Temperature-Dependent Cell Detachment on Pluronic Gels

ARTICLE *in* BIOMACROMOLECULES · MARCH 2005

Impact Factor: 5.75 · DOI: 10.1021/bm0494850 · Source: PubMed

6 AUTHORS, INCLUDING:

Akon Higuchi
National Central University
194 PUBLICATIONS **3,451** CITATIONS
SEE PROFILE

Tae Moon Tak
Seoul National University
30 PUBLICATIONS **1,986** CITATIONS
SEE PROFILE

Temperature-Dependent Cell Detachment on Pluronic Gels

Akon Higuchi,*,† Taro Yamamoto,† Kaichiro Sugiyama,† Shizue Hayashi,†
Tae Moon Tak,‡ and Tsutomu Nakagawa§

Department of Applied Chemistry, Seikei University, 3-3-1 Kichijoji Kitamachi, Musashino, Tokyo 180-8633, Japan

Received August 28, 2004; Revised Manuscript Received December 17, 2004

Cell culture on gels made of poly(ethylene oxide) and poly(propylene oxide) (Pluronic), which has a lower critical solution temperature around 30 °C, could be performed for 48 h. However, the Pluronic gels were highly hydrophilic and tended to dissolve in the culture medium. We achieved temperature-dependent detachment of cells from Pluronic gels containing or lacking extracellular matrix (ECM) by cooling the gels to 4 °C. Using normal human umbilical vein endothelial cells (HUVECs) grown on and released from Pluronic gels lacking ECM, we further found that the expression ratio of the surface markers CD34 and CD105 was twofold higher than for cells grown on polystyrene and removed with trypsin. In addition, the expression ratios for CD34 and CD105 on HUVECs cultivated on the Pluronic gels containing higher concentrations of ECM were lower, which may be due to ECM coating of the cell surface and, thus, interference with antibody binding. In summary, temperature-dependent detachment of cells from Pluronic gels allows the isolation of cells under mild conditions. This can be a powerful tool for surface marker analysis by flow cytometry.

Introduction

Pluronic, poly(ethylene oxide) (PEO)−poly(propylene oxide) (PPO)−PEO triblock copolymer, has been widely studied as a material for pharmaceutical and biomedical applications.[1−8] This copolymer shows the property of amphiphilicity and undergoes self-assembly into micelles and a lyotropic liquid crystal gel phase due to the hydrophilic PEO and hydrophobic PPO blocks.[1−3] Pluronic and related copolymers[9,10] have been approved for use by the United States Food and Drug administration and used extensively in the pharmaceutical industry as a vehicle for the controlled release of drugs and as a coating for wounds to promote healing and protect against microbial infection.[4−6]

In our previous study, we examined plasma protein adsorption and platelet adhesion to polysulfone membranes coated with Pluronic with varying PEO block lengths. The results suggested that the bioinert property of PEO segments in the Pluronic, which is ascribed to their high flexibility in aqueous medium, suppresses the adsorption of plasma proteins and platelets.[11]

Pluronic F127, which has similar properties as poly(*N*-isopropylacrylamide), reversibly interconverts around 30 °C, its lower critical solution temperature (LCST), from a soluble to an insoluble form in aqueous solution, when the concentration of Pluronic in aqueous solution is more than 15 wt %. Several researchers[12−17] have reported that cells can adhere and grow on slightly hydrophobic surfaces made of poly(*N*-isopropylacrylamide) above its LCST and that cells detach without enzymatic digestion below the LCST. This phenomenon is explained by the change in hydration of poly(*N*-isopropylacrylamide) with temperature. However, most of the studies related to temperature-dependent cell adhesion and detachment are limited to those using poly(*N*-isopropylacrylamide) surfaces. This knowledge has been employed for the preparation of cell sheets.[14−17]

In our previous study,[18] we investigated light-induced changes in cell attachment to poly(spiropyran-*co*-methyl methacrylate) membranes. Spiropyran is a nonionic photoresponsive molecule that is changed by UV irradiation to a hydrophilic polar, zwitterionic merocyanine isomer, which can then be converted back to the nonionic form by irradiation with visible light. Using poly(spiropyrane-*co*-methyl methacrylate)-coated glass plates and patterned light irradiation, we were able to cause selective UV light-induced detachment of platelets and mesenchymal stem cells.[18] This light-induced cellular detachment was not observed using cells grown on poly(methyl methacrylate)-coated glass plates, and it is the result of the change from the nonpolar spiropyran to the polar zwitterionic merocyanine isomer.

Herein, we describe cell culture and the temperature-dependent regulation of cell detachment on Pluronic F127 gels, which show reversible soluble/insoluble changes in aqueous solution in response to a temperature alteration across the LCST.

