine was synthesized from TREN according to the procedure previously reported.[44] Normal human dermal fibroblasts expressing green fluorescent protein (GFP-NHDFs) and human umbilical vein endothelial cells expressing red fluorescent protein (RFP-HUVECs) were obtained from Angio-Proteomie (Boston, MA, USA). Alexa Fluor-labeled phalloidin was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Other cells and culture media were purchased from Lonza (Basel, Switzerland).

2.2 Preparation of polymer-blend fiber substrates and PIPAAm-brushed thermoresponsive microfibers

Thermoresponsive microfibers were prepared by electrospinning polymer-blends of PVBC and PBMA in different ratios and subsequent atom transfer radical polymerization (ATRP) of IPAAm for PIPAAm modification on the surface of the fiber (Fig. 1A). The prepared thermoresponsive microfibers were used as temperature-modulated cell separation materials (Fig. 1B).

First, PVBC was prepared via radical polymerization of VBC. The polymerization inhibiter in the VBC monomer was removed by passing through the inhibitor-remover column. Then, VBC (50 mL, 0.350 mol) and AIBN (164.2 mg, 0.593 mmol) were dissolved in 200 mL toluene. Oxygen in the monomer solution was removed by freeze-pump-thaw cycling, and polymerization of VBC was performed at 70 °C for 20 h. After polymerization, purified PVBC was obtained via reprecipitation with methanol. The average molecular weight of the prepared PVBC was determined using gel permeation

chromatography (GPC). Commercially available PBMA (inherent viscosity of 0.470–0.560 dL/g) was used as the complementary component of microfibers.

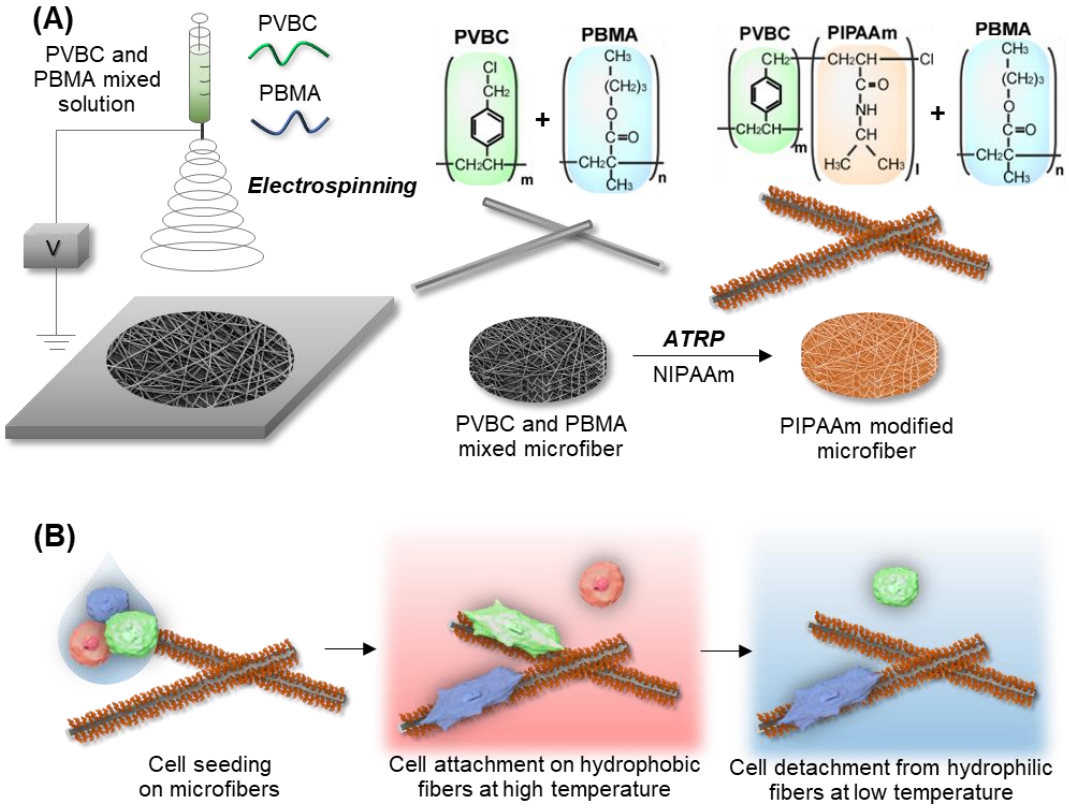

Fig. 1 Schematics showing (A) preparation of thermoresponsive microfibers and (B) cell separation processes.

Initially, PVBC and PBMA (1.8 g in total, 30 w/w%) were dissolved in 3.16 mL (4.2 g) of dichloromethane at PVBC:PBMA weight ratios of 100:0, 75:25, 50:50, 25:75, and 0:100 at 25 °C overnight. Microfibers of each polymer were electrospun using an electrospinning apparatus (NANON; MECC, Fukuoka, Japan). The spinning conditions were as follows: 30 kV applied voltage, 2.0 mL/h polymer solution feed ratio, 22 G syringe, 60 s spinning time, 25 °C, 30 cm distance between syringe tip and collector, and a fixed collector.

The reaction solvent for ATRP modification of PIPAAm was optimized particularly focusing on non-solubility of microfibers by changing its composition of methanol and water. The electrospun

PBMA microfibers were immersed into the mixed solvents (100:0, 80:20, 50:50, 20:80, 0:100 methanol:water) and incubated at 25 °C for 1 h with continuous shaking. After incubation, the microfibers were dried under reduced pressure, and their morphologies were observed using scanning electron microscopy (SEM; VE9800; Keyence, Osaka, Japan) and gold sputter deposition.

