Cell Sheet Detachment from Poly(N-vinylcaprolactam-co-N-isopropylacrylamide) Grafted onto Tissue Culture Polystyrene Dishes

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Abstract: Fabrication of functional tissue constructs using sandwiched layers of cultured cells could prove to be an attractive approach to tissue engineering. Rapid detachment of cultured cell sheets is a very important recovery method that permits facile manipulation of the sheet and prevents functional damage. To induce hydrophilic and hydrophobic structural changes on the required culture substrate in response to a culture temperature alteration, poly(N-vinylcaprolactam-co-N-isopropylacrylamide) (PNVCL-co-PNIPAAm) was grafted onto tissue culture polystyrene (TCPS) dishes through electron beam irradiation. Chemical analyses using ATR-FTIR spectroscopy revealed that PNVCL-co-PNIPAAm was successfully grafted onto the surfaces of the TCPS dishes. The TCPS dishes grafted with PNVCL-co-PNIPAAm were utilized for the cell sheet detachment experiments. Approximately 150 min was required to detach most of the cell sheets from the PNVCL-co-PNIPAAm-grafted TCPS surfaces.

Keywords: human fibroblast, poly(N-isopropylacrylamide), poly(N-vinylcaprolactam), hydration, cell detachment, cell culture

Introduction

Most mammalian cells must adhere to a solid substrate or supporting scaffold in order to proliferate and manifest their function [1-3]. In these in vitro cell culture systems, an efficient recovery of cells from the culturing substrate is an essential process for their further utility. Generally, recovery of cultured cells from the substratum requires treatment with a proteolytic enzyme such as trypsin. The enzymatic treatment inflicts damage to the cell membranes by hydrolyzing various membrane-associated proteins, resulting in impairment of the cell function [4-6]. Therefore, cultured cells sensitive to an enzymatic treatment cannot be subcultured, and only primary cells are utilized for various research purposes [7].

Poly(N-isopropylacrylamide) (PNIPAAm), a thermo-sensitive polymer, has a low critical solution temperature (LCST) of ca. 32 °C in water. PNIPAAm is fully hydrated with an extended chain conformation below 32 °C; it is extensively dehydrated and compact over 32 °C [8]. Cross-linked PNIPAAm and its copolymers have been developed as thermal on-off switching polymers for drug permeation and release using this mechanism [9-11]. Another temperature-responsive and biocompatible polymer that has been studied for therapeutic purposes is poly(N-vinylcaprolactam) (PNVCL), which is widely used in hair-care and cosmetic applications [12]. Applications of PNVCL in biomedical materials, in the stabilization of proteases, and in controlled drug delivery and drug release have been published, for example by Peng and Wu [13], Markvicheva and coworkers [14], and Vihola and coworkers [15]. PNVCL collapses when the temperature exceeds 32 °C [16] and, therefore, the thermo-sensitive PNVCL has, presumably, similar characteristics to PNIPAAm. PNVCL is a chemical analogue of polyvinylpyrrolidone (PVP), a well-known and widely used pharmaceutical excipient [17-20]. However, some evidence of PVP’s dose- and time-dependent toxicity has been found recently [21].
<table>
<thead>
<tr>
<th>Sample name</th>
<th>Weight ratio of monomers</th>
<th>Remarks $^a$ (amount of grafting, mg/cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIPAAm</td>
<td>100</td>
<td>● (1.8)</td>
</tr>
<tr>
<td>IV73</td>
<td>70 30</td>
<td>● (2.1)</td>
</tr>
<tr>
<td>IV55</td>
<td>50 50</td>
<td>×</td>
</tr>
<tr>
<td>IV37</td>
<td>30 70</td>
<td>×</td>
</tr>
<tr>
<td>PNVCL</td>
<td>- 100</td>
<td>▲ (2.2)</td>
</tr>
</tbody>
</table>

$^a$ ●, Homogeneously grafted onto TCPS surfaces; ×, heterogeneously grafted onto TCPS surfaces; ▲, homogeneously grafted onto TCPS surfaces, but not good for cell detachment.