Experimental Section

Materials. Pluronic F127 (molecular weight =120 000 g mol^{-1}, $PEO_{99}-PPO_{65}-PPO_{99}$) was supplied by Asahi Elec-

* To whom correspondence should be addressed. Tel.: +81-422-37-3748. Fax: +81-422-37-3748. E-mail: higuchi@ch.seikei.ac.jp.
† Seikei University.
‡ Seoul National University.
§ Meiji University.

10.1021/bm0494850 CCC: $30.25
Published on Web 01/15/2005

tric Industry Co., Ltd. Solutions of 0.5 mg/mL fibronectin (FN; F003, from human plasma, Asahi Technoglass Co., Ltd., Tokyo, Japan) and 5 mg/mL collagen (COL; Cellgen IPC-15, type I from calf skin, Koken Co., Ltd., Tokyo, Japan) were used as received.

Other chemicals were of reagent grade and were used without further purification. Ultrapure water was used throughout the experiments.

Preparation of Pluronic Gels. Pluronic F127 was dissolved in water at 4 °C, and a Pluronic solution of 30 wt % was prepared. After the Pluronic solution was sterilized at 120 °C for 20 min, a Pluronic solution of 500 μL was inserted into each well of 24-well tissue culture plates. A 10 μg/mL or 100 μg/mL FN solution and/or 0.5 mg/mL COL solution was also inserted into the wells of 24-well tissue culture plates containing Pluronic solution for the preparation of Pluronic gels containing FN, COL, and FN+COL, respectively. The Pluronic solutions were dried under a vacuum at 37 °C for 24 h. The gels containing FN, COL, and FN+COL were denoted as PL−FN, PL−COL and PL−FN+COL gels, respectively. PL−FN1 and PL−FN2 gels contained 0.19 and 1.9% FN, respectively. PL−COL gels contained COL of 18.8%. PL−FN+COL gels contained 0.19% FN and 18.8% COL. The gels containing no extracellular matrix (ECM) were denoted as PL gels.

The weight loss of Pluronic gels in water was measured after the Pluronic gels were dried under a vacuum at 37 °C for 24 h as follows. (1) Two milliliters of water was injected into the dry Pluronic in 24-well tissue culture plates at 37 °C. The weight of dry Pluronic was denoted as $W(0)$. (2) Every hour, four gels in the 24-well tissue culture plates were dried under a vacuum at 37 °C for 24 h, after the supernatant solution in the 24-well tissue culture plates was decanted off. (3) The weight of dry Pluronic was measured, and the weight loss of the gels was calculated as

$$\text{weight loss ratio (\%)} = [W(0) - W(t)]/W(0) \qquad (1)$$

where W(t) indicates the weight of dry Pluronic after immersion in water for t hours.

Cell Cultivation. L929 cells (Dainippon Seiyaku Co., Ltd.), derived from a mouse connective tissue fibroblast, were maintained in RPMI 1640 media (JRH Bioscience, Lenexa, KS) supplemented with 25 mg/L streptomycin sulfate, 3.5 mg/L benzylpenicillin potassium, and 10% fetal bovine serum (FBS, JRH Bioscience, Lenexa, KS) in a 5% CO_2 atmosphere at 37 °C.[19] Normal human umbilical vein endothelial cells (HUVEC, C2517, Takara Bio, Inc.) were maintained in EGM-2 media (Takara Bio, Inc.) supplemented with 5% FBS and EGM-2 SingleQuots (Takara Bio, Inc.) containing antibiotics and growth factors in a 5% CO_2 atmosphere at 37 °C. The cell lines were used between passage number 5 and passage number 10 in the following experiments.

The cell number was characterized by observation of the cells using an inverted microscope (Diaphoto TMD300, Nikon Co., Tokyo) equipped with a charge-coupled device (CCD) video camera, ARGUS 20 (Hamamatsu Photonics K.K.) and a temperature-regulated box.[19−21]

Cell Cultivation on Pluronic Gels. After UV irradiation (30-cm distance, 10 W, GL10, Stanley Co., Tokyo) of the Pluronic gels for 10 min for sterilization, L929 or HUVEC cells in suspensions (cell densities 1×10^4/cm^2 and 2 mL) were injected into the dried Pluronic gels in the tissue culture plates and were incubated in the CO_2 incubator in a 5% CO_2 atmosphere at 37 °C. For detachment test of the cells, 24-well tissue culture plates containing cells on the Pluronic gels were located on the ice water at 0 °C or on the water at 37 °C. The concentration of the Pluronic in the culture medium was calculated to be 7.5 wt %, when the Pluronic gels were completely dissolved into the culture medium below the LCST of the Pluronic. After 10 min, the medium was agitated with a Pasteur pipet and was exchanged with 2 mL of cultivation medium. Then, the numbers of residual cells were counted using an inverted microscope (Diaphoto TMD300, Nikon Co.) equipped with a CCD video camera, ARGUS 20 (Hamamatsu Photonics K.K.), digital camera (Camedea, Olympus Co.), and a temperature-regulated box. The cell number was obtained from the averages of four different experiments on each gel using four independent gels prepared from the same conditions (total $n = 16$).