ATRP was performed for PIPAAm modification of the microfibers. To this end, IPAAm (339 mg, 3 mmol) was dissolved in a mixed solvent of methanol (24 mL) and water (6 mL) in a 100 mL flask. The solution was deoxygenated by bubbling argon gas for 30 min. Subsequently, CuCl (19.8 mg, 0.2 mmol) and $CuCl_2$ (2.63 mg, 0.02 mmol) were added to the solution. The flask was sealed and transferred in a glove bag. Consecutively, microfibers (300 mg) in a 50 mL glass vessel and Me_6TREN (50.7 mg, 0.225 mmol) in a sealed glass tube were also placed in the glove bag, and vacuuming and refilling with argon gas was conducted. Then, Me_6TREN and the monomer solution was mixed and poured to the microfibers. In addition, ECP (2.46 μL, 0.018 mmol) was also added as a free initiator to obtain free PIPAAm for molecular weight analysis. The reaction was proceeded at 25 °C for 1 h with continuously shaking of the sealed glass vessel. After being rinsed with the methanol/water mixed solvent and then with water, the microfibers were immersed overnight in EDTA solution at 25 °C with continuous shaking to remove the cupper catalyst. Subsequently, the microfibers were immersed overnight in water at 25 °C with continuous shaking to remove EDTA. Finally, the microfibers were dried at 25 °C under reduced pressure. The free PIPAAm from ECP in the ATRP reaction solvent mixture was dialyzed (MWCO, 1 kDa) against EDTA solution and subsequently water. The purified polymer was obtained upon freeze-drying.

2.3 Characterization of polymer-blend fiber substrates and PIPAAm-brushed thermoresponsive microfibers

The morphologies of all prepared microfibers were observed using scanning electron microscopy (SEM; VE9800; Keyence, Osaka) at 2 kV of accelerating voltage with gold sputter

deposition. Fiber diameter and fiber mat thickness were also measured from the obtained SEM images using Image J software (National Institutes of Health, Baltimore, MD, USA).

The mechanical properties of the polymer-blend microfiber substrates were investigated using a tensile tester (EZ-S, Shimadzu, Kyoto, Japan) with a load cell of 5-N upper limit. The fiber mat was cut into 40 × 10 mm pieces. The distance between the grippers was set at 30 mm. The tension rate was 1.0 mm/min. Strain energy was obtained from the area under the stress-strain curve. Young's modulus was obtained from the slope of the stress-strain curve at the point of initial 0.5 % stress (PVBC:PBMA 100:0, 75:25, 50:50, and 25:75) and initial 0.1 % stress (0:100). Focusing on the fiber of PVBC:PBMA = 25:75 because of its superior mechanical properties, its composition was precisely analyzed with ^{1}H-NMR. The fibers were dissolved in chloroform-*d* (10 mg/mL) and the ^{1}H-NMR spectrum was measured (400 MHz, Varian, Palo Alto, USA). For comparison, the ^{1}H-NMR spectra of PVBC, PBMA, and mixture of PVBC and PBMA at the ratio of 25:75 were also measured.

The PIPAAm grafting on the polymer-blend microfiber substrates was confirmed using FT-IR spectroscopy and X-ray photoelectron spectroscopy by comparing the spectra of microfibers before and after ATRP. Respective FT-IR spectra were measured by attenuated total reflection Fourier transform infrared spectroscopy (ATR/FT-IR; FT/IR-6600, JASCO, Tokyo). Surface elemental analysis was performed using X-ray photoelectron spectroscopy (XPS; K-alpha, Thermo Fisher Scientific).

The molecular weight of the PIPAAm brush was indirectly determined by measuring free PIPAAm from ECP using a GPC system (GPC-8320, Tosoh, Tokyo, Japan). The columns used were serially connected TSK gel series columns (Super AW2500, Super AW3000, and Super AW4000). *N,N*-dimethylformamide containing 50 mM lithium chloride was used as eluent.

The thermoresponsive-modulation of the surface wettability for the PIPAAm-brushed microfibers was analyzed with contact angle measurement in the sessile drop method (DSA 100S, KRUSS, Hamburg, Germany). The contact angle was measured at 20 °C and 37 °C one second after a water droplet (2 μL) was placed on the microfibers.

2.4 Cell adhesion and detachment assays

Temperature-dependent adhesion/detachment of each cell type on the prepared microfibers was separately examined using normal human dermal fibroblasts (NHDFs), human umbilical vein endothelial cells (HUVECs), or human skeletal muscle myoblasts (HSMMs). Culture mediums for these cells are summarized in Table S1. The prepared microfiber mat (30 mg) was placed onto a 35-mm Petri dish after sterilization by exposure to UV light for 15 min. The appropriate culture medium for each cell type (300 μL) was added, and then cell suspension (300 μL, 3.33×10^4 cells/mL) was seeded and incubated at 37 °C for 3 h (denoted as the 37 °C sample). Incubation at 37 °C for 3 h and subsequently at 20 °C for 1 h was also conducted (20 °C sample). After incubation, each cell sample was fixed with 4 % paraformaldehyde (PFA, 2 mL, 25 °C, 30 min). After treatment with 0.2 % Triton X-100 (25 °C, 20 min), rinsing with PBS, and blocking by 5 % bovine serum albumin (BSA) solution (25 °C, 60 min), fluorescent staining using Hoechst 33342 (500-fold diluted with 1 % BSA, nucleus staining) and Alexa Fluor 568-labeled phalloidin (400-fold diluted with 1 % BSA, F-actin staining) was conducted under light shielding conditions (25 °C, 60 min). Fluorescence images were captured using a fluorescent microscope (TE2000-U, Nikon, Tokyo, Japan). The ratio of the extended cells to all cells—extended and round shapes—captured in the image was calculated as the cell adhesion ratio.