To our knowledge, evaluation of the putative cytotoxicity of PNVCL polymers has not been published previously. The biocompatibility of synthetic polymers has been improved by grafting hydrophobic polymers with hydrophilic chains, e.g., poly(ethylene oxide) (PEO), or with some other water soluble polymers. The grafting creates a steric repulsion against protein adsorption and, thus, increases the biocompatibility of the polymer [22].

In this study we fabricated TCPS dishes grafted with PNVCL-co-PNIPAAm through electron beam irradiation. The product showed hydrophilic and hydrophobic surface property alterations in response to temperature changes. A grafted TCPS substrate permits rapid two-dimensional cell sheet manipulation by facilitating a rapid water movement to the interface between the cell sheets and the TCPS surfaces, producing a cell sheet release.

**Experimental**

**Materials**

$N$-Vinylecaprolactam (NVCL, 98 %, Aldrich Chemical Co., Germany) and $N$-isopropylacrylamide (NIPAAm, 97 %, Aldrich Chemical Co., Milwaukee, WI, USA) were purified by recrystallization from $n$-hexane/toluene (Duksan Pure Chemicals, Seoul, Korea). TCPS dishes (Nuncron$^\text{TM}$ surfaces) were purchased from NUNC (Rosilde, Denmark). Trypsin/0.01 %-EDTA solution, 200 U/mL pенициллин, 200 µg/mL streptomycin, fungi zone, and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Gibco BRL (Grand Island, NY). Trypan blue (0.4 % 100 mL), T-8154, and sodium bicarbonate were purchased from the Sigma Chemical Co. Water was distilled and deionized using a Milli-Q System (Waters, Millipore, USA). All other chemicals were of reagent grade and used without any further purification.

**Preparation of PNVCL-co-PNIPAAm-grafted TCPS Dishes**

NVCL and NIPAAm monomers were dissolved in 2-propanol at a concentration of 50 % (wt/wt). The monomer weight ratios (NIPAAm/NVCL) were 10:0, 7:3, 5:5, and 3:7. The weight ratios of monomers in the initial mixtures are summarized in Table 1. These monomer solutions (30 µL) were spread uniformly over the surface of the TCPS (Nuncron$^\text{TM}$ surfaces) and then irradiated using a conventional electron beam accelerator (ELV-4, EB-tech Co. Ltd., Daejeon, Korea) at a radiation dose of 300 kGy (acceleration voltage of 0.7 MeV under atmosphere). The monomers were polymerized and covalently grafted onto the TCPS surfaces through this electron beam irradiation process. Unreacted monomer and ungrafted polymers were removed by washing extensively with cold water. The PNVCL-co-PNIPAAm-grafted TCPS dishes were dried under vacuum at room temperature.

**Surface Characterization**

The amount of PNVCL-co-PNIPAAm grafted onto the TCPS dishes was determined by attenuated total reflection-Fourier transform IR spectroscopy (ATR-FTIR, Bruker TENSOR 37, Germany). The control substrate of PS has strong absorption bands attributed to aromatic groups at 1600 cm$^{-1}$. When PNVCL-co-PNIPAAm was grafted onto each substrate, an amide absorption band appeared in the region near 1650 cm$^{-1}$. The peak intensity ratio ($I_{1650}/I_{1600}$) was used to determine the amount of PNVCL-co-PNIPAAm grafted on each surface, using a calibration curve of a known PNVCL-co-PNIPAAm amount cast on a TCPS from solution.

**Water Contact Angle Measurements**

The samples of the PNVCL-co-PNIPAAm-grafted and -ungrafted TCPS dishes were cut into 1.0 × 1.0 cm pieces to measure their water contact angles. Water contact angles were determined using a sessile drop method at 20 and 37$^\circ$ with a FACE contact angle meter (image processing type CA-X, Kyowa Interface Science, Saitama, Japan). All samples were measured three times and averaged. Water contact angles at 20 and 37$^\circ$ are presented as mean values ($n = 3$) and standard deviations.

