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Multilayered Mouse Preosteoblast MC3T3-E1 Sheets Harvested from Temperature-Responsive Poly(*N*-isopropylacrylamide-*co*-acrylamide) Grafted Culture Surface for Cell Sheet Engineering

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ABSTRACT: A novel temperature-responsive tissue culture surface was prepared using ultraviolet irradiation to graft poly(*N*-isopropylacrylamide-*co*-acrylamide) (PNIAM-*co*-AM) onto commercial tissue culture surfaces. The physical properties of the grafted surfaces were confirmed by Fourier Transform Infrared Spectroscopy, contact angle measurement, and Atomic Force Microscopy. The grafted surface was also tested and shown to be nontoxic using mouse preosteoblast MC3T3-E1 cells. The cells grew as successfully on the grafted surface as those on ungrafted surface. When the temperature was reduced from 37 to 10°C for 30 min, followed by 20°C for 60 min, the confluent cells could be detached as a continuous sheet. A histological examination showed that the harvested cell sheet preserved tight junctions and extracellular matrix proteins, allowing the sheet to adhere to other cell sheets as multilayers. The resulting multilayered sheets were in good condition, as indicated by the LIVE/DEAD stain. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 129: 3061–3069, 2013

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INTRODUCTION

The use of cell sheet technology for regenerative medicine has been progressing rapidly in recent years.[1–3] This technology provides an alternative to the use of biodegradable scaffold materials and the injection of single cell suspensions.[4,5] A large scaffold construct is not ideal because cell death normally occurs at the center of the construct due to insufficient oxygen, nutrients, and removal of metabolic waste.[6] Thus, it is not suitable for complex tissues such as cardiac muscle, liver, and kidney.[7] In the case of the single cell injection, the shape and location of the injected cells are difficult to control; thus, the cells cannot be retained around the target organs or tissues.[4,8] Cell sheet engineering, in which the cells form a sheet-like construct, has been developed to overcome these problems.[6,9,10]

Cell sheet technology depends on a temperature-responsive surface, which can control cell attachment and detachment from the culture surface by simply changing temperature.[2,3,6,8] Temperature-responsive culture plates are generally prepared by surface modification with poly(*N*-isopropylacrylamide), PNIAM, and its derivatives.[11] The PNIAM structure consists of isopropyl and amide groups, which allow it to undergo a hydrophilic-hydrophobic transition at the low critical solution temperature (LCST) at 32°C.[1–3,12,13] Previous studies have shown that various types of cells can attach, spread, and proliferate on the temperature-responsive culture surface at 37°C, similar to those cultured on ungrafted tissue culture polystyrene (TCPS) dishes.[14] The highly confluent cells cultured on the temperature-responsive surface can be detached as a cell sheet when the temperature is reduced below the LCST due to rapid hydration and swelling of the grafted PNIAM surface.[1–3,6,8,13] Therefore, tight junctions and extracellular matrix (ECM) proteins are preserved because there is no need for the conventional enzymatic treatment that degrades attachment proteins.

Plasma polymerization, electron beam (EB), and UV irradiation are the radiation-induced grafting methods, which have been proposed to graft NIAM monomers onto tissue culture surfaces to fabricate temperature-responsive culture dishes. The plasma polymerization is a one-step method to fabricate the temperature responsive PNIAM surface. However, it is not suitable for large-scale production because of difficulties related to continuous treatment and size.[11] Alternatively, PNIAM-coated culture dishes can be prepared by EB irradiation.[9,11,15–17] Unfortunately, this fabrication process is expensive due to the requirement of specialized equipment and well-trained personnel, which may not be possible to carry out in most laboratories.[11] Recently, commercially available temperature-responsive culture dishes, in which a homopolymer of PNIAM is grafted by EB irradiation, are available under the name "UpCell^{TM}" (CellSeed, Tokyo, Japan). Although various sizes of UpCell products are available, the largest surface area of UpCell surfaces is only limited to 56.7 cm^2 and restricted to circular shapes (petri dishes with 100 mm diameter). This can be problematic if a larger cell construct in other geometries for tissue regeneration is needed. Furthermore, the cost of UpCell^{TM} cultureware is almost 5 times that of regular tissue culture dishes, making the construction of multilayered cell sheets especially costly. Therefore, a more economical technique to graft PNIAM onto tissue-culture surfaces is necessary.

The UV irradiation method has been proposed as a simple, inexpensive, and versatile approach to create temperature-responsive surfaces.[12,18] The drawback of this method is that UV irradiation is not as effective as EB irradiation in radical polymerization of *N*-isopropylacrylamide (NIAM) monomer and grafting the thin film onto polystyrene substrates. To overcome this problem, copolymerization of NIAM and another monomer with a reactive side chain, such as acrylamide (AM) was suggested. AM processes an active amide (-NH_2) group that allows covalent bonding to other molecules even when polymerized at low irradiation intensity.[19–21] Furthermore, AM monomers improve the mechanical strength of the copolymer without affecting the temperature-responsive property of the poly(*N*-isopropylacrylamide) (PNIAM).[22]

In this study, we propose an alternative technique to manipulate single and multilayered mouse pre-osteoblast MC3T3-E1 sheets from the thermo-responsive polymer, poly(*N*-isopropylacrylamide-*co*-acrylamide), PNIAM-*co*-AM, grafted surface. The monomers of NIAM and AM were grafted onto polystyrene substrates using UV irradiation. The physical properties of the grafted surface were characterized and the surface was subsequently used to culture and create multilayered cell sheets.

