Articles

Cell Sorting Technique Based on Thermoresponsive Differential Cell Adhesiveness

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Cell sorting of specific target cells from a mixture of different cell types is a prerequisite for development of functional engineered tissues based on stem-cell and tissue engineering. This paper presents a new method of cell sorting that uses a mixture of thermoresponsive cell-adhesive and non-cell-adhesive substances. The former substance is poly(*N*-isopropylacrylamide)-grafted gelatin (PNIPAM−gelatin) and the latter is PNIPAM. Graded cell adhesion, produced by mixed coating of these thermoresponsive substances at an appropriate mixing ratio, clearly differentiated the adhesive potentials of two bovine vascular cell types (endothelial cell and smooth muscle cell). The sequential procedures of detachment at room temperature and subsequent replating at 37 °C on dishes coated with a mixed coating with the same composition as that employed previously yielded remarkably pure target cells, as determined using confocal laser scanning fluorescence microscopy. This method, leading to harvesting of target cells, is characteristic of simple manipulation with no cell damage. Such advantages are expected to facilitate stem-cell and tissue engineering.

Introduction

Cell sorting of target-specific cells to be harvested from a multicellular tissue or a multicellular aggregate, in which functionally addressable cells are localized in subtissues of an organized tissue, is an essential task for recently evolved and developing regenerative medical fields, including tissue engineering and stem-cell engineering. Existent cell-sorting techniques for isolation of cells include[1−3] (1) density-gradient isolation under a centrifugal force field; (2) monoclonal antibody-based isolation, which includes fluorescence activated cell sorting (FACS) using fluorescence-labeled monoclonal antibody for specific surface cell marker under a flowing state; and (3) antibody-immobilized immunomagnetic beads. The first technique has been used routinely for cell subpopulations of lymphocytes in blood and has been used recently for cell sorting of stem cells, precursors, and progenitor cells. For cases in which a surface-specific marker was already identified for a target cell, its antibody-based techniques 2 and 3 have proven to be powerful and effective. A new technique must be developed for cases where a surface-specific marker has not been identified.

This paper describes and demonstrates a new technique driven by the principle of thermoresponsive differential cell adhesion using a series of graded or gradient cell adhesion substrates that comprise a mixture of poly(*N*-isopropylacrylamide), PNIPAM, as non-cell-adhesive coating and PNIPAM-grafted gelatin (PNIPAM−gelatin) as cell-adhesive coating. The technique uses cell-type-dependent differences in cell adhesion on graded adhesive coatings and recovers adhered cells at room temperature without using enzymatic digestion at room temperature. The latter thermoresponsive cell detachment, the technique which was first demonstrated on electron-beam-driven polymerized PNIPAM-modified dishes, was pioneered by Okano and his colleagues[4] and has since evolved into *cell sheet engineering*, which has shown a new avenue of therapeutic tissue engineering.[5,6]

In this paper, manipulation of a series of different strengths of cell adhesion was demonstrated on a coating of a mixture of cell-adhesive and non-cell-adhesive thermoresponsive polymers (PNIPAM-grafted gelatin and PNIPAM) at different mixing ratios. (Note that PNIPAM−gelatin was originally developed for reversible substrate-coatable or injectatble artificial extracellular matrix.[7−12]) The sequential replating of adhered cells has resulted in cell sorting from a mixture of two bovine vascular cell types: endothelial cells (ECs) and smooth muscle cells (SMCs).

Experimental Section

Materials. Figure 1 shows schematic illustrations of the preparation procedure and chemical structures of PNIPAM−gelatin, which was prepared via a living photoradical polymerization technique. Details of the preparation and characterization methods for PNIPAM−gelatin were described in our previous paper.[7−9] Briefly, PNIPAM−gelatin was