Surface Marker Analysis. Surface marker expression of CD34 and CD105 on HUVEC cells was analyzed using flow cytometry (Coulter EPICS XL; Beckman-Coulter Co.).[22,23] Preparation of dyeing of HUVEC cells with anti-CD34 antibody and anti-CD105 antibody was performed as follows:

(1) The medium containing HUVECs was decanted off, after the medium was centrifuged at 1500 rpm for 5 min. A total of 7 μL of FcR block solution (130-059-901, Miltenyi Biotec, Inc.) was added onto the cell pellets, and the cells were dissolved into FcR block solution using a Boltex mixer (VX-100; Montreal Biotech, Inc.). (2) A total of 20 μL of anti-CD34 antibody conjugated with fluorescein isothiocyanate (555821, BD Biosciences Pharmingen) and 20 μL of CD105 antibody conjugated with phycoerythrin (A07414, Beckman-Coulter Co.) was added into the cell suspension solution, which was further incubated at 0 °C for 30 min. (3) The cell solution was centrifuged at 1500 rpm for 5 min, after 2 μL of phosphate buffer solution containing 0.1% NaN_3 and 0.5% bovine serum albumin (solution A) was added into the cell solution. (4) The cell solution was decanted off. A total of 10 μL of propidium iodide solution (51-66211E, BD Biosciences Pharmingen) and 200 μL of solution A were added onto the cell pellets.

Results and Discussion

Cell Culture of Pluronic Gels. Thick Pluronic gels (0.15 g/2 cm^2) were prepared by injection of 0.5 mL of 30 wt % Pluronic solution into 24-well tissue culture plates and were dried under a vacuum at 37 °C for 24 h in this study. The cell growth and morphology of L929 cells cultured on several Pluronic gels or a control polystyrene (PSt) tissue culture plate were examined by phase contrast microscopy as indices of cell behavior and function. The cells on the PSt tissue culture plate (Figure 1a) gave relatively high numbers of filopodia and a flattened morphology as reported previously.[19] On the other hand, the cells on Pluronic gels with

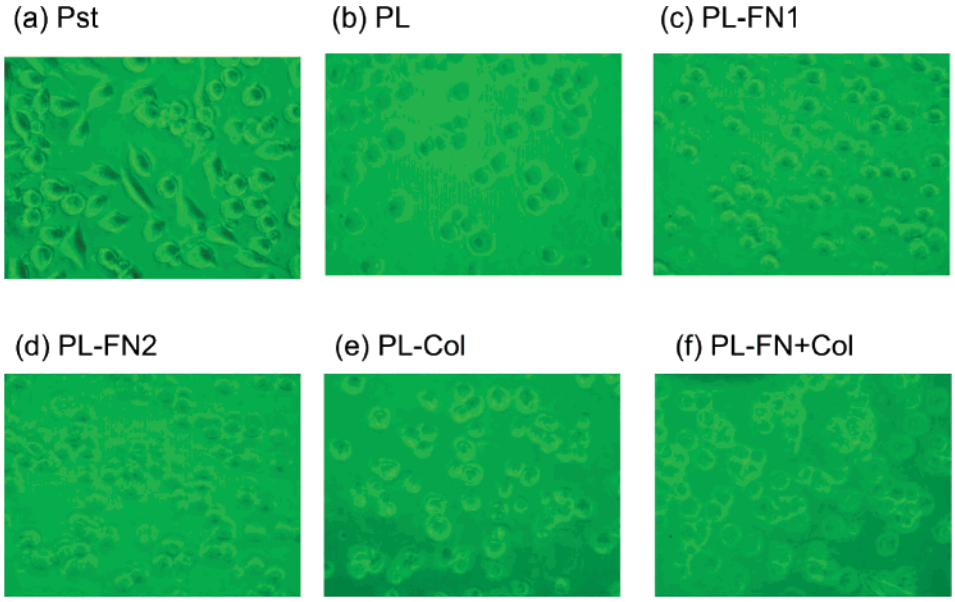

Figure 1. Phase contrast micrographs of L929 cells cultured on PSt tissue culture plates (a), PL gels (b), PL−FN1 gels (c), PL−FN2 gels (d), PL−COL gels (e), and PL−FN+COL gels (f) in the media containing 10% FBS after 12 h of incubation. A total of 2×10^4 cells (i.e., 1×10^4 cells/cm^2) were inoculated initially on each Pluronic gel and PSt culture plate.