EXPERIMENTAL

Materials

N-isopropylacrylamide (NIAM) was purchased from Sigma-Aldrich and was re-crystallized at −5°C in *n*-hexane. AM, *N*,*N*′-Methylenebisacrylamide (MBAM) and potassium periodate (KIO_4) were purchased from Aldrich and were used without further purification. Minimum Essential Medium (MEM), Trypsin-EDTA, Fetal Bovine Serum (FBS), penicillin-streptomycin (Pt/St), Amphotericin B, Trypan Blue, LIVE/DEAD Viability/Cytotoxicity Assay Kit (L-3224), and Vybrant MTT cell proliferation kit were purchased from Invitrogen (Grand Island, NY). Dimethyl sulfoxide (DMSO) was purchased from Amresco (Solon, OH). Matrigel basement membrane was purchased from BD science (San Jose, CA). Treated, nonpyrogenic, sterile commercial TCPS dishes, 35 × 10 mm^2 style, were purchased from Corning (Tewksbury, MA). Commercial tissue culture 6-well plates and UpCell^{TM} 6 well plates were purchased from Thermo Scientific, (Yokohama, Japan).

Poly(NIAM-co-AM) Grafted Culture Surface Preparation and Characterization

The preparation of PNIAM-*co*-AM grafted surfaces using UV polymerization technique and the resulting surface characterization are described. Briefly, commercial 6 well plates were first pre-irradiated by a UV lamp (UVGL-58 handheld; 6W, 265 nm, without filter) for 30 min to activate the surface. In the following step, an aqueous solution containing 0.0565 g of NIAM (1 mol L^{-1}) and 0.0367 g of AM (1.04 mol L^{-1}) monomers, 1.54 × 10^{-3} g of MBAM (20 mmol L^{-1}) as a crosslinker, and 5.75 × 10^{-4} g of KIO_4 (5 mmol L^{-1}) as a photoinitator was added to each culture surface and left to equilibrate overnight. After 24 h, the solution was removed, and these dishes were exposed to UV light (6W, 256 nm, without filter) for another hour. The PNIAM-*co*-AM grafted surfaces were rinsed with ethanol to remove the remaining monomer and dried at room temperature under a vacuum condition for 24 h. These copolymer grafted surfaces were then chemically sterilized using acidic ethanol (70% EtOH, pH = 2) and were washed twice in sterile phosphate buffered saline (PBS) before use in the cell culture studies. In all experiments, ungrafted commercial six well plates were used as a control.

The PNIAM-*co*-AM grafted surfaces were characterized using Fourier Transform Infrared (FTIR) Spectroscopy, contact angle measurements, and Atomic Force Microscopy (AFM). The surface of samples was scratched into powder and pressed into pellets using KBr. The pellets were examined using FTIR Spectroscopy (Thermo Nicolet 6700). Attenuated Total Reflection (ATR) in combination with FTIR was also applied to investigate the grafted surfaces. The ATR accessory was equipped with a ZnSe ATR crystal. The surfaces were placed over the ATR crystal and maximum pressure was applied. The FTIR spectrometer was equipped with a KBr beamsplitter. The surface properties of PNIAM-*co*-AM grafted surfaces were characterized using a contact angle-measuring device following the sessile drop method at the temperatures ranging from 10 to 45°C. AFM characterization was carried out at 5 and 45°C in deionized water using Atomic Force Seiko Instrument SPA 400 Microscope in dynamic force microscopy mode. A cantilever SI-DF3 was used with a nominal spring constant of $C = 1.7$ Nm^{-1} and a resonance frequency of 28 kHz.

Cell Culture

Mouse preosteoblast MC3T3-E1 cells (passage 10–20 for all experiments) were provided by Faculty of Medicine, Chulalongkorn University (Thailand). The cells were maintained in MEM medium supplemented with 10% FBS, 1% Pt/St, and 1% Amphotericin B, at 37°C under a CO_2 (5%) atmosphere. Cell

morphology was photographed under a bright-field inverted microscope (Sundrew MCXI600, Vienna, Austria).

In Vitro Toxicity of PNIAM-co-AM Grafted Surface

The toxicity of the grafted copolymer was evaluated using a colorimetric method MTT test based on ISO 10993-5 (Biological evaluation of medical devices–Part 5). MC3T3-E1 cells were transferred to 96 well plates at a density of 3×10^2 cells/cm^2 and allowed to grow for 3 days. Afterwards, the culture media were discarded and replaced with the media previously incubated with PNIAM-*co*-AM grafted copolymer film. At the predetermined time point, the supernatant was removed and replaced with the working solution (MTT assay solution in phenol red free MEM). The plates were incubated for 1 h and protected from light. The formazan salt resulting from the reduction of MTT was dissolved in DMSO. The absorbance from the wells was measured at 570 nm with a microplate reader (TECAN Model Infinite M 200). The result was reported as percentage of relative viable cell number compared with the control in which the cells were incubated with fresh medium and calculated according to eq. (1).

$$\%\ \text{Relative viable cells} = \frac{\text{cell concentration in sample well plate}}{\text{cell concentration in sample control plate}} \times 100 \quad (1)$$

Effect of Incubation Time at Low Temperature on the Cell Viability

Since the cell detachment step was performed in a low temperature chamber without CO_2 supplementation, the effects of low temperature and lack of CO_2 on the cells needed to be investigated. MC3T3-E1 cells were seeded on the copolymer grafted surface and cultured at 37°C for 2 days in MEM medium. Afterward, the cells were incubated at 10 and 20°C for 30, 60, 90, and 120 min. At each time point, the cells were stained with LIVE/DEAD Viability/Cytotoxicity Assay Kit (L-3224, Invitrogen). The cell morphology was observed using a fluorescence microscope (Olympus BX60) and photographed to determine cell viability. The cell viability was expressed as percent survival, as shown in eq. (2):

$$\%\ \text{Cell viability} = \frac{\text{live cells (green)}}{\text{total cells number (live cells} + \text{dead cells)}} \times 100 \quad (2)$$

Values represent the mean ± standard deviation (SD) of six areas.