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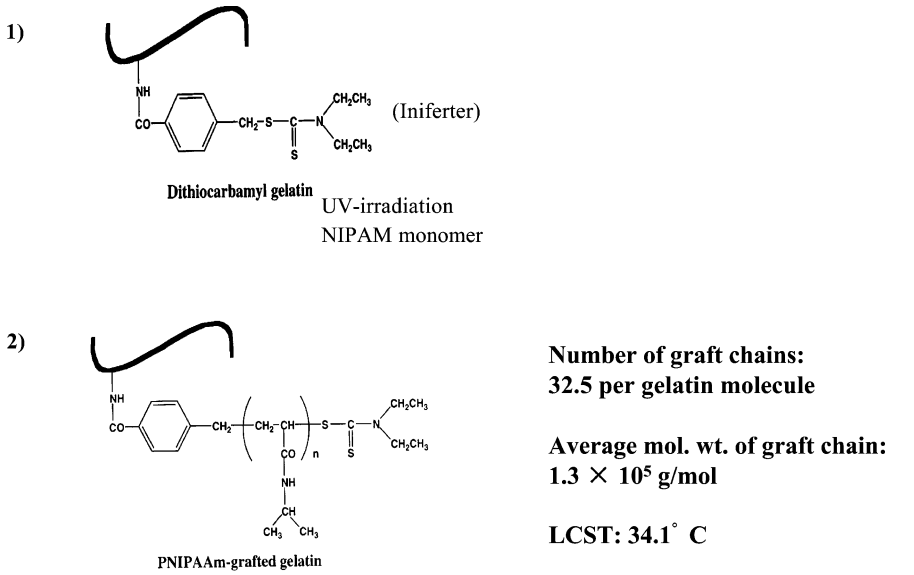

Figure 1. Schematic of generation of PNIPAM−gelatin: (1) dithiocarbamated gelatin was derivatized on lysine residues of gelatin (9.5×10^5 g/mol), (2) quasiliving photopolymerization of *N*-isopropylacrylamide under ultraviolet light irradiation. (3) Schematics for PNIPAM graft-polymerized gelatin [Molecular weight of PNIPAM graft chain, 5.0×10^4; number of graft chains, 25.9 per gelatin molecule; lower critical solution temperature (LCST), 34.3 °C at 0.1 wt % aqueous solution. Details of preparation were described in a previous report.

prepared using UV-light-induced quasiliving radical polymerization of NIPAM on dithiocarbamyl-group-derivatized gelatin [extracted from bovine bone (9.5×10^4 g/mol); Nitta Gelatin Inc., Osaka, Japan]. Dithiocarbamation was carried out in an aqueous solution using the condensation reaction between the amino groups of lysine residues of gelatin and *N*,*N*-dithiocarbamylbenzoic acid in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (water-soluble condensation agent).

The molecular parameters and temperature-dependent solubility in aqueous solution, along with the characterization procedures, were reported in previous papers. The graft chains in the PNIPAM-grafted gelatin were estimated as 25.9 per gelatin molecule; the molecular weight of graft chains was estimated as 5×10^4. The PNIPAM (MW $= 4.8 \times 10^4$) was prepared using conventional redox polymerization of a NIPAM monomer in water. The low critical solution temperatures (LCSTs), as determined from temperature-dependent turbidity change, were 32.0 °C for PNIPAM and 34.3 °C for PNIPAM−gelatin in respective aqueous solutions at 1.0 wt % concentration. The sterization of PNIPAM and PNIPAM−gelatin were carried out by precipifation in ethanol from respective aqueous solutions.

Cell Culture. The culture medium used was Dulbecco's modified Eagle's medium (DMEM; Gibco Invitrogen Co.) supplemented with 15% fetal bovine serum (FBS; Gibco Invitrogen) and antibiotics (penicillin, streptomycin, and amphotericin B). The ECs and SMCs were harvested from a bovine aorta according to methods described in our previous paper.[14] Aqueous solutions of PNIPAM−gelatin and PNIPAM were mixed at different mixing ratios to a final concentration of 0.1 wt %. Then, 400 μL of the mixed solution was poured into untreated polystyrene dishes (diameter, 35 mm; 1000-05; Iwaki Glass Co. Ltd., Tokyo, Japan) and air-dried under minimal air flow in a clean bench.