Temperature-modulated cell separation on the PIPAAm-brushed microfibers was examined for HUVEC, NHDF, and HSMM cells. The myoblast culture medium SkGM-2 (300 μL) was first added to the microfibers. Then, the mixed suspension of RFP-HUVEC, GFP-NHDF, and HSMM in SkGM-2 (300 μL, 1.0×10^4 cells/mL each) was seeded on the microfiber mat, and incubated at 37 °C for 3 h (37 °C sample) or at 37 °C for 3 h followed by incubation at 20 °C for 1 h (20 °C sample). After incubation, cell fixation, treatment with Triton X-100, and Alexa Fluor405-phalloidin staining were conducted, as mentioned above. Subsequently, microscopic observations using a fluorescent microscope (TE2000-U) and a confocal laser scanning microscope (FluoView FV1200, Olympus, Tokyo) were performed and

cell adhesion ratios were calculated.

Statistical analysis was performed with an unpaired Student *t*-test with a statistical significance of $P < 0.05$. All presented values were the mean and standard deviation of 3 measurements.

3. Results and discussion

3.1 Improved mechanical properties of microfiber substrates ascribed to PBMA-blending

Our GPC measurements showed that the averaged molecular weight of the prepared PVBC was 6.20×10^4, whereas the molecular weight of purchased PBMA was calculated[45] from its inherent viscosity as $1.10–1.36 \times 10^5$; these values were considered as sufficiently large for stable electrospinning. Accordingly, FE-SEM observation revealed that all prepared microfibers of homopolymer and polymer-blends exhibited uniform structures and smooth surfaces irrespective of the mixed ratios of PVBC and PBMA (Fig. 2). The diameters of the fibers—20.0 to 28.2 μm (Table 1)—were thick enough for individual cells to adhere to single fibers, as reported previously.[39] In addition, the mesh sizes of all microfiber mats were roughly estimated to be tens to several hundreds of micrometers (Fig. 2A-1 to 2E-2); cells could pass between the microfibers to reach the interior of the fiber mats.

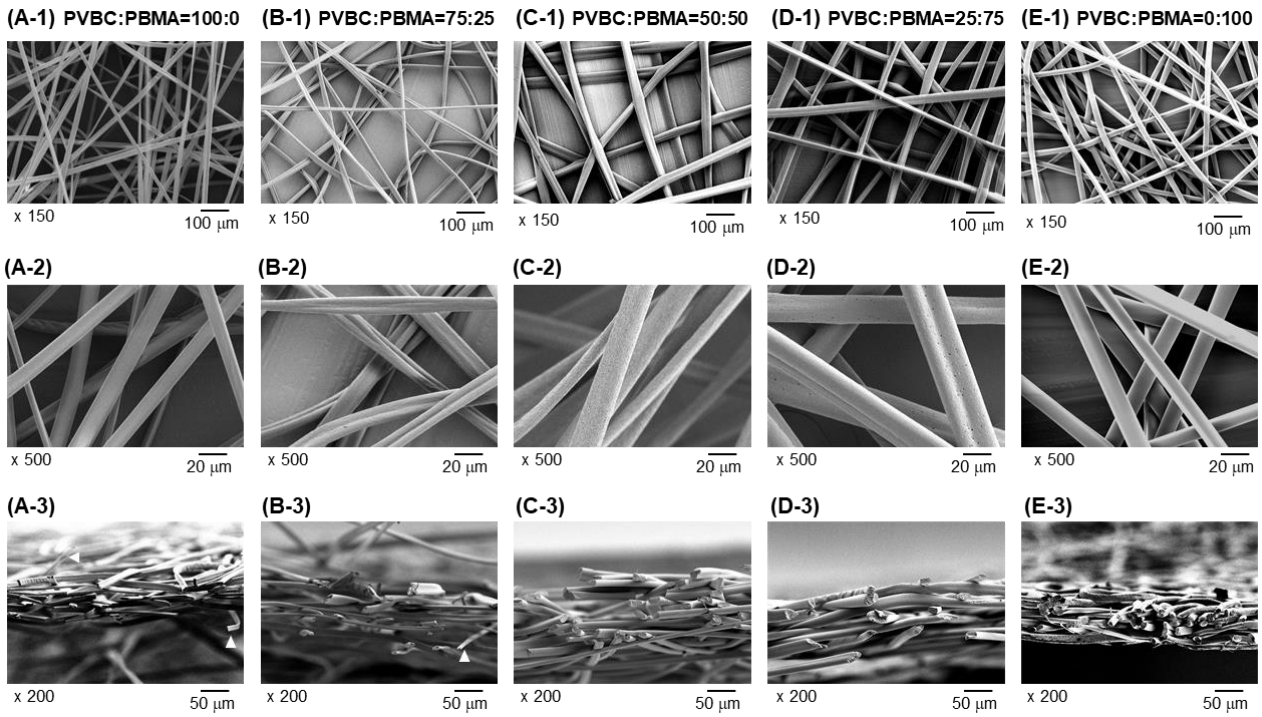

Fig. 2 SEM images of microfibers of homopolymer and polymer-blends of PVBC and PBMA in the ratio of 100:0 (A), 75:25 (B), 50:50 (C), 25:75 (D), and 0:100 (E). (1) Low magnification image, (2) high magnification image, and (3) cross-sectional image. Arrowheads in (A-3) and (B-3) indicate fibers protruding from the fiber mat.

Table 1 Microfiber diameters and thickness of prepared fiber mats

Code a)	Polymer composition	Fiber diameter (μm) b)	Fiber mat thickness (μm) b)
A	PVBC : PBMA = 100:0	22.7 ± 2.8	216.4 ± 26.6
B	PVBC : PBMA = 75:25	22.6 ± 3.0	152.6 ± 31.4
C	PVBC : PBMA = 50:50	22.9 ± 3.6	129.4 ± 14.9
D	PVBC : PBMA = 25:75	28.2 ± 3.0	118.2 ± 13.6
E	PVBC : PBMA = 0:100	20.0 ± 1.5	108.6 ± 10.0

a) Corresponding to those in Fig. 2. b) Determined from SEM images.