**Cell Culture**

Normal human fibroblasts (HF) were aseptically isolated from a foreskin donated by the Urology Department of Hanyang University Hospital in Seoul, Korea, as in previous methods [23,24]. The fibroblast culture medium was composed of Dulbecco’s modified Eagle’s medium (DMEM, Gibco BRL, Rockville, Maryland, USA) and 10 % fetal bovine serum (FBS, Gibco BRL).
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Figure 1. ATR-FTIR spectra of PNVCL-co-PNIPAAm-grafted TCPS dishes and control substrate.

Detachment of Single Cells and Cell Sheets from PNVCL-co-PNIPAAm-Grafted Surfaces

HF s were plated on each surface at a density of $3 \times 10^4$ cells/cm$^2$ and cultured for 2 h at 37 °C for spreading on each grafted surface. For detachment of single HF s, spread cells were transferred to a CO$_2$ incubator equipped with a cooling unit fixed at 20 °C. After 30, 60, 90, 120, and 150 min of incubation at 20 °C, the cell morphology was observed using a phase-contrast microscope and photographed. The rounded and spread cells in the photographs were counted, and the percentage of rounded cells to the total number of cells counted was determined as a mean value ($n = 3$) and standard deviation. Ungrafted TCPS dishes were used as controls. The percentage recovery of single cells from PNVCL-co-PNIPAAm-grafted TCPS dishes was compared to that of PNIPAAm-grafted TCPS dishes.

HF s were plated onto each surface at a density of 1.3 times confluence ($1.3 \times 10^6$ cells/dish) and cultured at 37 °C. After 24 h of incubation, the unattached cells were removed by medium exchange. Cell sheets were cultured for 8 days after reaching confluence; each plate was transferred to the CO$_2$ incubator equipped with a cooling unit fixed at 20 °C and periodically removed to acquire photographs during detachment. The photographs were scanned into a computer system for analysis. Software was used to measure the area of each detached cell sheet. The areas of detached cell sheets relative to the in situ confluently cultured cell sheet area were calculated and averaged from four photographs of each sample.

Table 2. Water Contact Angles of the PNVCL-co-PNIPAAm-Grafted and Ungrafted Surfaces Estimated Using a Sessile Drop Method ($n = 3$)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$\theta_{H_2O}$ 20 °C</th>
<th>$\theta_{H_2O}$ 37 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCPS surface</td>
<td>65.8 ± 0.9°</td>
<td>66.0 ± 2.1°</td>
</tr>
<tr>
<td>PNVCL-co-PNIPAAm surface</td>
<td>55.3 ± 1.2°</td>
<td>66.2 ± 3.3°</td>
</tr>
</tbody>
</table>

Statistical Analysis

The data were analyzed by ANOVA using SAS (Release 6.12, SAS Institute Inc., Cary, NC, USA); differences among the mean values were processed by Duncan’s multiple range tests. Values of $p < 0.05$ were statistically considered.

Results and Discussion

Surface Characterization

ATR-FTIR spectra of PNVCL-co-PNIPAAm-grafted TCPS dishes and ungrafted ones are shown in Figure 1. The ungrafted PS has strong absorption bands attributed to aromatic groups at 1600 cm$^{-1}$. When PNVCL-co-PNIPAAm was grafted onto each substrate, an amide absorption band appeared in the region near 1650 cm$^{-1}$. The peak intensity ratio ($I_{1650}/I_{1600}$) was used to determine the amount of PNVCL-co-PNIPAAm grafted onto the TCPS surfaces. The amount of graft was similar regardless of the composition of the monomer, as shown in Table 1.

Table 2 shows water contact angle data from each surface; the values were obtained using the sessile drop method at 20 and 37 °C. TCPS dishes grafted with PNVCL-co-PNIPAAm (IV73) exhibited decreasing contact angles upon lowering the temperature from 37 to 20 °C, while control surfaces had negligible contact angle changes with changing temperature. This result indicates that TCPS dishes grafted with PNVCL-co-PNIPAAm (IV73), which are hydrophobic at higher temperature, become markedly more hydrophilic in response to a temperature reduction due to spontaneous hydration of surface-grafted PNVCL-co-PNIPAAm. By lowering the temperature, such a hydrophobic or hydrophilic polymer change and polymer matrix swelling induces the detachment of cultured cells from PNVCL-co-PNIPAAm-grafted surfaces.