After the cell-suspended culture medium was poured into the coated dishes at 37 °C, with subsequent 3-h culture, nonadherent cells were removed from the dishes by gentle aspiration at 37 °C. Then, the dishes were left to stand at room temperature (20 °C) for 10 min. After all adhered cells were detached completely and floating in the medium, these cells, subjected to two cycles of sequential process of centrifugal sedimentation (800 rpm/5 min) and resuspension in fresh culture medium, were reseeded on the dish coated with the mixture of the same composition as the previous one at 37 °C. The sequential procedures of cell attachment, removal of nonadherent cells, detachment using thermoresponsive phase transition, resuspension with culture medium, and replating were conducted. Determination of populations of ECs and SMCs at each cycle of procedure was conducted by treatment with fluorescence-labeled acetylated low-density lipid (LDL) (Santa Cruz Biotechnology Inc., Santa Cruz, CA), which is uptaken only by ECs, followed by counting of stained cells under confocal laser scanning fluorescence microscopy (CLSM) (Radiance 2000, Bio-RAd Laboratories Inc., Hercules, CA) and counting all the cells under phase-contrast microscopy (Nikon Inc., Tokyo, Japan).

An in situ fluorescence viability study was carried out using a live−dead viability/cytotoxicity kit (Molecular Probes, Eugene, OR) with combined calcein derivative and ethidium homodimer and determined by CLSM along with the manufacturer's instruction.

Results

Aqueous solutions containing a mixture of cell-adhesive PNIPAM−gelatin and non-cell-adhesive PNIPAM at graded mixed ratios were thin-layer-coated on dishes and air-dried. Bovine endothelial cells (ECs) and smooth muscle cells (SMCs) were seeded respectively at 37 °C on each coated dish at an equal cell number (3×10^5 cells/cm^2). After 3-h culturing, nonadherent cells were washed gently away. The adhered cells were visually counted on randomly selected phase-contrast microsope images (at least 50 photos). After standing at room temperature for 10 min, all adherent cells become round and sooner or later detach spontaneously, in most cases, or by gentle pipetting. Finally, all the adhered cells detach and float in the medium. No adherent cells were observed on sole PNIPAM coating and high PNIPAM-rich mixed coating. The degree of cell adhesion increased rapidly by a small increment of PNIPAM−gelatin content in the mixed coating and reached full adhesion at high PNIPAM−gelatin-rich compositions, irrespective of cell type. Regardless of cell type, the cell density at the full adhesion was almost same as those on the tissue culture dish (see dotted line in Figure 2A).

Figure 2B shows an enlarged figure of the adhesion potential at the low PNIPAM−gelatin content region of the mixed coating. The onset of cell adhesion occurred at approximately 2.0 wt % for ECs and 2.9 wt % for SMCs. A further increase in the content of PNIPAM−gelatin in the mixed coating