The thickness of the fiber mat was demonstrated to be monotonically decreased with increasing the PBMA ratio (Table 1). Cross-sectional SEM images in Fig. 2 revealed that substrates containing no (PVBC:PBMA = 100:0) or low PBMA (75:25) had microfibers protruding out of the mat planes, whereas fibers assembled and accumulated closely in PBMA-rich mats (25:75, 0:100). The PBMA-scarce/PVBC-rich mats were shown to have a rigid, hard texture, which broke easily, leading to

formation of thick mats. In contrast, PBMA-rich mats were observed to be non-brittle and flexible. This could be attributed to the softness of PBMA with low T_g (ca. 25 °C, molecular weight 9.37×10^4)[46], enabling dense accumulation and formation of thinner mats with fiber deformation, such as curving, bending, squashing, and so forth.

The non-brittle softness of PBMA-rich microfibers was verified in the tensile test (Fig. 3 and Fig. S1). The strain-stress curves of the PBMA-scarce (PVBC:PBMA = 100:0, 75:25) microfiber mats showed coarse profiles. These mats exhibited remarkably low ultimate tensile strength, with averages values being less than 0.1 MPa. The strain at the break point of the homo-PVBC, as a polystyrene derivative—ca. 2 %—was observed to be similar to that of polystyrene[47] despite differences in the shapes of samples. To examine how mechanical properties were enhanced by blending PBMA at each ratio, we performed two-group comparison tests between a PVBC homo-polymer as a reference sample and each PVBC-PBMA polymer blend using Student's *t*-test. Both tensile strength and Young's modulus values were high with significantly difference in the PVBC:PBMA = 25:75 blend ($p < 0.01$), while those value were significantly different in the 50:50 blend ($p < 0.05$) but not in the 75:25 blend. All average values of tensile strength of PBMA rich microfibers became 0.4–0.5 MPa. These values were slightly lower than those reported for polycaprolactone (PCL) and polylactic acid (PLLA)-*co*-PCL fiber mats, but not as deviated considering the differences of the materials.[48] Furthermore, the Young's modulus appeared to become higher in PBMA-rich microfiber mats. The values were shown to be in the tens of the MPa order. In addition, the strain energy of microfibers increased with the PBMA content in microfibers. In particular, homo-PBMA microfibers exhibited a significantly high strain energy ascribed to the large strain value at almost the same ultimate tensile strength—ca. 0.6 % (Fig. S1). We assumed that the higher content of PBMA with ductility, as well as the higher density of fiber mats (Fig. 2) probably contributed to the substantial improvement of these mechanical properties. Evaluation of their mechanical properties suggested that the mechanical strength of microfibers increased with PBMA content. However, microfibers composed of only PBMA did not have an ATRP initiation site for

subsequent PIPAAm modification. Thus, the PVBC:PBMA = 25:75 composite microfibers were used for subsequent investigations, because of their exhibited substantial mechanical strength, ideal for handling in the chemical modification with PIPAAm and cell separation experiments.

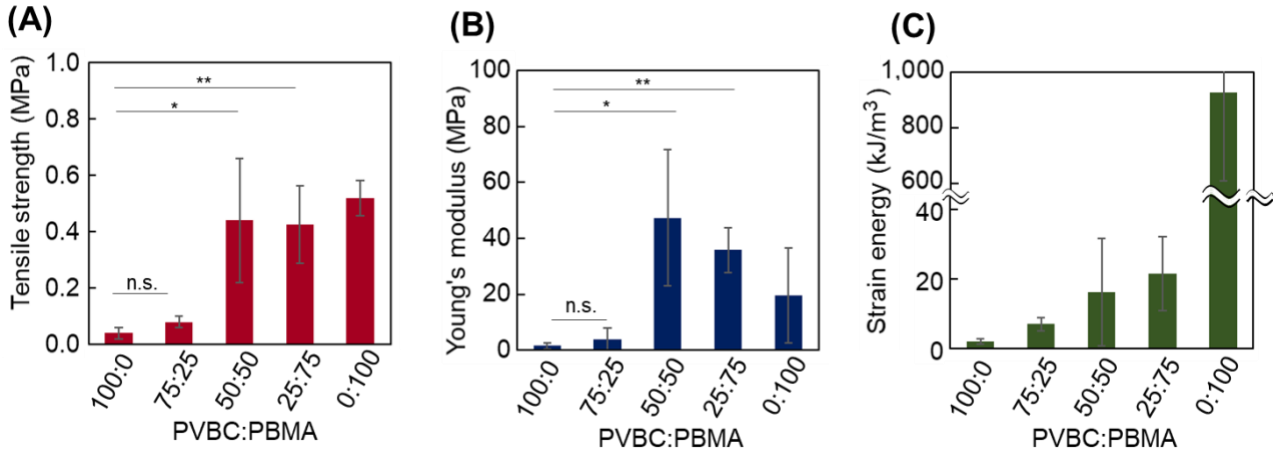

Fig. 3 Mechanical properties of microfiber mats with various polymer compositions. (A) Tensile strength, (B) Young's modulus, and (C) strain energy (** $p < 0.01$; * $p < 0.05$; n.s. indicates differences that are not significant with $p > 0.05$, $n = 3$).

To confirm the actual polymer composition, the ^{1}H NMR spectrum of prepared microfibers was measured and compared to that of a 25:75 (w/w) PVBC and PBMA mixture (Fig. S2). The ratio of methylene peaks of PVBC to that of PBMA was almost the same between microfibers and mixed PVBC and PBMA, clearly indicating that the composition of the polymer of the prepared microfibers was almost the same as that of the feed polymer at electrospinning.