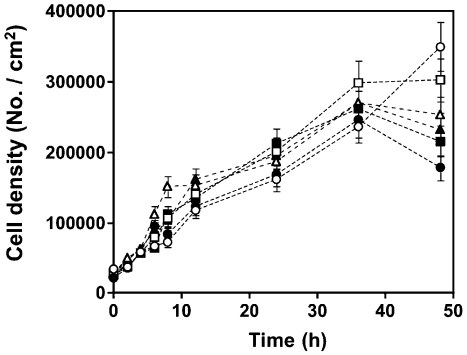

Figure 2. Growth curves of L929 cells cultured on PSt tissue culture plates (○), PL gels (●), PL−FN1 gels (□), PL−FN2 gels (■), PL−COL gels (△), and PL−FN+COL gels (▲) in the media containing 10% FBS. A total of 2×10^4 cells (i.e., 1×10^4 cells/cm^2) were inoculated initially on each Pluronic gel and PSt culture plate. Data are expressed as means ±SD of four independent measurements. Some of them are hidden within the symbols.

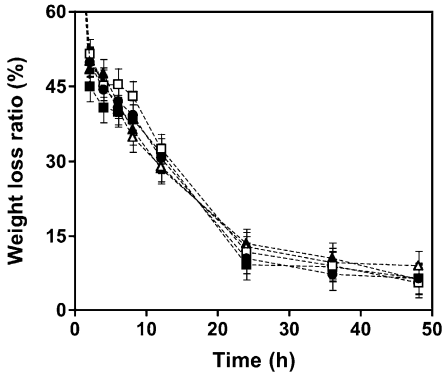

Figure 3. Time dependence of the weight loss ratio of PL gels (●), PL−FN1 gels (□), PL−FN2 gels (■), PL−COL gels (△), and PL−FN+COL gels (▲) in water at 37 °C. Data are expressed as means ±SD of four independent measurements. Some of them are hidden within the symbols.

or without added ECM proteins (FN and/or COL) were round in shape, indicating that they did not adhere well to the Pluronic gels (Figure 1b−f).

Figure 2 shows the density of L929 cells cultured on the Pluronic gels or PSt tissue culture plates for 48 h. All cells grew until 36 h of cultivation. The cells on Pluronic gels containing ECM (PL−FN1, PL−FN2, PL−COL, and PL−FN+COL gels) grew relatively faster than those on the Pluronic gels lacking ECM (PL gels) or on an uncoated PSt tissue culture plate. This may be due to the stimulation of L929 cell growth by specific binding to ECM proteins, because we also found that the cells grew well on the membranes prepared with ECM proteins in the previous study.[21] After 36 h, cell growth on Pluronic gels was suppressed, but as previously described,[19] growth on the PSt tissue culture plate continued after 36 h. Furthermore, the cell density on Pluronic gels lacking ECM proteins decreased dramatically after 36 h of cultivation. These studies indicate that the addition of ECM proteins enhances cell growth on Pluronic gels.

Durability of Pluronic Gels. We suspected that the suppression of cell growth on Pluronic gels after 36 h of cultivation was due to the dissolution of the Pluronic gels in the culture medium. Therefore, we assessed the weight loss of Pluronic gels in water at 37 °C (Figure 3). The Pluronic gels were found to be highly hydrophilic and tended to dissolve in the water, although 5% of Pluronic remained in water after 48 h of immersion. We found that the speed of weight loss of Pluronic gels decreased gradually over time in Figure 3. This is explained as the concentration of Pluronic in the aqueous phase increases over time because of dissolution of Pluronic gels into the aqueous phase. High concentration of Pluronic in the aqueous phase tended to prevent the dissolution of Pluronic gels into the aqueous phase. Furthermore, the round morphology of the L929 cells on Pluronic gels and the inhibition of growth after 36 h of cultivation can be explained by the high hydrophilicity of Pluronic gels because cell growth on highly hydrophilic surfaces is known to be strongly suppressed.[19]

Temperature-Dependent Cell Detachment. Pluronic gels were dried under a vacuum at 37 °C before cell culture