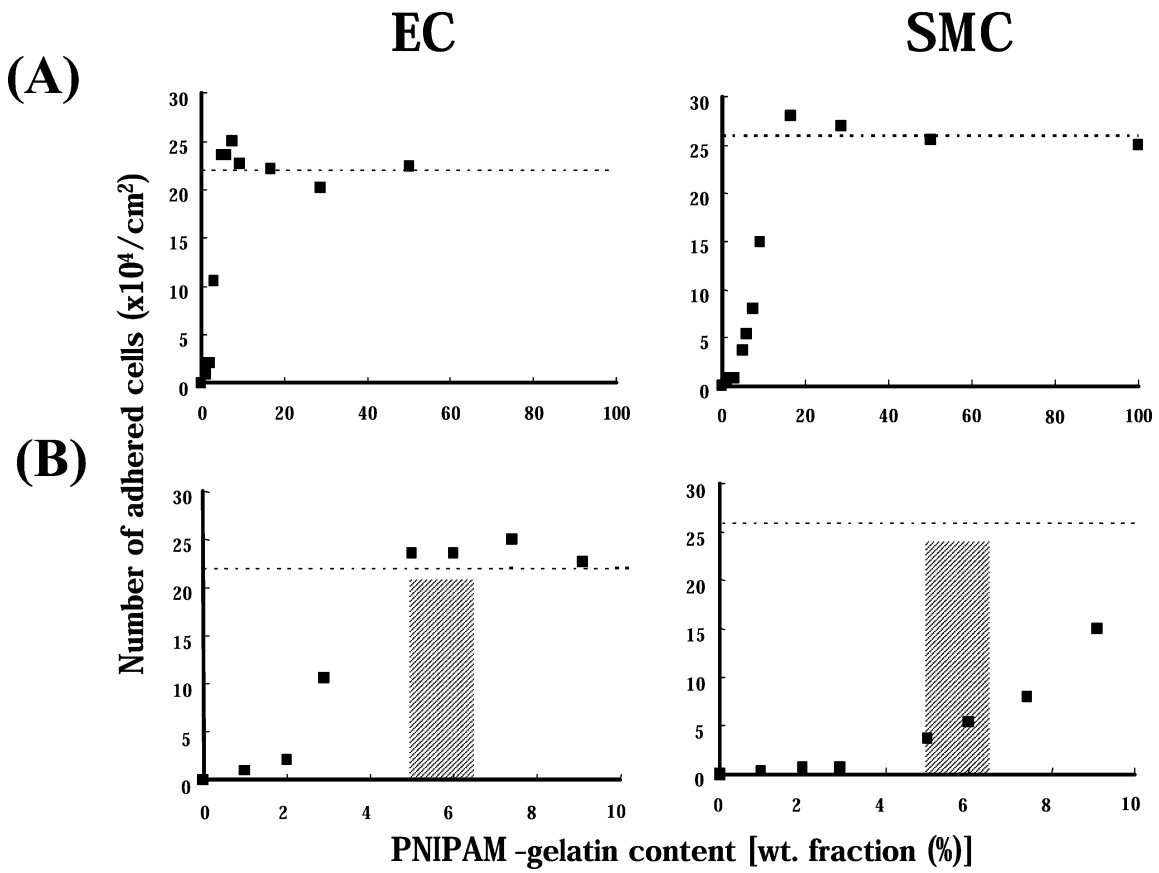

Figure 2. Cell adhesion vs graded compositions of mixed coatings as a function of PNIPAM−gelatin weight fraction (A, full range; B, up to 10% wt fraction). (Left) Endothelial cells (EC). (Right) Smooth muscle cells (SMC). Note that a shadowed window shows compositions exhibiting high adhesion of EC and low adhesion of SMC. Coating A was designated as the mixed coating with 5.0 wt % of PNIPAM−gelatin and 95.0 wt % of PNIPAM; coating B was designated the mixed coating with 6.5 wt % of PNIPAM−gelatin and 93.5 wt % of PNIPAM. Dotted lines show the number of adhered cells on tissue culture dishes (control).

increased the cell adhesion for both cell types. Full adhesion occurred at the PNIPAM−gelatin content in the mixed coating of approximately 5 wt % for ECs and 17 wt % for SMCs.

As shown clearly in Figure 2B, a marked difference exists in the cell adhesion potential between EC and SMC at graded mixed coatings from approximately 2 to 17 wt % of PNIPAM−gelatin content. The adhesion potentials of both cell types increased rapidly with PNIPAM−gelatin content in this region, but its increase rate was much higher for EC than for SMC. For that reason, the utilization of this *window* region in the mixed coatings (shadowed region in Figure 2B), enabling differentiated adhesion potentials between EC and SMC, might achieve cell sorting.

Practical cell sorting was demonstrated using two mixed coatings as follows: coating A contains 5.0 wt % of PNIPAM−gelatin and 95.0 wt % PNIPAM, and coating B contains 6.5 wt % of PNIPAM−gelatin and 93.5 wt % of PNIPAM. First, the cell mixture at the equal cell number (5×10^4 cells/cm^2) was plated on a coating dish. After 3-h culture at 37 °C, nonadherent cells were removed by gentle washing. The recovered cells, which were harvested by standing at room temperature for 10 min followed by two cycles of centrifugal sedimentation/resuspension in fresh culture medium, were replaced on the other mixed coating dish with the same mixing ratio as previously at 37 °C.