Single Cell and Cell Sheet Detachment

After a short culture time, the seeded HFs were well attached and spread on the PNVCL-co-PNIPAAm-grafted (IV73) and ungrafted surfaces. The degrees of cell attachment and spreading on the control TCPS surfaces and PNVCL-co-PNIPAAm-grafted TCPS dish surfaces (IV73) were nearly identical. After 2 hr of incubation at 37 °C,
more than 90% of the seeded cells were attached and spread on both surfaces, as shown in Figure 2.

Figure 3 shows the percentage of detached single cells from both the control TCPS dishes and PNVL-co-PNIPAAm-grafted TCPS surfaces as a function of the incubation time in the culture medium at 20°C. Spread cells were detached from the grafted surfaces at 20°C, but almost none of the cells cultured on the ungrafted surfaces were detached. PNVL-co-PNIPAAm is hydrated below its LCST, producing an expanded, swollen, hydrophilic surface. On the PNVL-co-PNIPAAm-grafted TCPS dishes, water required to hydrate PNIPAAm at a lower temperature can readily penetrate the culture matrix from only the periphery of each cell to the interface between the cell and grafted copolymer chains. Recovered detached cells adhere to and grow readily on other PNVL-co-PNIPAAm-grafted surfaces at 37°C, indicating no damage to either the structural or functional aspects of the cells (data not shown). This control of cell detachment and attachment by altering the temperature was reversible and reproducible.

Longer terms like single cells, were also readily detached from the temperature-responsive grafted surfaces. After the cells reached confluence, monolayer cell sheets were eventually formed. These sheets detached from the surfaces intact, maintaining cell-cell junctions by lowering the culture temperature below the LCST. Moreover, viable cell sheets that detached from the grafted TCPS surfaces by reduced temperature treatment were recovered together with the extracellular matrix (ECM) produced and deposited during cell culture. Retention of the intact ECM is necessary to preserve the cell sheet continuity. Thus, intact, viable cell sheet detachment through lower temperature treatment could prove useful for constructing three-dimensional tissue-like structures by fabricating sandwiches of cell sheets and associated ECM.

Figure 4 shows the changes in the remaining area of cell sheets on both control TCPS and PNVL-co-PNIPAAm-
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Figure 5. Photographic images of the time-dependent detachment of confluent cultures of human fibroblasts from a PNVCL-co-PNIPAAm-grafted surface at 20 °C.

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grafted TCPS (IV73) as a function of the incubation time in the culture medium at 20 °C. A period of 150 min was required to detach ca. 98% of the cell sheets on the PNVCL-co-PNIPAAm-grafted TCPS surfaces.

Cell sheets on the PNVCL-co-PNIPAAm-grafted TCPS surfaces initially detached slowly, probably because the water required to hydrate the PNVCL-co-PNIPAAm-grafted TCPS could only penetrate from the cell sheet periphery. The detached cell sheets recovered from the PNVCL-co-PNIPAAm-grafted TCPS surfaces were photographed as shown in Figure 5. A complete cell sheet was recovered from the surface of the PNVCL-co-PNIPAAm-grafted TCPS.

Conclusions

PNVCL-co-PNIPAAm-grafted TCPS dishes were effectively applied to detach viable HF sheets from culture surfaces. The grafted surface architecture had no adverse effect on cell attachment or proliferation and allowed access of water from beneath and from the periphery of the cultured cell sheets. Water in the PNVCL-co-PNIPAAm-grafted TCPS surfaces could penetrate only from the outside edges of the cell sheet. Rapid two-dimensional intact cell sheet recovery, with no damage from lower temperature treatment, and further manipulation of detached cell sheets into tissue constructs should prove an interesting means to fabricate three-dimensional tissue-like structures by sandwiching viable layers of detached cell sheets of various types in vitro.

References