Construction of Single and Multilayered Cell Sheets

To construct a cell sheet, 3 mL of preosteoblasts at densities of 3×10^5 - 6×10^5 cells/cm^2 were seeded on the control and copolymer grafted surfaces. The cells were cultured for 3–4 days until they reached 100% confluency. Before the low-temperature treatment, the surfaces were washed with sterile PBS to remove nonadherent cells. A hydrophilically modified poly(vinylidenedifluoride) (PVDF) membrane (Durapore membrane filter, pore size 5.0 μm; Millipore Corporation) was placed over the confluent cell layer. The hydrophilic nature of membrane allowed it to stick to the cell layer. An additional 100 μL of the culture medium was added over the membrane to avoid drying of the cell layer. To release confluent cells as an intact sheet, the cultures were incubated at temperatures of 10°C for 30 min, followed by 20°C for 60 min. The membrane, together with the cell sheet, was peeled off using forceps. They were then transferred either to a new culture surface, resulting in a single layer cell sheet, or to another confluent cell culture for multilayered cell sheet construction. The sheets, still attached to the PVDF membrane, were transferred to a basement membrane coated TCPS dish (BD Matrigel™ Basement Membrane Matrix, BD science) and incubated at 37°C for 1–2 h to promote attachment to the new basal layer. Finally, the PVDF membrane was gently peeled off after adding fresh medium to the edge of culture dish, and the cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. The area of cell attachment and cell detachment were calculated using the tracing and analysis tools of ImageJ 1.44 software. The percentage of cell detachment was expressed as a percentage of the detached area and calculated following eq. (3):

$$\%\ \text{Cell detachment} = \frac{\text{detached areas of cell sheets recovered from the surface}}{\text{total area(attached area} + \text{detached area)}} \times 100 \quad (3)$$

Data are reported mean ± SD.

Cell Morphology with Scanning Electron Microscope (SEM)

Mouse preosteoblasts cells were cultured at low (3×10^2 cells/cm^2) and high (3×10^4 cells/cm^2) cell density on PNIAM-*co*-AM grafted 35 mm petri dishes for 3 days. The petri dishes were then cut into squares of 1.5×1.5 cm^2 before being fixed in glutaraldehyde (2.5% in 0.1*M* phosphate buffer pH 7.2) overnight at 4°C. These surfaces were extensively rinsed in phosphate buffer and dehydrated with a series of ethanol solution (30, 50, 70, and 95% for 10 min each and absolute ethanol 3 times, 10 min each time). Afterwards, the specimens were placed in a critical point dryer (Balzers model CPD 020) before being mounted on SEM specimen stubs, coated with gold in a sputter coater (Balzers model SCD 040), and visualized using SEM (JEOL, model JSM-5410LV).

Viability of the Retrieved Cell Sheet

To analyze the growth potential of the cell after the transfer manipulations, the viability of the retrieved cell sheet construct was assessed. After the harvested cell sheets were cultured on the protein coated TCPS surface at 37°C for 24–48 h, the cell constructs were stained with LIVE/DEAD® Assay Kit to determine the cell viability as described previously.

Histological Analysis

For cross-sectional observation, frozen sections of multilayered sheets were prepared using the Tissue-Tek OCT compound (Sakura Fine Technical, Tokyo, Japan). The frozen embedded cell sheets were sectioned using a cryostat (Leica CM3050S) at −20°C. Thin (5 μm) cross-sectional slices were then fixed with 95% ethanol. These samples were subject to Hematoxylin and Eosin (H&E) staining performed by a conventional method. Briefly, the cross-sectional slice surfaces were first stained with hematoxylin and later immersed in the first OG-6 counterstain for keratin staining and in the second EA (Eosin Azure)

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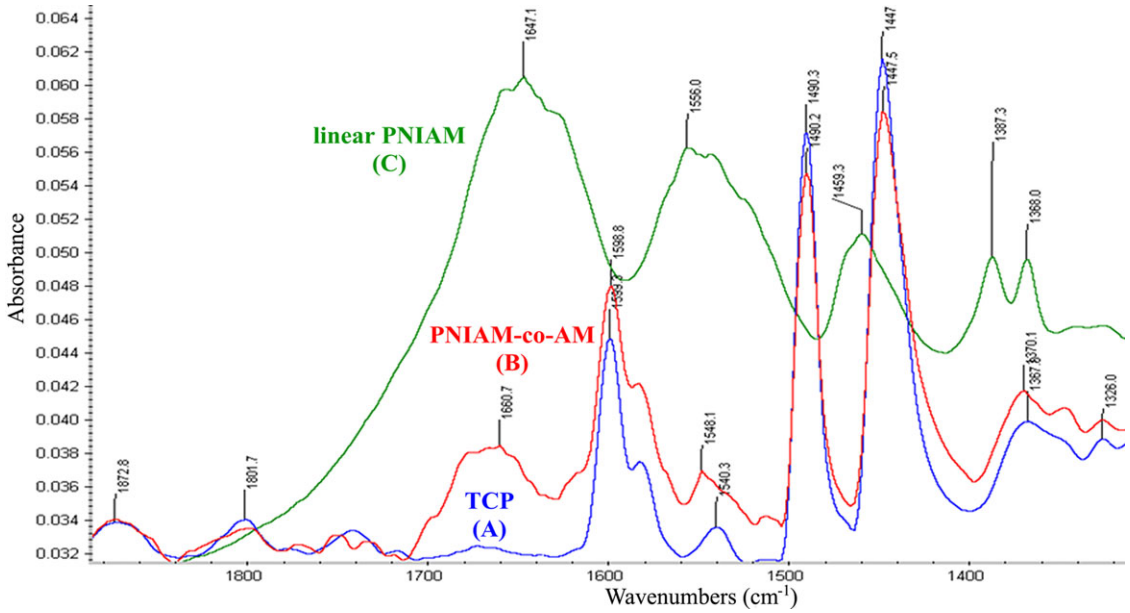