This repeated sequence of replating, gentle removal of nonadherent cells, and harvesting at room temperature enabled the enhanced concentration of a more adhesive cell type, EC, as follows. Figure 3A illustrates the plating-cycle-dependent cell enrichment effect on coating A, determined from phase-contrast microscopic images of cells and fluorescence images of fluorescent LDL-staining specific to ECs (only ECs uptake LDL). The total number of adhered cells was determined by phase-contrast microscopy and ECs were determined from fluorescence microscopy. Cells, appearing in the phase-contrast microscopic photo but not in the CLSM photo, were defined as SMCs. A markedly high cell number was noted for ECs irrespective of the replating cycle, whereas the cell number of adhered SMCs decreased with the replating cycle. The degrees of impurity in harvested cells, defined as the number of SMCs to the total number of adhered cells, were as follows. For coating A (Figure 3A), the degree of impurity was 0.11 at the first plating and 0.03 at the second replating. By the third replating, eventually no SMCs were observed. For coating B (Figure 3B), the degree of impurity at the first plating was 0.33, but with an increase in the replacement cycle, the SMC population decreased rapidly; at the fifth plating, no SMCs were observed. However, the reduction rates of number of adhered SMCs upon replating cycle were smaller than that expected from Figure 2B. This may be derived from the slight change in PNIPAM−gelatin content at the outermost layer of the mixing coating. Such subtle deviation in composition at the outermost surface from that in the mixing solution may be caused by coating conditions such as drying speed.

As for the cell viability, the live−dead assay showed that, irrespective of replating cycle (examined up to fourth plating) or regardless of cell type, dead cells were less than a few percent of the harvested cells, whereas populations of dead cells harvested by conventional trypsin treatment were higher: approximately several percent or more for both EC and SMC.

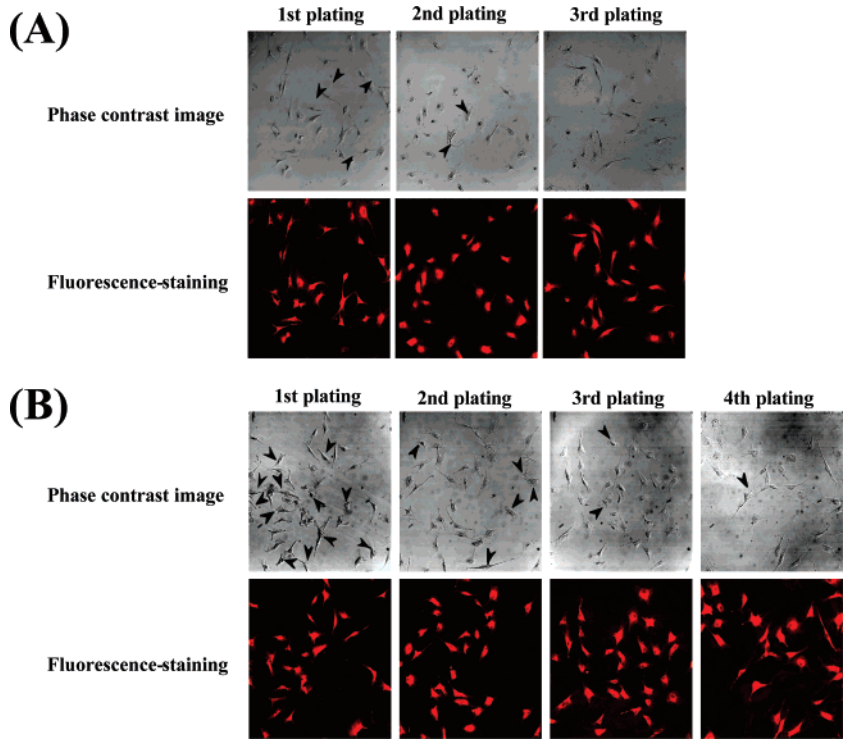

Figure 3. Phase-contrast and fluorescence microscopic images of cells in coating A at first to third replating cycle and in coating B at first to fourth replating cycles. The fluorescent images were obtained by treatment of fluorescence-labeled acetylated LDL specifically uptaken only by ECs. Red cells are identified as ECs and nonstained cells (marked arrow on phase contrast microscope in the upper figure) are SMCs.