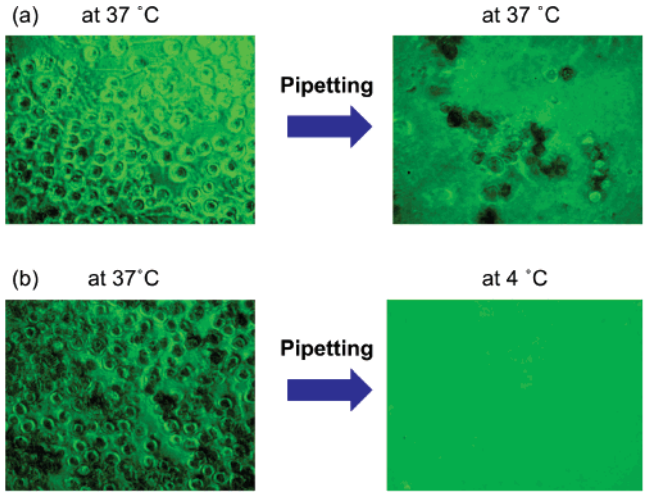

Figure 4. Temperature-dependent detachment of L929 cells on PL gels. PL gels were kept at 37 °C (a) or Pluronic gels were cooled to 4 °C (b) and subsequently the culture media were pipetted up and down. A total of 2×10^4 cells (i.e., 1×10^4 cells/cm^2) were inoculated initially on each Pluronic gel.

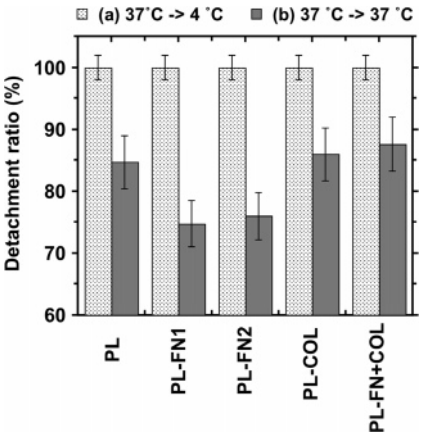

Figure 5. Detachment ratio of L929 cells cultured on PL, PL−FN1, PL−FN2, PL−COL, and PL−FN+COL gels in the media containing 10% FBS when Pluronic gels were cooled to 4 °C (a) or Pluronic gels were kept to 37 °C (b) and subsequently the culture media were pipetted up and down. A total of 2×10^4 cells (i.e., 1×10^4 cells/cm^2) were inoculated initially on each Pluronic gel. Data are expressed as means ±SD of four independent measurements.

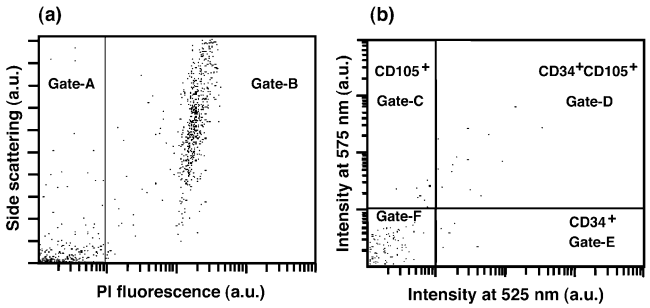

Figure 6. Flow cytometric scattergrams of HUVEC cells detached from PL gels at the fluorescent intensity of PI fluorescence and intensity of side light scattering (a) and at the fluorescent intensities of 525 and 575 nm (b). A total of 2×10^4 cells (i.e., 1×10^4 cells/cm^2) were inoculated initially on each Pluronic gel.

experiments. L929 cells were cultivated on several Pluronic gels for 12 h to allow extensive attachment. Thereafter, we examined temperature-dependent cell detachment. Figure 4 shows L929 cells on Pluronic gels before and after being cooled to 4 °C. L929 cells were rarely observed on the surface of the Pluronic gels after they were cooled to 4 °C and washed with culture medium (Figure 4b). The detachment of L929 cells from the Pluronic gels is due to the dissolution of Pluronic gels into the culture medium by a reduction in temperature below the LCST of Pluronic F127, because no detached cells were observed on the PSt 24-well tissue culture plate after being cooled from 37 to 4 °C. On the other hand, some L929 cells remained attached when the cells were treated in the same way (i.e., pipetting of the culture medium) but kept at 37 °C (Figure 4a). This indicates that some L929 cells detached from the Pluronic gels even at 37 °C. This is probably due to weak cell attachment to the highly hydrophilic Pluronic gels.

We further calculated the detachment ratio from the number of cells on the Pluronic gels before and after cooling to 4 °C:

$$\text{detachment ratio} = (1 - N_a/N_b) \times 100\ (\%) \qquad (2)$$

where N_b and N_a are the numbers of cells attached before and after washing with culture medium. Figure 5 shows the detachment ratio of L929 cells on the various Pluronic gels. When the L929 cells were cooled to 4 °C, they completely detached from all of the Pluronic gels after gentle pipetting of the culture medium to remove unbound cells. In contrast, some of cells remained when the Pluronic gels were kept at 37 °C during the procedure. These results clearly show the temperature-dependent detachment of L929 cells from Pluronic gels.

Flow Cytometry Analysis of Cells. Temperature-dependent detachment of cells from the surface of Pluronic gels allows isolation of cells under very mild conditions; the cells do not require the addition of trypsin to be removed from the growth substrate. This can be a powerful tool for surface marker analysis by flow cytometry because the ECM and cell adhesion molecules can be preserved.