Figure 1. FTIR spectra of (A) ungrafted TCPS, (B) PNIAM-*co*-AM grafted TCP surface and (C) linear PNIAM, for wavenumbers ranging from 1800 to 1400 cm^{-1}. The peak at around 1660 cm^{-1} represents the secondary amide in the AM copolymer. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

counterstain. The surfaces were then decolorized using 95% ethanol. Afterwards, these surfaces were cleaned with xylene twice, mounted with permanent mounting medium, and examined using a phase contrast microscope (Olympus BX 60).

RESULTS AND DISCUSSION

Preparation and Characterization of PNIAM-*co*-AM Grafted Surface

The presence of PNIAM-*co*-AM on the tissue culture surface was first confirmed by FTIR. Figure 1 displays the spectra of linear PNIAM, PNIAM-*co*-AM grafted, and ungrafted tissue culture surfaces between 2000 and 1200 cm^{-1}. The spectrum of PNIAM-*co*-AM grafted TCP dish shows the secondary amide (C—O stretching) at 1663.1 cm^{-1}, and secondary open chain amide at 1551.2 cm^{-1}. These peaks are absent in the spectrum of the ungrafted TCPS but they are found in that of linear PNIAM, indicating that PNIAM-*co*-AM was successfully grafted on the TCPS surfaces by ultraviolet irradiation.

Changes in the temperature-dependent wettability of PNIAM-*co*-AM grafted culture surface were investigated in comparison with that of the ungrafted surface by the contact angle measurement (Figure 2). The contact angle of the ungrafted surface remained high between 95° and 98° regardless of the temperature variation, indicating the surface's hydrophobic characteristic. In contrast, the contact angle of the grafted surface was significantly increased from 58° to 75° when the temperature was raised from 30 to 35°C, respectively. Below 30°C, the LCST of the copolymer, the PNIAM-*co*-AM grafted surface exhibited hydrophilic surface properties due to the spontaneous hydration of surface-grafted molecules in response to temperature reduction.[23,24] Above the LCST, the PNIAM chain collapsed, exposing more isopropyl groups and increasing the surface's hydrophobicity.[24] The contact angles observed in this study were consistent with those of the Biazar et al.[25] in which the ungrafted polystyrene substrate had a contact angle of 91°–94°, while the contact angles of PNIAM grafted surface were 55° at 10°C and 61° at 37°C.

The surface topography of the grafted surfaces was examined at 5 and 45°C using AFM, as shown in Figure 3. The root-mean-square roughness of the PNIAM-*co*-AM grafted surface at 45°C was 9.35 nm, while that of the copolymer film at 5°C was 14.8 nm. An increase in the surface roughness at low temperature was probably a result of the extension of the hydrated polymer chain.[2]

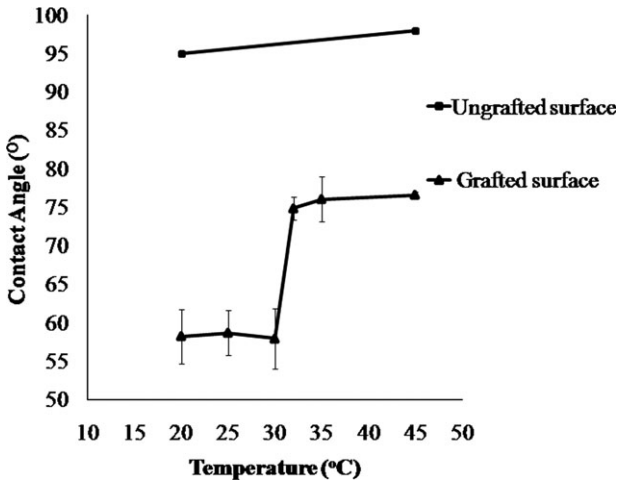

Figure 2. Contact angles of the PNIAM-*co*-AM grafted surface and ungrafted surface at various temperatures.