Table 1. Cell Sorting Based on Differential Cell Adhesiveness[a]

replating cycle	EC (cell number)	SMC (cell number)	SMC/ total cell number
Coating A: PNIPAM−Gelatin, 5.0 wt %; PNIPAM, 95.0 wt %			
1	433	52	0.11
2	536	18	0.03
3	427	0	0
Coating B: PNIPAM−Gelatin, 6.5 wt %; PNIPAM, 93.5 wt %			
1	435	218	0.33
2	400	124	0.20
3	351	44	0.11
4	370	20	0.05
5	350	0	0

[a] Cell number is determined by counting adhered cells in phase-contrast microscopic and confocal scanning microscopic photos (cells/mm^2).

Discussion

Cell sorting from a multicellular tissue or tissue fluid such as bone marrow is the first step of fabrication of functional tissues via tissue engineering using adult, stem, and progenitor cells, which determine the quality of engineered tissues. Immunological isolation using FACS or magnetic beads has proven to be a very reliable technique when a cell-specific surface marker is identified and its antibody is conventionally available. A significant difference in cell density or cell size enables fractionation or purification of a target cell type.

In this paper, we proposed a differential cell-adhesive thermoresponsive substrate technique. A given cell adhesion is prepared easily by coating a mixture of non-cell-adhesive PNIPAM and cell-adhesive PNIPAM−gelatin at a given mixing ratio. Because both polymers exhibit thermoresponsive phase transition at lower critical solution temperature (LCST) at 32.0 °C for PNIPAM and 34.1 °C for PNIPAM−gelatin, respectively, the mixed coating precipitates in water at physiological temperatures, but is dissolved spontaneously and desorbed away from the coated surface below LCST. This physicochemical detachment of cells without enzymatic digestion, which often causes membrane damage, enables harvesting of vital intact cells; it is claimed by Okano and colleagues as damage-free harvesting of cells or tissues, as verified on a thermoresponsive PNIPAM-polymerized surface.[5] Our results also supported the evidence that little cell damage was observed even after the forth replating cycle for both cell types. This was verified by live−dead assay.

Increased repeated replating yields a higher purity of sorted cells (Table 1). The *n*th repeated replating cycle will engender impurity of $(m)^n$ if the degree (or fraction) of impurity is *m* at the first plating. For example, the degree of impurity is 0.1 at the first plating, the degree of impurity will be 0.01 at the second plating, 0.001 for at the third plating, and 0.0001 at the forth one. For that reason, in principle, a tremendous purification is feasible through repeated plating cycles if an appropriate mixing ratio enables large differential cell adhesion.

The reason why there is some difference in PNIPAM−gelatin content-dependent cell adhesion between cell types is not clear, but it is highly anticipated that differential cell adhesiveness results from differences in density (or number) of cell membrane adhesion receptor (integrin) that molecularly recognizes adhesive proteins such as fibronectin upon cell adhesion.

This paper first demonstrates the cell sorting based on differential cell-adhesive thermoresponsive coatings. Optimized cell adhesiveness for a target cell can be easily manipulated with an appropriate mixing ratio of cell-adhesive and non-cell-adhesive themoresponsive materials. In addition to the mixing ratio, PNIPAM−gelatin has molecular parameters manipulating cell adhesion, which include graft chain density and graft chain length. Our previous study showed that such precision control of graft architecture is feasible.[8,12,13] Future study will elucidate the usefulness of this novel harvesting technique for providing

high purity of cells used for tissue and stem cell engineering, where functional cells play a pivotal role in regenerative tissues.

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