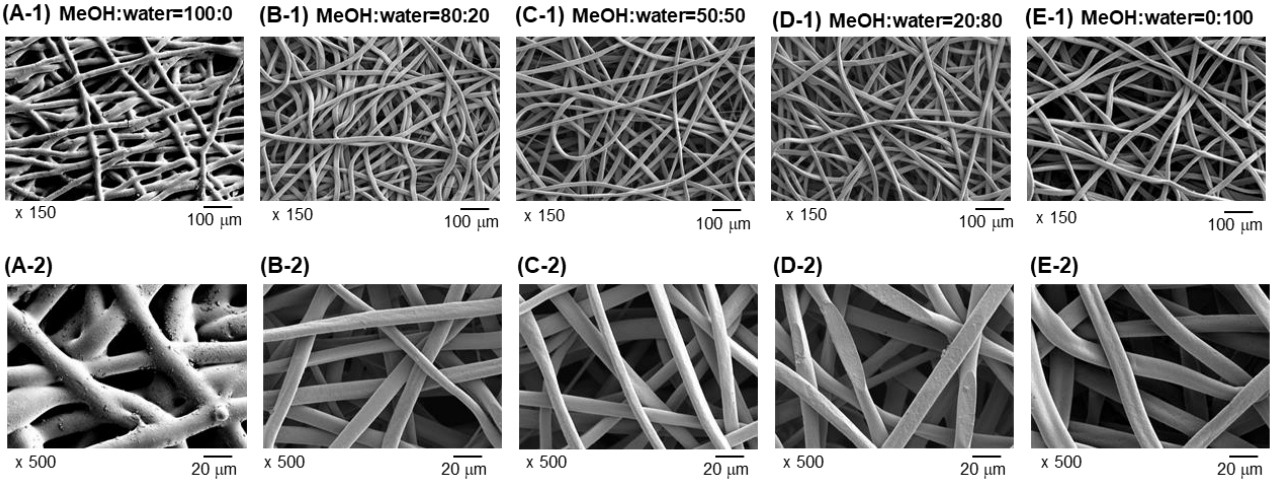

Fig. 4 Fiber morphologies after immersing in reaction solvent of ATRP. (A) 100:0, (B) 80:20, (C) 50:50, (D) 20:80, and (E) 0:100 of the solvent mixture methanol:water. (1) low and (2) high magnification images.

Next, ATRP was performed for the PIPAAm modification of microfibers. In previous studies on PIPAAm modification of several substrates, including polystyrene by ATRP, 2-propanol, methanol, and a mixture of methanol and water were used as reaction solvents.[38, 39, 49, 50] According to these studies, we anticipated that solvents with relatively low polarity might dissolve microfibers during ATRP. Hence, we observed the morphologies of microfibers of PBMA after immersing in the mixed solvents of methanol and water in various ratios (Fig. 4). As anticipated, microfibers immersed in methanol were observed to be partially dissolved and fused together. In contrast, the morphologies of microfibers immersed in methanol:water = 80:20, 50:50, 20:80, and 0:100 did not change. Among these solvents, water (0:100) was shown to be inappropriate as an ATRP reaction solvent for PIPAAm modification, because water could not sufficiently penetrate into the interior of the hydrophobic PVBC:PBMA = 25:75 microfiber mat; PIPAAm modification on microfibers could not occur inside the mat. In addition, water was noted to accelerate the reaction rate of ATRP, and an excessively high reaction rate might lead to uncontrolled polymerization.[50] Thus, an 80:20 methanol:water ratio was used as solvent for ATRP.

To examine the PIPAAm modification on microfibers, we performed surface elemental analysis of microfibers before and after ATRP using XPS (Table 2). Accordingly, we noted that higher nitrogen to carbon ratio was obtained in PIPAAm-modified microfibers than in unmodified ones, due to the clearly detected nitrogen of the modified PIPAAm. This result suggested that microfibers were successfully modified by PIPAAm using ATRP. Additionally, we measured the FT-IR spectrum of PIPAAm modified microfibers (Fig. S3), where small peaks were observed at 1600 cm^{-1}, attributed to the amide bond of IPAAm. In contrast, no such peaks were detected in unmodified microfibers, directly indicating that PIPAAm was successfully modified on microfibers through the ATRP procedure. The molecular weight of free PIPAAm synthesized from ECP by ATRP in the reaction solvent was recovered and characterized using GPC in order to estimate that of the PIPAAm modified on microfibers (Table 2). The averaged molecular weight M_n and polydispersity M_w/M_n were shown to be

2500 and 1.14, respectively. The small polydispersity was indicative of controlled polymerization under the ATRP conditions.

The thermoresponsive property of the microfibers was investigated using the contact angle measurement in a sessile drop method (Fig. 5 and Fig. S4). The water droplets on the microfiber mats exhibited a spherical structure, due to the trapped air in the microfiber mats, as explained by the Cassie-Baxter state.[51, 52] Compared with that of unmodified microfibers, the PIPAAm-modified microfibers exhibited a slight hydrophilic property. This was assumed to result from the fact that modified PIPAAm rendered the microfibers hydrophilic. In addition, PIPAAm-modified microfibers were demonstrated to be significantly more hydrophobic at 37 °C than at 20 °C (Fig. S4), whereas the contact angles of unmodified microfibers scarcely changed. This result indicates that PIPAAm-modified microfibers exhibited temperature-responsive changes in hydrophobicity, and thus could be used as thermoresponsive cell adhesion and detachment substrates.

Table 2 Characterization of microfibers before and after PIPAAm modification.

Code	Elemental composition (%) a)				N/C ratio	M_n b)	M_w/M_n b)
	C	N	O	Cl			
Unmodified Fiber	80.4	0.02	19.3	0.24	0.25×10^{-3}	-	-
PIPAAm modified fiber	80.9	0.91	17.8	0.40	11.2×10^{-3}	2500	1.14

a) Determined using XPS. b) Determined using GPC for free PIPAAm copolymerized in ATRP.