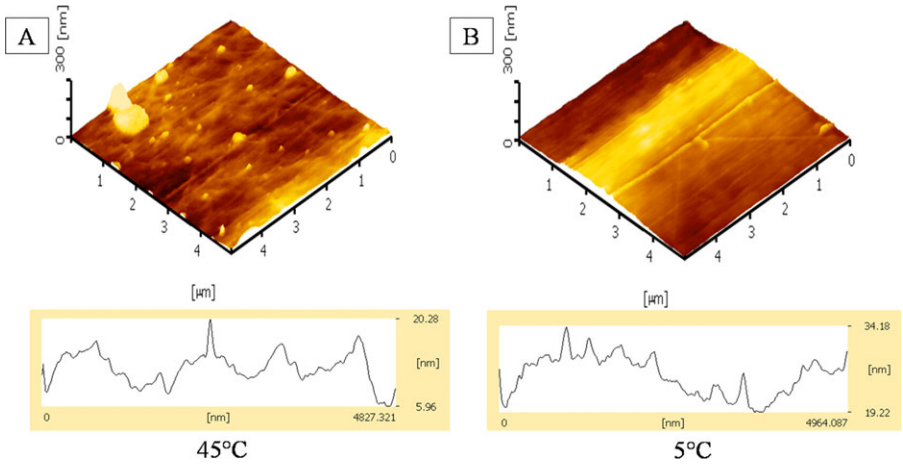

Figure 3. Atomic force microscopic images and height traces obtained from *in situ* measurement of PNIAM-*co*-AM grafted TCPS (A) at 45°C and (B) at 5°C. The scan area is 5 × 5 μm^2. The height profile is a horizontal line passing 3 μm from the front of the picture. A cantilever SI-DF3 was used with a nominal spring constant of $C = 1.7$ Nm^{-1} and a resonance frequency of 28 kHz. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

In Vitro Toxicity of PNIAM-*co*-AM Grafted Surface

To use the PNIAM-*co*-AM grafted surface for tissue culture, the toxicity of the modified surface must be determined. The effect of the copolymer on the cells' metabolic activity was determined using the MTT test. In this experiment, mouse preosteoblast MC3T3-E1 cells were treated with the cell culture media previously incubated with PNIAM-*co*-AM grafted copolymer film for 7 days. The cell viability was tested every day. As shown in Table I, the cell viability of MC3T3-E1 cells incubated with the spent medium was 96.5 ± 0.001% on the first day and was reduced to 73.73 ± 0.02% after 1 week incubation. According to ISO 10993-5: Biological evaluation of medical devices–Part 5, any materials that yield the cell viability of <70% are considered toxic.[23] In this study, the cell viability was above 70%, indicating that PNIAM-*co*-AM polymer film was not cytotoxic. The morphology of MC3T3-E1 cells showed fibroblast-like structure similar to that on the control sample (data not shown).

Effect of the Low Temperature Conditions on the Cell Viability

Because the cell detachment step was performed in a low temperature chamber without CO_2 supplementation, the impact of low temperature and lack of CO_2 on MC3T3-E1 cell viability needed to be investigated. As shown in Table II, the cell viability was close to 100% upon incubation at 10 and 20°C for 120 min, indicating that the cells viability was not affected by the low temperature treatment.

Cell Culture on PNIAM-*co*-AM Grafted Culture Surface

Changes in cell morphology and the detachment of the cells from the culture surface are used as an indicator of cell survival.[26] The ability of PNIAM-*co*-AM surface to support cell attachment was examined by a typical morphological observation. MC3T3-E1 cells were plated onto the PNIAM-*co*-AM grafted and ungrafted surfaces. The images clearly showed that the MC3T3-E1 cells on the grafted copolymer exhibited uniform fibroblast-like morphology, in the same manner as those observed on the ungrafted surfaces (Figure 4). The growth rates of the cells cultured on both surfaces were found to be similar, yielding ~2 × 10^6 cells/cm^2.

Cell Sheet Detachment

Okano et al. have proposed a two-step mechanism of cell detachment from temperature-responsive grafted surfaces. First, the grafted copolymer chains are hydrated and maintain expanded conformations at lower temperatures resulting in reduced interactions between the cells and grafted culture surfaces (passive step).[2] Second, an active cellular metabolic process enables the changes in their membrane shape, consuming internal metabolic energy to fully self-detach from temperature-responsive surfaces (active step).[2,27,28] However, by decreasing temperature, the metabolism of the cells is suppressed, inhibiting the morphological changes necessary for cell detachment.[2,27] Therefore, the optimal conditions to detach a complete cell sheet needs to be determined balancing between the hydration of the grafted surfaces and the maintenance of the active cell metabolism. In this study, mouse preosteoblast

Table I. The *In Vitro* Toxicity of Cell Culture Media Previously Incubated with PNIAM-*co*-AM Grafted Copolymer Film on Mouse preosteoblast MC3T3-E1 Cells After 7 Days of Incubation ($n = 6$)

% Cell viability at day 1–day 7						
Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
96.5 ± 0.001	91.2 ± 0.08	83.4 ± 0.05	82.7 ± 0.01	76.4 ± 0.01	74.5 ± 0.01	73.7 ± 0.02

Table II. The Percentage of Viable MC3T3-E1 Cells on the PNIAM-*co*-AM Grafted Surface Incubated at 10°C and 20°C

Incubation time	30 min	60 min	90 min	120 min
Lowering temperature to 10°C	99.81 ± 0.19	99.52 ± 0.60	98.29 ± 0.70	97.45 ± 1.86
Lowering temperature to 20°C	100	100	100	98.69 ± 0.92

MC3T3-E1 cells were allowed to reach over 100% confluence to achieve strong cell-cell junctions at 37°C. Then, for noninvasive cell harvest of preosteoblast MC3T3-E1 sheets, the incubation temperature was reduced to below 32°C. The cell detachment was performed using two conditions: (1) 90 min incubation at 20°C or (2) 30 min incubation at 10°C followed by additional incubation at 20°C for 60 min. Furthermore, the percentages of cell detachment achieved with and without a PVDF membrane were also compared.