To examine this possibility further, we performed flow cytometric analysis of surface markers on cells detached after cultivation on conventional PSt tissue culture plates or released from Pluronic gels by cooling. We used HUVECs for these experiments because they are known to express several surface markers, such as CD34 and CD105.[24−27] Figure 6 shows the flow cytometric scattergrams of HUVECs cultured on Pluronic gels. Following selection of viable cells by propidium iodide staining,[28] the expression of the surface antigens of $CD34^-CD105^+$ (gate C in Figure 6b), $CD34^+CD105^+$ (gate D in Figure 6b), and $CD34^+CD105^-$ (gate E in Figure 6b) was determined. Therefore, the cells analyzed in gate A in Figure 6a were evaluated using the protocol employed for Figure 6b. Figures 7 and 8 show the expression ratio of $CD34^-CD105^+$, $CD34^+CD105^+$, and $CD34^+CD105^-$ of HUVECs cultivated on PSt tissue culture plates and several types of Pluronic gels as evaluated by flow cytometry. The expression ratio was calculated from the flow cytometric data as follows:

$$\text{expression ratio} = (N_{CD}/N_{total}) \times 100 \qquad (3)$$

where N_{total} is the total number of cells and N_{CD} is the number

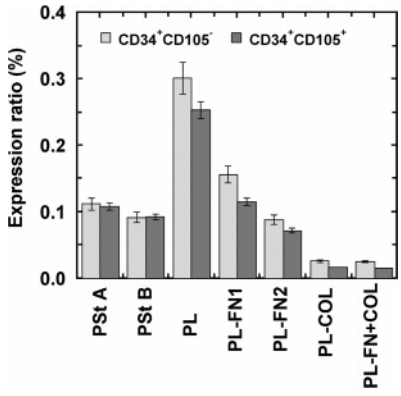

Figure 7. Expression ratio of $CD34^+CD105^-$ and $CD34^+CD105^+$ of HUVEC cells detached from PSt culture plates (PSt A, PSt B) and PL, PL−FN1, PL−FN2, PL−COL, and PL−FN+COL gels evaluated from the flow cytometry. The cells were incubated for 12 h before the cell detachment using the trypsin digestion method (PSt A, PSt B) and thermo-induced method (PL, PL−FN1, PL−FN2, PL−COL, and PL−FN+COL gels). The cell detached from the PSt culture plate contained 0% (PSt A) or 0.19% FN (PSt B) in the cell solution. A total of 2×10^4 cells (i.e., 1×10^4 cells/cm^2) were inoculated initially on each Pluronic gel and PSt culture plate. Data are expressed as means ±SD of four independent measurements.

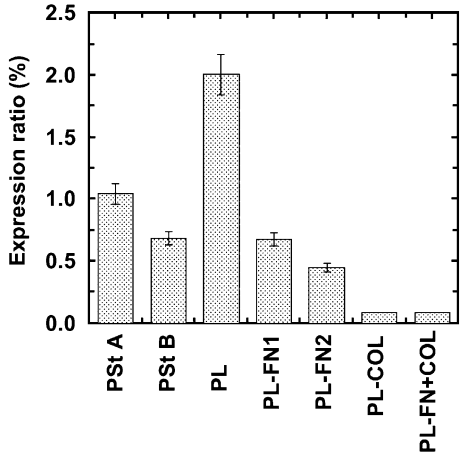

Figure 8. Expression ratio of $CD34^-CD105^+$ of HUVEC cells detached from PSt culture plates (PSt A, PSt B) and PL, PL−FN1, PL−FN2, PL−COL, and PL−FN+COL gels evaluated from the flow cytometry. The cells were incubated for 12 h before the cell detachment using the trypsin digestion method (PSt A, PSt B) and thermo-induced method (PL, PL−FN1, PL−FN2, PL−COL, and PL−FN+COL gels). The cell detached from the PSt culture plate contained 0% (PSt A) or 0.19% FN (PSt B) in the culture media. A total of 2×10^4 cells (i.e., 1×10^4 cells/cm^2) were inoculated initially on each Pluronic gel and PSt culture plate. Data are expressed as means ±SD of four independent measurements.

of cells expressing the specific cell surface marker analyzed in the flow cytometry. The HUVECs cultivated on the PSt culture plates were detached by digestion with trypsin, and the cells cultivated on the Pluronic gels were detached by cooling to 4 °C.