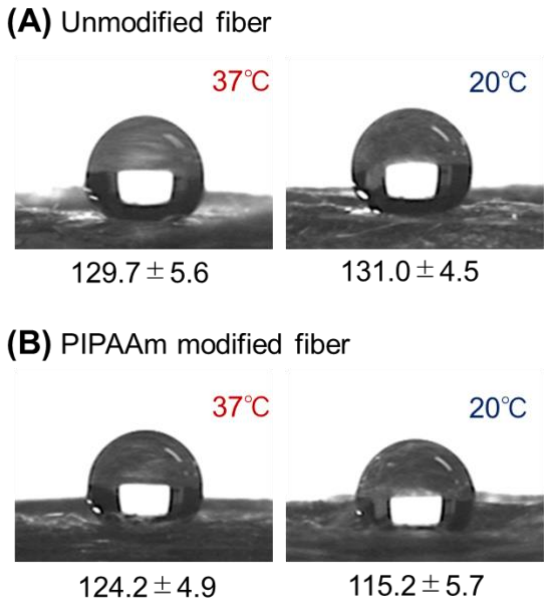

Fig. 5 Contact angle measurement in a sessile drop method for microfibers before (A) and after (B) PIPAAm modification.

3.2 Cell adhesion properties on thermoresponsive microfibers

We further examined the adhesion behavior of three types of cells, namely HUVEC, NHDF, and HSMM cells, on the thermoresponsive microfibers (Fig. 6 and 7). As control experiments, cell morphology on polystyrene tissue culture dishes—absolute positive controls clearly showing adhered cell morphology—and unmodified microfibers were observed (Fig. S5, S6 and S7). These cell types are actually used in the fabrication of cardiovascular and muscular engineered tissues.[3, 53-56] Thus, effective separation of these cells would be useful for tissue engineering applications. To clearly evaluate its adhesion behavior, each cell type was individually seeded and cultured on thermoresponsive microfibers at 37 °C. As a result, we observed that NHDF and HSMM cells adhered well on these microfibers; fluorescently stained F-actin fibers were observed in the elongated shape (Fig. 6). In contrast, punctate F-actin signals in HUVEC cells suggested weak adhesion. Accordingly, the number of HUVEC cells on the microfiber substrate was shown to be apparently small. These results reasonably reflected the inherent property of each cell type. Previous studies have also shown that NHDF and HSMM cells

exhibited good adhesion on non-thermoresponsive micro-patterned substrates and electrospun fibers; they were reported to be noticeably elongated along these substrates, whereas HUVEC cells did not.[38, 39, 57, 58] We also examined the effect of the temperature change on the behaviors of cells attached on the prepared thermoresponsive fibers. Most of the adhered NHDF cells were shown to get detached from microfibers following the subsequent incubation at 20 °C, as the modified PIPAAm on microfibers became hydrophilic. However, HSMM cells were noted to be still adhered to microfibers at 20 °C, probably because of their strong adhesion property. Thus, HSMM cells were demonstrated to not detach from microfibers even at low temperature. We have also reported the inherent strong adhesion of HSMM cells compared with those of HUVEC and NHDF cells on other thermoresponsive substrates—the micro-patterned culture substrates; HSMM cells was reported to adhere to the micro-patterned substrates via actin stress fibers.[38]

It is noteworthy that PBMA-containing thermoresponsive microfibers in this study exhibited the higher cell adhesion and detachment property for NHDF cells, compared with those of previously reported PBMA-non-containing thermoresponsive microfibers.[39] These results indicated that the prepared microfibers in this study have excellent potency as temperature modulated cell separation materials. This could be probably attributed to the lower density of PIPAAm. Previous reports indicated that PIPAAm brush-modified substrates in the low density exhibited an enhanced cell adhesion property compared with that of dense PIPAAm brush.[32, 33] In the present study, the density of PIPAAm brush on microfibers would become considerably low, because the composition of PVBC was only 25 %; the previous type of PIPAAm-modified microfibers was prepared using PVBC alone as substrate. Thus, cell adhesion to microfibers would be enhanced compared with that of the previously developed microfibers.

Additionally, we also observed the cell adhesion behavior of the unmodified microfibers to examine the effect of blending PBMA and PVBC (Fig. S5 and S6). Cells scarcely adhered to unmodified microfibers, even at 37 °C. Since PVBC microfibers exhibited good adhesion properties toward HUVECs and NHDFs in a previous report,[39] blending PBMA and PVBC may not enhance cell

adhesion. The good cell adhesion properties of the PIPAAm brushes grafted with PVBC/PBMA blend microfibers could be attributed to the low density of modified PIPAAm on the microfiber substrate.

We also examined cell number per unit area on microfibers as another method of evaluating the cell adhesion properties toward each cell type (Fig. S8), because cell adhesion ratio does not consider unattached cells on the fibers. The results showed that cell number exhibited almost the same tendency as did cell adhesion ratio. However, a large standard deviation was observed in cell numbers, indicating that this evaluation method produces widely variable results. Thus, in this study, the cell adhesion ratio was used, enabling reliable statistical evaluation with high reproducibility of the cell adhesion properties of each cell type on the microfibers (Fig. 7).

In addition, during the adhesion and detachment processes, we did not observe the same remarkable changes in cell morphology suggesting cell death that have been reported previously.[39] This indicates that the prepared microfibers have no cytotoxicity and can be utilized as cell separation materials.