Table III shows the percentages of the area of the cell sheet recovered from the PNIAM-*co*-AM grafted surface using these different conditions. When harvesting a cell sheet without the PVDF membrane, the cell monolayer surface had to be gently agitated with culture medium to lift-up the intact cell layers. Significantly, lower detached area of the cell sheet was obtained with condition (1), as compared with condition (2). It is possible that the temperature used in condition (1), 20°C, was not low enough to accelerate rapid hydration of the polymer chain. As a result, the majority of the cell sheet remained attached to the grafted surface. However, the two-step incubation process employed in condition (2) initially induced the rapid PNIAM surface hydration at 10°C and later increased the cellular metabolism in morphological changes at 20°C. Figure 4(D) shows the morphology of the cell sheet on the grafted surface detached without a membrane using condition (2). It is clear that the cell sheet was released from the sheet periphery toward the interior. Note that the cells on the control TCPS surface remained adherent regardless of temperature [Figure 4(C)].

When the cell sheets were detached from the grafted surfaces, they contracted because of strong cell–cell interaction.[24] To prevent the shrinking and folding of the cell sheet, the use of membranes as supporting materials was investigated. The PVDF membrane was used to achieve complete detachment and to transfer the cell sheets to other cell sheets for multilayer sheet construction, achieving adhesion with the underlying ECM proteins. By utilizing the PVDF membrane for cell harvesting, significantly larger areas of the cell sheets were detached using both conditions (Table III). Almost a complete cell sheet (96.19%) was recovered with the PVDF membrane when the cell culture was incubated at 10°C for 30 min, followed with 20°C for 60 min. Thus, this condition was used to construct a double-layered preosteoblast sheet. Over 97.79 % of the double-layered sheet detached from the PNIAM-*co*-AM grafted surface within 90 min, whereas no cells detached from the ungrafted surface.

The commercial UpCell™ temperature-responsive culture 6-well plate was also used to construct a single cell layer for

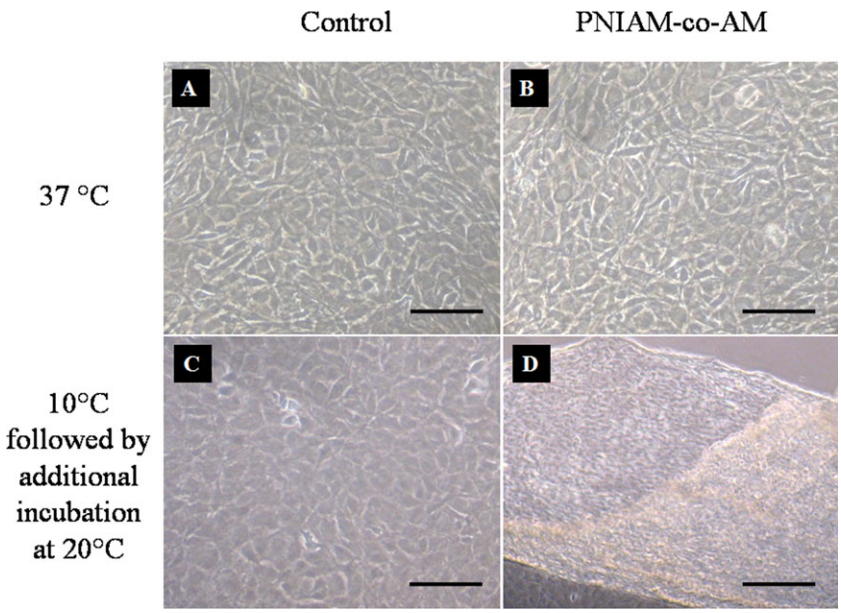

Figure 4. MC3T3-E1 cell adhesion on the control (A) and PNIAM-*co*-AM grafted surface (B) after a 72 h culture at 37°C. The morphology of MC3T3-E1 cells on the ungrafted surface (C) and the grafted surface (D) incubated at 10°C for 30 min, followed by additional incubation at 20°C for 60 min. The cells detached spontaneously from the grafted surface as a cell sheet. Scale bar = 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table III. The Conditions Used to Detach the Preosteoblast Cells from the PNIAM-*co*-AM Grafted Surfaces and the Area of the Detached Cell Sheet

Single cell layer detachment	% Area detached/35 cm^2	
Conditions; temperature and incubation time	Without PVDF	With PVDF
(1) 20°C (90 min)	30.50 ± 5.61	61.28 ± 7.48
(2) 10°C (30 min) followed by additional incubation at 20°C (60 min)	85.80 ± 8.24	96.19 ± 0.24

Data are expressed as the mean of three samples with SD. Note that PVDF = poly(vinylidenedifluoride) membrane.

comparison.[16] The mouse preosteoblast cells were shown to adhere, spread, and proliferate on the commercial UpCell™ surface similarly to those on a commercial ungrafted 6-well plate at 37°C. Intact cell sheets could be harvested when the temperature was reduced to 20°C for 30 min (data not shown). Faster cell sheet recovery was probably a result of a denser and uniform PNIAM layer on the UpCell™ surface, prepared by the deposition of NIAM using EB irradiation induced graft polymerization. This strategy results in the simultaneous polymerization of the monomer and covalent grafting of the rising polymer chains on the substrate.[29] Consequently, the PNIAM grafted surface is likely to be more consistent and homogeneous, leading to more hydration even at higher temperatures.