The expression ratio of $CD34^-CD105^+$, $CD34^+CD105^+$, and $CD34^+CD105^-$ for HUVECs cultivated on the Pluronic gels containing no ECM (PL gels) was approximately twofold higher than for cells cultivated on the PSt tissue culture plate. Except for cells cultivated on the Pluronic gels containing a low amount (0.19%) of FN, HUVECs detached from the Pluronic gels containing ECM had approximately equal or lower expression ratios than cells detached from PSt tissue culture plates. The expression ratios for $CD34^-CD105^+$, $CD34^+CD105^+$, and $CD34^+CD105^-$ from HUVECs cultivated on the Pluronic gels containing higher concentrations of ECM showed lower surface marker expression ratios.

Given these results, we further examined the effect of ECM on the expression of surface markers by flow cytometry. We assessed the expression ratio of the surface markers in HUVECs grown for 12 h on PSt tissue culture plates with and without 0.19% FN in the cell solution. Following detachment with trypsin, the expression ratios of $CD34^-CD105^+$, $CD34^+CD105^+$, and $CD34^+CD105^-$ for HUVECs cultivated on the PSt culture plate containing 0.19% FN in the cell solution were lower than that for cells grown on plates in the absence of FN ($p < 0.05$ from Student's t test). This may be due to an interference of antibody binding by ECM attached to the cell surface. This effect could also lead to the reduced expression ratios of the surface markers on the Pluronic gels containing ECM compared to those on the PL gels as found in Figures 7 and 8.

Conclusion

In the current studies, we found that cells can be cultured on Pluronic gels for 48 h, although the Pluronic gels were highly hydrophilic and tended to dissolve in the culture medium. We also achieved temperature-dependent detachment of cells from Pluronic gels containing or lacking ECM.

The expression ratio of surface markers on HUVECs cultivated on Pluronic gels containing no ECM was twofold higher than for cells detached from PSt plates by the conventional trypsin digestion method. Also, culture of HUVECs on Pluronic gels containing higher concentrations of ECM resulted in reduced expression ratios for CD34 and CD105. This may be due to interference of the ECM proteins with antibody binding by coating of the cell surface.

Nishida et al. investigated tissue-engineered epithelial-cell sheets that were fabricated ex vivo by culturing harvested cells for 2 weeks on temperature-responsive cell-culture surfaces with 3T3 feeder cells.[29] They used poly(*N*-isopropylacrylamide) on temperature-responsive cell-culture surfaces. The Pluronic F127 gels would be also useful in the preparation of tissue-engineered epithelial or another type of cell sheets in the future. Temperature-dependent detachment of cells on the Pluronic gels allows the isolation of cells under mild conditions. This could be a powerful tool for surface marker analysis using flow cytometry. In addition, the preparation of Pluronic gels is simple and does not require polymer synthesis because Pluronic F127 is commercially available. We now plan on generating insoluble Pluronic gel surfaces to allow long-term (i.e., more than 1 week) cell culture.

Acknowledgment. We gratefully acknowledge financial support through a Grant-in-Aid for Scientific Research on Priority Areas (B, "Novel Smart Membranes Containing Controlled Molecular Cavity", No. 13133202, 2001-2004) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. We also thank financial support through the Science Research Promotion Fund from Japan

Private School Promotion Foundation. This research was partially supported from a Grant-in-Aid for Exploratory Research (No. 14655136) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. This research was also supported from Asahi Glass Foundation and Salt Science Foundation.