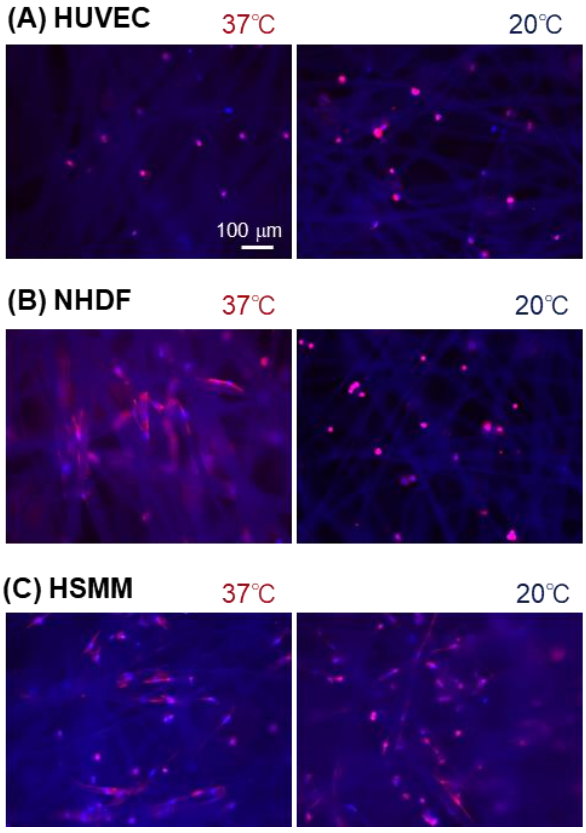

Fig. 6 Cell morphologies on PIPAAm-modified microfibers. (A) HUVEC, (B) NHDF, and (C) HSMM cells. Red: F-actin, blue: nuclei. Microfibers were observed in faint blue background owing to autofluorescence. Images on the left column (37 °C) show cells after incubation at 37 °C for 3 h. Images on the right column (20 °C) were obtained after incubation at 37 °C for 3 h, followed by incubation at 20 °C for 1 h.

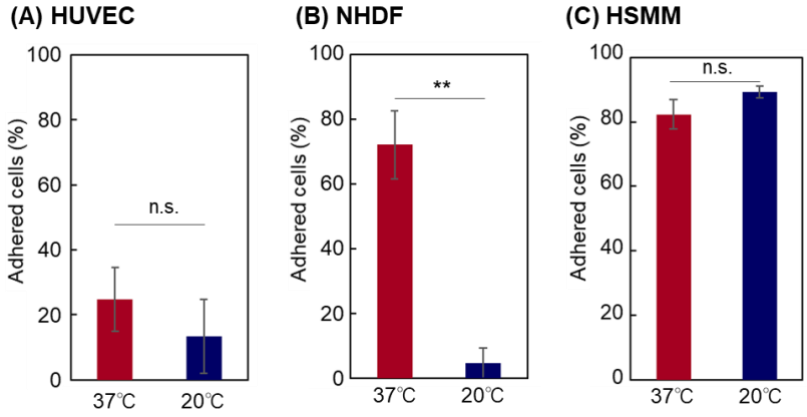

Fig. 7 Percentage of adhered cells on PIPAAm-modified microfibers. (A) HUVEC, (B) NHDF, and (C) HSMM cells. 37 °C: after incubation at 37 °C for 3 h, 20 °C: after incubation at 37 °C for 3 h, followed by incubation at 20 °C for 1 h. (** $p < 0.01$; n.s. indicated not significant in $p > 0.05$, $n = 3$).

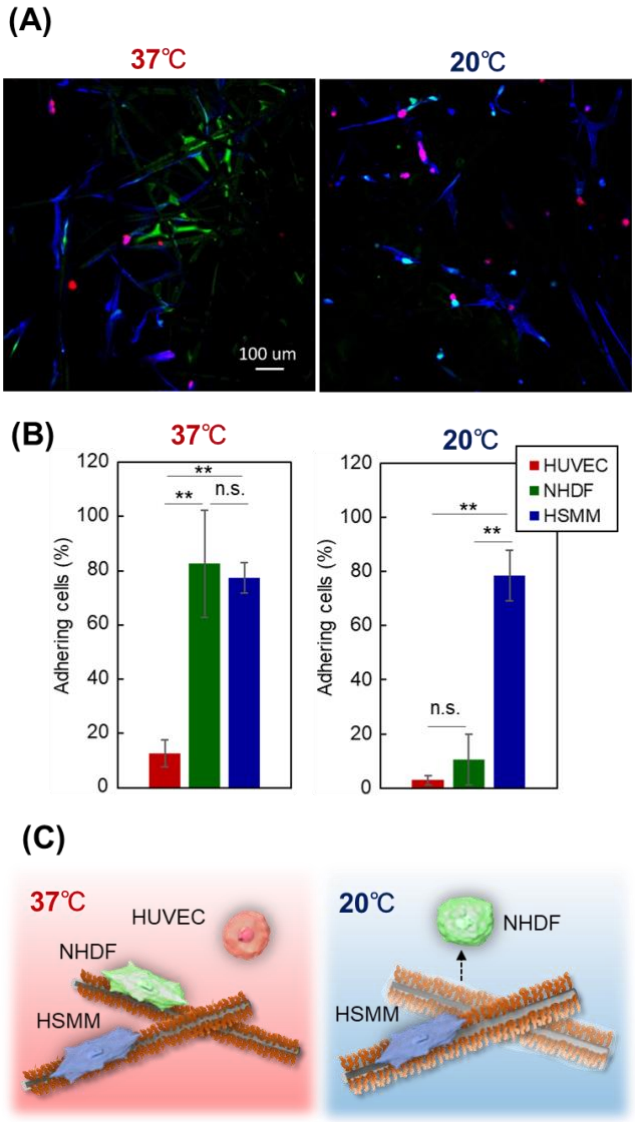

Fig. 8 Cell separation using PIPAAm-modified microfibers. (A) Fluorescence images of cell suspension mixture on microfibers. Red; HUVEC, green; NHDF, and blue; HSMM cells. (B) Ratio of cells adhering to microfibers. (C) Illustration showing temperature-modulated cell separation using thermoresponsive microfibers. (** $p < 0.01$, n.s. indicates differences that are not significant $p > 0.05$; $n = 3$).

