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Thermoresponsive polymer-modified microfibers for cell separations

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ABSTRACT

Thermoresponsive polymer-modified microfibers were prepared through electrospinning of poly(4-vinylbenzyl chloride) (PVBC) and subsequent surface-initiated atom transfer radical polymerization for grafting poly(*N*-isopropylacrylamide) (PIPAAm). Electrospinning conditions were optimized to produce large-diameter (20 μm) PVBC microfibers. The amount of PIPAAm grafted on the microfibers was controlled *via* the IPAAm monomer concentration. The microfibers exhibited thermally controlled cell separation by selective adhesion of normal human dermal fibroblasts in a mixed cell suspension that also contained human umbilical vein endothelial cells. In addition, adipose-derived stem cells (ADSCs) exhibited thermally modulated cell adhesion and detachment, while adhesion of other ADSC-related cells was low. Thus, ADSCs could be separated from a mixture of adipose tissue-derived cells simply by changing the temperature. Overall, the PIPAAm-modified microfibers are potentially applicable as temperature-modulated cell separation materials.

Statement of Significance

Thermoresponsive poly(*N*-isopropylacrylamide) (PIPAAm) polymer-modified poly(4-vinylbenzyl chloride) (PVBC) microfibers were prepared *via* electrospinning of PVBC, followed by surface-initiated ATRP. They formed effective thermally-modulated cell separation materials with large surface areas. Cells adhered and extended along the modified microfibers; this was not observed on previously reported PIPAAm-modified flat substrates. The cellular adhesion enabled separation of fibroblast cells, as well as that of adipose-derived mesenchymal stem cells, from mixtures of similar cells. Thus, the temperature-controlled thermoresponsive microfibers would be potentially useful as cell separation materials.

1. Introduction

Regenerative medicine has become a promising field. In particular, cell-based regenerative medicine where cells are transplanted into the human body has been investigated as an effective therapy for patients who cannot be treated with chemically synthesized drugs. To improve transplantation efficiency, various techniques have been investigated, such as direct injection of cell suspensions [1], fabrication and transplantation of cellular tissue using biodegradable scaffolds [2,3], and cell sheets prepared in thermoresponsive cell culture dishes [4–7]. Because cell injection therapy requires purified cell suspensions, cell separation methods are important for effective treatments. Also, when considering the fabrication of cellular tissue *in vitro*, variable cell compositions *via* cell separation and cell mixing would be important for functional cellular tissue fabrication [8–10]. Various types of cell separation methods have been developed [11–14]. Medical applications require simple and safe methods rather than complicated and overly specific ones. Separation using thermoresponsive polymer-modified surfaces is one of the strongest candidates [15–17]. Poly(*N*-isopropylacrylamide) (PIPAAm), a well-known thermoresponsive polymer, exhibits a temperature-dependent phase transition at its lower critical solution temperature of 32 °C [18], which is near body temperature. PIPAAm becomes hydrophilic at low temperatures due to hydration, and becomes hydrophobic at high temperature due to dehydration. The polymer-modified surface thus

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changes its hydrophilicity/hydrophobicity reversibly with the ambient temperature. PIPAAm has been used in various biomedical applications such as temperature-regulated drug and gene deliveries [19–21], temperature-modulated chromatography [22], biosensors and actuators[23–25], and cell culture substrates for cell sheet fabrication [26–28]. In particular, temperature-controlled cell adhesion/detachment on a PIPAAm-modified surface has been used for cell separations. PIPAAm-brush-modified glass substrates can separate human cells used in cardiovascular tissue engineering *via* differences in cell detachment rates [15]. Also, various cell types with different effective detachment temperatures from hydrophobic PIPAAm copolymer brushes were separated by multi-step temperature changes [16]. By incorporating cationic monomers into the PIPAAm main chains, cationic brush-modified glass substrates were used for selective adhesion and subsequent recovery of human bone marrow mesenchymal stem cells [17]. In summary, these reports demonstrate that cells can be separated using temperature-induced changes of PIPAAm or its copolymer-modified glass plates.

The above cell separation methods have many advantages. They do not require modification of cells surfaces, unlike fluorescence-activated or magnet-activated cell sorting [29–31]. Therefore, native non-labeled cells can be recovered. The thermoresponsive surfaces can recover adhered cells without using digestive enzyme such as trypsin, thus preserving cellular activity, membrane proteins, and extracellular matrices [32,33]. In addition, recovered cells from a PIPAAm-modified surface have been safely transported for clinical applications [4–6]. Because cell separation can be performed simply by changing the temperature, it does not required complex procedures. The simplicity is useful for preparation of cell suspensions for *in vitro* tissue fabrication *via* tissue engineering. Finally, the methods can be applicable to rough cell separations from tissues in a living organism for transplantation as a suspension or a fabricated tissue where non-labeled and safe cell separation are required.

Despite the above advantages, PIPAAm-modified glass plates have a limited surface area for separating cells [15–17]. Larger surface areas would allow separations of the greater number of cells required to fabricate large three-dimensional tissues for transplantation therapy. To increase the active surface area, we have used nano- or microfiber mats prepared by electrospinning methods. They have been previously investigated as functional scaffolds or cell culture substrates, and have much larger surface areas for cell adhesion relative to those of flat culture plates [34–38]. Thus, if PIPAAm-modified fiber mats could be prepared, they would be efficient thermally modulated cell separation materials.