Cell Morphology with SEM

To observe the process of single cell and cell sheet detachment from PNIAM-*co*-AM grafted surface, a SEM study was carried out with MC3T3-E1 cells cultured on the copolymer films and the control ungrafted surfaces. Figure 5(B–D) shows that, at 37°C, MC3T3-E1 preosteoblasts on PNIAM-*co*-AM grafted surface exhibit fibroblast morphology and adhesion patterns similar to those on the ungrafted surface [Figure 5(A)]. The high density MC3T3-E1 cells on the copolymer grafted surfaces clearly show the formation of cell tight junctions. When the temperature was reduced to 20°C, the shrinkage of the cells on the polymer film, because of cytoskeleton tensile forces, was apparent [Figure 5(F)]. At high cell density, the reorganization of the actin cytoskeleton caused the folding of membrane protein at the edge of the cell layer, pulling the adjacent cells away from the surface and detaching the cells as an intact sheet [Figure 5(G,H)]. In contrast, the cells on the ungrafted surface remained flat and spread without any morphological change [Figure 5(E)].

In adherent cells, the cells are flat and spread because of the balance between the tensile stress of the ECM proteins (external force) and the pulling forces exerted by the cytoskeletal dynamics (internal force).[30] At low temperatures, the rapid hydration of the PNIAM-*co*-AM polymer chain eliminates the tight anchorage of the ECM proteins deposited on the substrate, resulting in the detriment of force equilibrium. Therefore, the force exerted by the cytoskeleton causes cell contraction and deadhesion.[30] Unlike the grafted surface, the ungrafted surface possesses no surface property alteration with the temperature. Thus, the ECM proteins remained unchanged, adhering the cells closely to the substrate by providing an external force counteracting the internal force from the actin cytoskeleton, allowing the cells to remain flat and spread.

Multilayered Cell Sheets

The process to construct a multilayered preosteoblast sheet is illustrated in Figure 6. After the culture medium was removed, a hydrophillically modified PVDF membrane was placed onto the cell culture, immediately sticking to the cell sheet. The culture plate was then incubated at 10°C for 30 min to induce an extension of PNIAM-*co*-AM chain and 20°C for 60 min to activate the cellular metabolism. Afterwards, the PVDF membrane

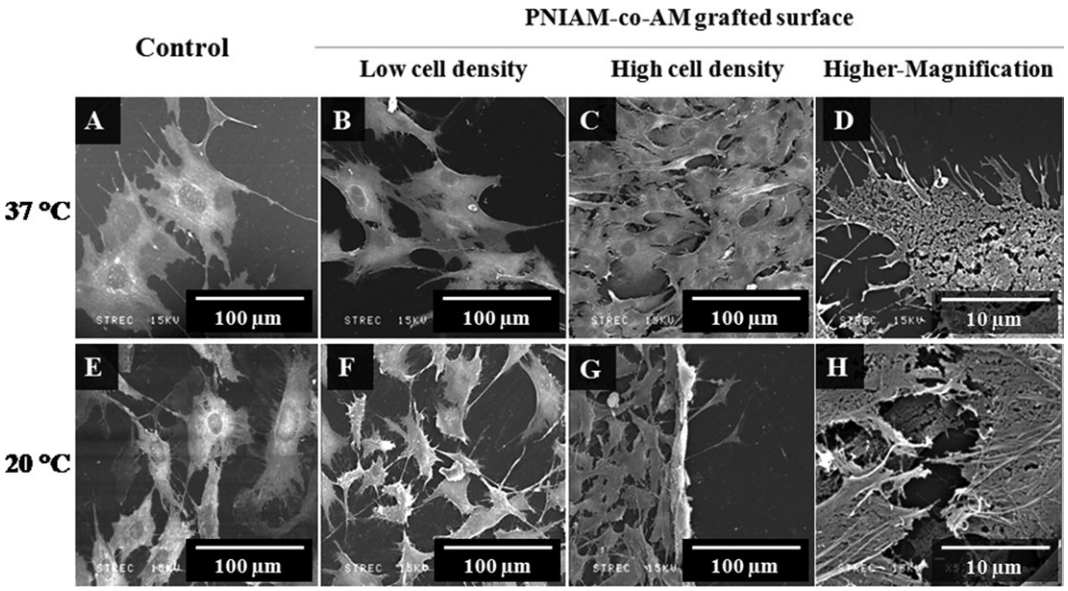

Figure 5. SEM images showing the MC3T3-E1 cell morphology on the control (A, E) and PNIPAM-*co*-AM grafted surface (B–D and F–H) at 37 and 20°C, respectively. Scale bars: 10 μm (A–F) and 100 μm (G and H).