References and Notes

(1) Shishido, S. M.; Seabra A. B.; Loh, W.; Ganzarolli de Oliveira, M. *Biomaterials* **2003**, *24* (20), 3543.
(2) Desai, M.; Jain, N. J.; Sharma, R.; Bahadur, P. *J. Surfactants Deterg*. **2000**, *3*, 193.
(3) Ivanova, R.; Alezandridis, P.; Lindman, B. *Colloids Surf., A* **2001**, *183*−*185*, 41.
(4) Liaw, J.; Lin, Y. *J. Controlled Release* **2000**, *68* (2), 273.
(5) Morishita, M.; Barichello, J. M.; Takayama, K.; Chiba, Y.; Tokiwa, S.; Nagai, T. *Int. J. Pharm.* **2001**, *212* (2), 289.
(6) Wang, Y. Y.; Hong, C. T.; Chiu, W. T.; Fang, J. Y. *Int. J. Pharm.* **2001**, *224* (1−2), 89.
(7) Orringer, E. P.; Casella, J. F.; Ataga, K. I.; Koshy, M.: Adams-Graves, P.; Luchtman-Jones, L.; Wun, T.; Watanabe, M.; Shafer, F.; Kutlar, A.; Abboud, M.; Steinberg, M.; Adler, B.; Swerdlow, P.; Terregino, C.; Saccente, S.; Files, B.; Ballas, S.; Brown, R.; Wojtowicz-Praga, S.; Grindel, J. M. *JAMA, J. Am. Med. Assoc*. **2001**, *286* (17), 2099.
(8) Varshney, M.; Morey, T. E.; Shah, D. O.; Flint, J. A.; Moudgil, B. M.; Seubert, C. N.; Dennis, D. M. *J. Am. Chem. Soc*. **2004**, *126* (16), 5108.
(9) Kim, M. S.; Seo, K. S.; Khang, G.; Cho, S. H.; Lee, H. B. *J. Biomed. Mater. Res.* **2004**, *70A* (1), 154.
(10) Jeong, B.; Bae, Y. H.; Kim, S. W. *J. Biomed. Mater. Res.* **2000**, *50* (2), 171.
(11) Higuchi, A.; Sugiyama, K.; Yoon, B. O.; Sakurai, M.; Hara, M.; Sumita, M.; Sugawara, S.; Shirai, T. *Biomaterials* **2003**, *24* (19), 3235.
(12) Uchida, K.; Sakai, K.; Ito, E.; Kwon, O. H.; Kikuchi, A.; Yamato, M.; Okano, T. *Biomaterials* **2000**, *21* (9), 923.
(13) Ratner, B. D.; Horbett, T.; Hoffman, A. S.; Hauschka, S. D. *J. Biomed. Mater. Res.* **1975**, *9* (5), 407.
(14) Tsuda, Y.; Kikuchi, A.; Yamato, M.; Sakurai, Y.; Umezu, M.; Okano, T. *J. Biomed. Mater. Res.* **2004**, *69A* (1), 70.
(15) Nishida, K.; Yamato, M.; Hayashida, Y.; Watanabe, K.; Maeda, N.; Watanabe, H.; Yamamoto, K.; Nagai, S.; Kikuchi, A.; Tano, Y.; Okano, T. *Transplantation* **2004**, *77* (3), 379.
(16) Shimizu, T.; Yamato, M.; Kikuchi, A.; Okano, T. *Biomaterials* **2003**, *13*, 2309.
(17) Harimoto, M.; Yamato, M.; Hirose, M.; Takahashi, C.; Isoi, Y.; Kikuchi, A.; Okano, T. *J. Biomed. Mater. Res.* **2002**, *62* (3), 464.
(18) Higuchi, A.; Hamamura, A.; Shindo, Y.; Kitamura, H.; Yoon, B. O.; Mori, T.; Uyama, T.; Umezawa, A. *Biomacromolecules* **2004**, *5*, 1770.
(19) Higuchi, A.; Tamiya, S.; Tsubomura, T.; Katoh, A.; Cho, C. S.; Akaike, T.; Hara, M. *J. Biomater. Sci., Polym. Ed.* **2000**, *11* (2), 149.
(20) Higuchi, A.; Takanashi, Y.; Tsuzuki, N.; Asakura, T.; Cho, C. S.; Akaike, T.; Hara, M. *J. Biomed. Mater. Res.* **2003**, *65A* (3), 369.
(21) Hara, M.; Adachi, S.; Higuchi, A. *J. Biomater. Sci., Polym. Ed.* **2003**, *14* (2), 139.
(22) Higuchi, A.; Yamamiya, S.; Yoon, B. O.; Sakurai, M.; Hara, M. *J. Biomed. Mater. Res.* **2004**, *68A* (1), 34.
(23) Higuchi, A.; Tsukamoto, Y. *J. Biomed. Mater. Res.* **2004**, *71A*, 470.
(24) Paunescu, V.; Suciu, E.; Tatu, C.; Plesa, A.; Herman, D.; Siska, I. R.; Suciu, C.; Crisnic, D.; Nistor, D.; Tanasie, G.; Bunu, C.; Raica, M. *J. Cell Mol. Med.* **2003**, *7* (4), 455.
(25) Unger, R. E.; Krump-Konvalinkova, V.; Peters, K.; Kirkpatrick, C. J. *Microvasc. Res.* **2002**, *64* (3), 384.
(26) Muller, A. M.; Hermanns, M. I.; Skrzynski, C.; Nesslinger, M.; Muller, K. M.; Kirkpatrick, C. J. *Exp. Mol. Pathol*. **2002**, *72* (3), 221.
(27) Mutin, M.; Dignat-George, F.; Sampol, J. *Tissue Antigens* **1997**, *50* (5), 449.
(28) Martin, S. J.; Reutelingsperger, C. P.; McGahon, A. J.; Rader, J. A.; van Schie, R. C.; LaFace, D. M.; Green, D. R. *J. Exp. Med.* **1995**, *182* (5), 1545.
(29) Nishida, K.; Yamato, M.; Hayashida, Y.; Watanabe, K.; Yamamoto, K.; Adachi, E.; Nagai, S.; Kikuchi, A.; Maeda, N.; Watanabe, H.; Okano, T.; Tano, Y. *New Engl. J. Med.* **2004**, *351* (12), 1187.

BM0494850