Since each cell type exhibited different adhesion and detachment properties in response to temperature changes, we examined the thermal control of cell separation using the prepared thermoresponsive microfibers. Cell suspension mixtures of RFP-HUVEC, GFP-NHDF, and HSMM at a ratio of 1:1:1 in myoblast culture medium (Fig. 8A and B) were seeded and cultured on the prepared microfibers. At 37 °C, NHDF and HSMM cells were observed to adhere and extend on microfibers, as they did in individual cultures, whereas HUVEC cells did not, showing punctate red-fluorescent signals

(Fig. 8A). Adhered NHDF cells were shown to detach from microfibers when the temperature was reduced to 20 °C, showing punctate green-fluorescent signals (Fig. 8A), and indicating that large amounts of NHDF cells could be recovered from the culture medium by lowering the temperature (Fig. 8B, C). In contrast, HSMM cells maintained adherence even at 20 °C (Fig. 8A, B).

These observations suggested that the prepared thermoresponsive microfibers in this study could be used to separate cells from a mixture simply by changing the temperature. Thus, these microfibers could be used as easy-to-use cell separation tools.

4. Conclusions

We developed thermoresponsive microfibers with enhanced mechanical properties for temperature-modulated cell separation, the by electrospinning a mixed polymer solution consisting of PVBC and PBMA, and subsequently modifying the microfibers with PIPAAm via ATRP. The microfibers were prepared by changing the composition of PVBC and PBMA, and consequently their mechanical properties were investigated. The PBMA-rich microfibers exhibited excellent mechanical properties. On the contrary, the PVBC-rich microfibers were fragile. Based on these results, the PVBC:PBMA ratio of 25:75 was selected as the optimal substrate for microfibers. The prepared microfibers were modified with PIPAAm via ATRP, and then using cells for cardiovascular and muscular tissue engineering we examined the thermoresponsive cell adhesion and detachment on these microfibers. When both individually cultured and cocultured, NHDF and HSMM cells were observed to adhere on microfibers at 37 °C, whereas HUVEC cells did not. After reducing the temperature to 20 °C, adhered NHDF cells detached from microfibers, whereas HSMM cells remained attached. These results indicated that high content of HUVEC and NHDF cells could be obtained from the medium at 37 °C and 20 °C, respectively, and these cells could be actually separated in coculture by simply changing the temperature. Thermoresponsive microfibers could be useful tools for separation of cells in applications of tissue engineering and regenerative medicine.

Acknowledgments

This research was partially supported by a Grant-in-Aid for Scientific Research (No. 26420714 and 19H02447 for KN) from the Japan Society for the Promotion of Science (JSPS), SENTAN (no. JPMJSN16B3) from the Japan Science and Technology Agency (JST), and research grant from the Uehara Memorial Foundation.

Conflict of interests

There is no conflict of interest.

Electronic Supplementary Information

Supplementary data can be found in the online version.

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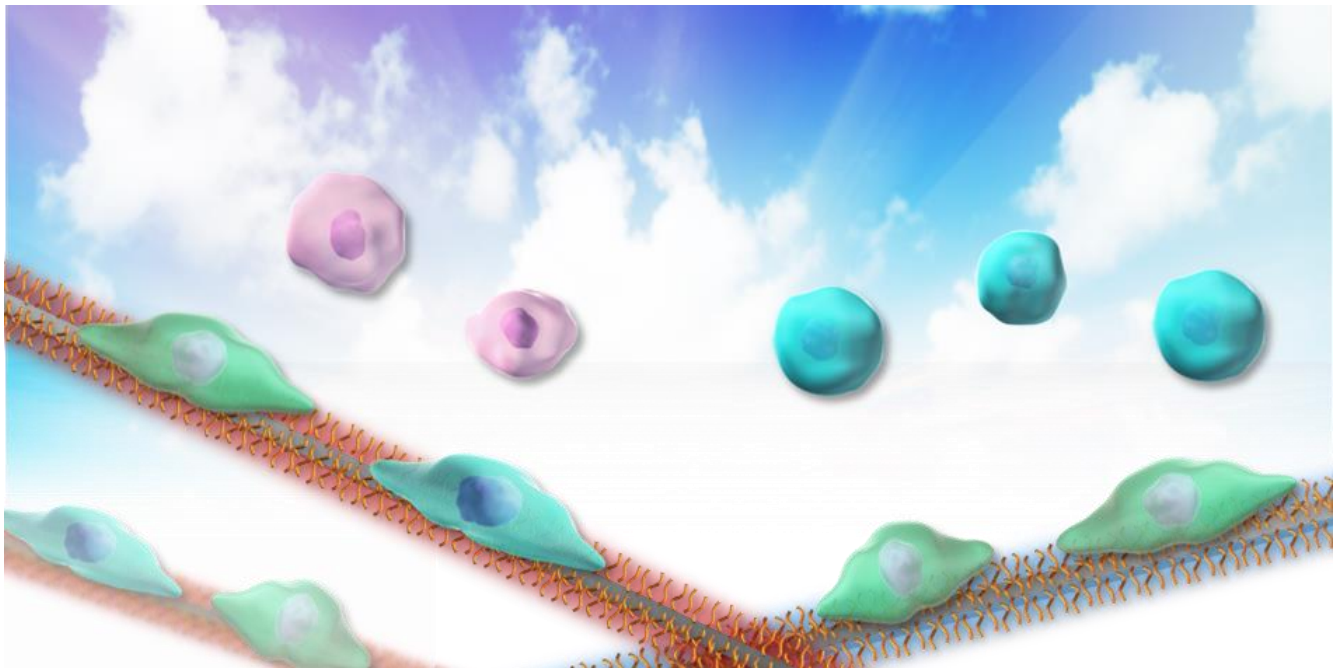
Graphical Abstract



Thermoresponsive microfibers with enhanced mechanical properties for temperature-modulated cell separation were developed by electrospinning a mixed polymer solution consisting of PVBC and PBMA, and by subsequently modifying the microfibers with PIPAAm via ATRP. Using the developed microfibers, thermally modulated cell separation for cardiovascular and muscular tissue engineering was achieved by incubation at 37 °C and 20 °C.