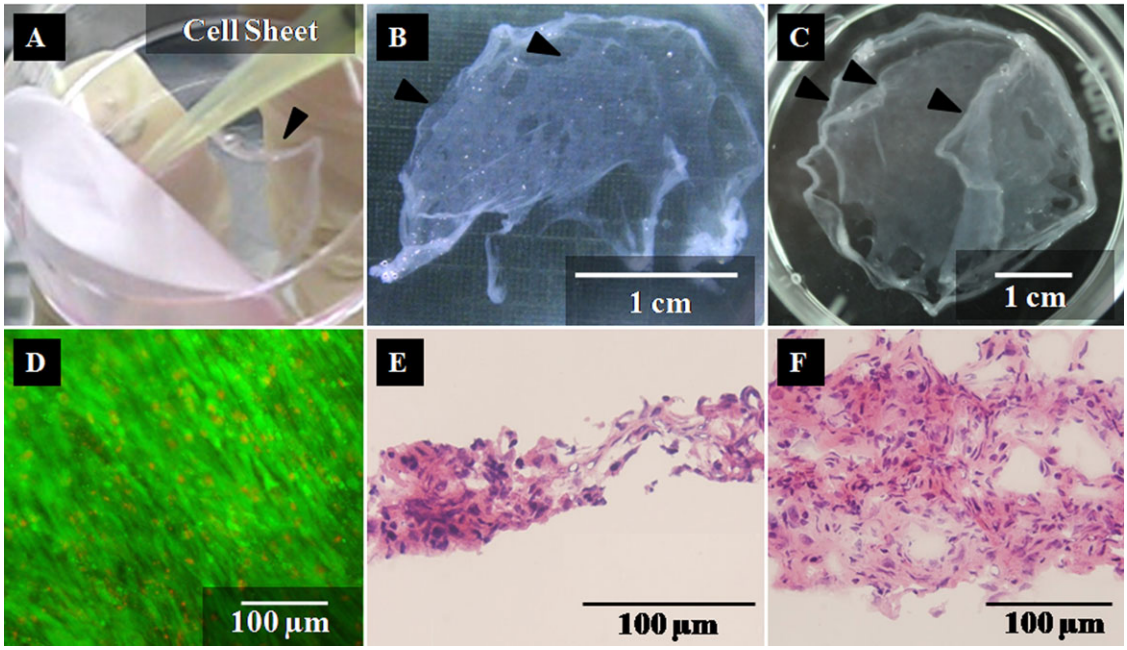

Figure 6. Construction of multilayered cell sheets; (A) preosteoblast MC3T3-E1 sheet and PVDF membrane were gently detached from the PNIAM-*co*-AM surface by reducing the temperature. The PVDF membrane was then peeled off from the cell sheet surface. (B) Double and (C) triple-layered preosteoblast sheets were cultured on the basement membrane protein coated surface at 37°C. (D) A live/dead stained image of the recovered multilayered MC3T3-E1 sheet detached from the PNIAM-*co*-AM grafted surface. The cross sections of (E) the double-layered and (F) the triple-layered preosteoblast sheets stained with H&E dye. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

was slowly peeled from the periphery of the well plate with tweezers, allowing the confluent cell sheet to be harvested as a single continuous cell sheet. The PVDF membrane/preosteoblast sheet was transferred to another cell sheet for stratification of the preosteoblast. For the cell sheet attachment, fresh culture medium was gently added to the edge of a matrigel-coated culture dish and the PVDF membrane was peeled off from the multilayered sheet [Figure 6(A)]. The harvested double- and triple-layer cell sheets are shown in Figure 6(B,C), respectively. Since the terminals of cytoskeleton were connected to ECM receptors, the deposited ECM was recovered from the dish surfaces by these forces,[14,31] providing necessary adhesion molecules to attach the cell layers to one another. Unfortunately, the shrinkage of the cell sheets was apparent after the PVDF membrane was removed, resulting from cytoskeletal reorganization during cell detachment.

Viability of the Retrieved Cell Sheet

As the cell sheet maintains cell tight junctions and ECM proteins on the basal side of the harvested cells,[2,13,14] the sheet can adhere rapidly on to a new culture dish and maintain the typical morphology of the cells. The quality of the retrieved multilayer sheet was examined using morphological observations and live/dead staining. The multilayered sheets were left undisturbed and grown on a matrigel-coated TCPS at 37°C for 2 days. Figure 6(D) shows live/dead stained images of the recovered multilayered preosteoblast sheets. Almost all the cells in the multilayer sheet were viable as shown in green fluorescence with only a few dead cells in red fluorescent color. The preosteoblast cells in the stacked sheet had fibroblast-like structures, similar to those in a conventional monolayer culture. These results indicate that the mouse preosteoblasts in the multilayered sheet were still healthy and viable even after the low temperature treatment and the process of cell sheet manipulation.

Histological Analysis

The presence of intact ECM proteins in the multilayered mouse preosteoblast sheets was confirmed using H&E stain. Hematoxylin was used to stain the nucleic acids of the cells a deep blue-purple color. Eosin stains proteins nonspecifically. In a typical tissue, nuclei are stained blue, whereas the cytoplasm and ECM have varying degrees of pink staining.[32] Transverse sections of the harvested double-layered [Figure 6(E)] and triple-layered [Figure 6(F)] cell sheets stained with H&E revealed tissue-like organized structure with relatively abundant ECM proteins present throughout the sheets. The ECM proteins on the basal side of cell sheets could function as a glue to attach cell sheets onto the others.[14,17] As a result, the multilayered cell sheets recovered from the temperature-responsive surfaces and could be easily adhered to other cell sheets or surfaces.[9,17]

CONCLUSIONS

We have developed a method to harvest multilayered mouse preosteoblast MC3T3-E1 sheets from the PNIAM-*co*-AM synthesized by UV-induced polymerization. The temperature-responsive PNIAM-*co*-AM grafted culture surface exhibits reversible hydrophilic to hydrophobic characteristics with changes in temperature. The grafted surface was shown to be nontoxic, providing suitable surface to support cell attachment and proliferation similar to the control ungrafted surface. For

the construction of a cell sheet, MC3T3-E1cells were allowed to reach 100% confluency to ensure the establishment of cell tight junctions at 37°C on the PNIAM-*co*-AM surface. To harvest the cell sheet, the temperature of the grafted copolymer surface was reduced to 10°C for 30 min to allow the polymer chain to extend. Afterwards, the temperature was increased to 20°C for 60 min to activate the cellular metabolism responsible for cell detachment. The use of a hydrophilic PVDF membrane to harvest the cell sheets has been shown to significantly improve the outcome. This condition was used to harvest doubled and tripled layer cell sheets with greater than 97% cell sheet recovery. Because the ECM proteins under the cultured cells were harvested with the cells, the cell sheets were more adhesive to other cell sheets and to new culture surface.

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