Here, PIPAAm-modified microfibers were prepared through electrospinning of poly(4-vinylbenzyl chloride) (PVBC) and subsequent surface-initiated atom transfer radical polymerization (ATRP) of IPAAm. PVBC was used as the base material for the fibers, because chloride is an ATRP initiation site [39,40]. PVBC also has a structure similar to polystyrene, which is used for cell culture dishes. ATRP was used because it can precisely control the polymerization, and, specifically, the graft chain length. Also, surface-initiated ATRP forms densely packed structures, leading to a large quantity of grafted PIPAAm [41,42]. Adhesion and detachment of various cell types were examined on the modified microfibers by incubation at high and low temperatures. Because they are widely used in tissue fabrication, human umbilical vein endothelial cells (HUVECs) and normal human dermal fibroblasts (NHDFs) separations were investigated [43–46]. In addition, separation of human adipose-derived stem cells (ADSCs) and related cells that are important in regenerative medicine, such as adipocytes and human microvascular endothelial cells (HMVECs), were performed [47–51]. The potential of these microfibers for use as temperature-controlled cell separating materials was investigated.

2. Experimental

2.1. Materials

N-Isopropylacrylamide (IPAAm) was kindly provided by KJ Chemicals (Tokyo, Japan) and re-crystallized from *n*-hexane. 4-Vinylbenzyl chloride (VBC) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Polymerization inhibitor was removed by passing through a column designed for that purpose (Sigma-Aldrich). Toluene, 2,2′-azobis(isobutyronitrile) (AIBN), dichloromethane, 2-propanol, *N,N*-dimethylformamide (DMF), α-chloro-*p*-xylene, CuCl, ethylenediamine-*N,N,N,N*-tetraacetic acid (EDTA), tris(2-aminoethyl)amine (TREN), formaldehyde, formic acid, sodium hydroxide were purchased from Wako Pure Chemicals (Osaka, Japan). Tris[(2-dimethylamino)ethyl]amine (Me_6TREN) was synthesized according to a previously described procedure [52]. Cell culture dishes were purchased from Corning (NY, USA). Human umbilical vein endothelial cells labeled with red fluorescent protein (RFP-HUVECs) and normal human dermal fibroblasts labeled with green fluorescent protein (GFP-NHDFs) were purchased from Angio-Proteomie (Boston, MA, USA). Calcium and magnesium-free D-phosphate buffered saline [(PBS (−)] was purchased from Nacalai Tesque (Kyoto, Japan). Cell Tracker Green and Orange, and Alexa Fluor 405 were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Anti-CD31 antibody was purchased from Abcam (ab28364, Cambridge, UK). Other cells and cell culture media were purchased from Lonza (Basel, Switzerland).

2.2. Preparation of thermoresponsive microfibers

PVBC was synthesized by radical polymerization. VBC (50 mL, 0.355 mol) and AIBN (97.5 mg, 0.594 mol) were dissolved in toluene (150 mL). The solution was degassed by three freeze-thaw cycles and sealed with a stopcock. The polymerization proceeded at 70 °C for 20 h. The PVBC was purified by re-precipitation in a methanol/PVBC solution [20:1(v/v)] and dried under reduced pressure at room temperature.

PVBC microfibers were fabricated as follows by using an electrospinning apparatus (NANON, MECC, Fukuoka, Japan) [Fig. 1 (A)]. PVBC was dissolved in a mixed solvent of dichloromethane and DMF in the ratio of 10:0, 9:1, or 7:3 (v/v) at various concentrations (Table 1). Electrospinning was performed with an applied voltage of 15 kV and various polymer solution flow rates.

Thermoresponsive polymers were grafted on the PVBC microfibers *via* surface-initiated ATRP, creating various PIPAAm chain lengths [Fig. 1(B)]. The typical procedure is as follows. IPAAm (4.24 g, 37.5 mmol) was dissolved in 2-propanol (150 mL) in a 300-mL flask. The initial monomer concentration was 250 mM, 500 mM, or 1000 mM. The solution was deoxygenated by bubbling argon gas through it for 2 h. CuCl (98.3 mg, 0.993 mmol), Me_6TREN (255 mg, 1.11 mmol) and ATRP catalysts were added to the solution in an argon gas atmosphere. PVBC fibers (1.50 g) were placed in a Teflon vessel. The reaction solution in the flask, the PVBC microfibers in the vessel, and α-chloro-*p*-xylene (13.1 μL, 99.4 μmol) were placed into a glove bag purged with argon gas. All the reactants were mixed in the vessel containing PVBC fibers, and the vessel was sealed. ATRP proceeded at 25 °C for 16 h with continuous shaking. The microfibers were then filtered and rinsed by immersion in a solution of 10 mM EDTA (aq) and methanol [1:1 (v/v)] with continuous shaking overnight. Next, the microfibers were transferred to water and rinsed with continuous shaking for another two days; the water was changed daily. After rinsing, the PIPAAm-modified microfibers were freeze-dried and stored at room temperature. All sample surfaces were abbreviated as FVI-