Transactions of the Materials Research Society of Japan 25 [4] 899-902 (2000)

Acceleration of Cell Sheet Recovery for Tissue Engineering

Oh Hyeong Kwon, Akihiko Kikuchi, Masayuki Yamato, and Teruo Okano
Institute of Biomedical Engineering, Tokyo Women's Medical University,
8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan
Fax: 81-3-3359-6046, e-mail: tokano@lab.twmu.ac.jp

Modulation of cell sheets might be an important technology to achieve construction of 3-dimensional tissue mimicking structure by piling up of detached cell sheets. Rapid detachment of cell sheets is very important not only to modulate but also to prevent them from functional damages. To accelerate the hydrophilic/hydrophobic structural change in response to temperature alteration, poly(*N*-isopropylacrylamide)(PIPAAm) was grafted on a porous membrane by electron beam irradiation. Analyses by attenuated total reflection-Fourier transform infrared (ATR-FTIR) and electron spectroscopy for chemical analysis (ESCA) revealed that PIPAAm was successfully grafted on the surface of porous membrane. In the experiment of cell sheet detachment, the tissue culture polystyrene (TCPS) dishes grafted with PIPAAm were used to compare with PIPAAm-grafted porous membrane. It needed about 75 min to detach cell sheet completely from PIPAAm-grafted TCPS dish, while only 30 min was enough to detach cell sheet in the case of PIPAAm-grafted porous membrane. It is considered that in the case of porous membrane, water molecules could be supplied from underneath as well as periphery of a cell sheet, resulting in rapid hydration of grafted PIPAAm molecules and detachment of cell sheet, while in the case of TCPS dish water molecules could be supplied from only periphery of a cell sheet.

Key words: cell culture, bovine aorta endothelial cells, poly(*N*-isopropylacrylamide), porous membrane, water supply

1. INTRODUCTION

Extensive researches have been performed in the design of intelligent polymer which shows response to stimuli such as pH,[1] temperature,[2] ionic strength,[3] electric field or current,[4] photoirradiation[5] for application to drug delivery or enzymology.

Among the many intelligent polymers, poly(*N*-isopropyl-acrylamide)(PIPAAm) shows soluble hydrophilic state below its lower critical solution temperature (LCST), but becomes insoluble hydrophobic state above its LCST. Because of its unique thermal property changes, it has been widely studied in the fields of separation,[6] drug delivery,[7] cell and tissue engineering,[8] and other switching devices.[9]

We have fabricated tissue culture polystyrene (TCPS) dishes grafted with poly(*N*-isopropylacrylamide) (PIPAAm) that show hydrophilic/hydrophobic surface property alterations in response to temperature changes.[10-12] Cultured cell sheets form on these surfaces, and we have successfully recovered intact, viable cell sheets to construct 3-dimensional tissue-like structures. In previous reports, we have described the recovery of bovine aortic endothelial cell sheets from PIPAAm-grafted TCPS surfaces by low temperature treatment.[11,12] However, cell sheet detachment from surfaces of TCPS grafted with PIPAAm is slow, occurring gradually from the sheet periphery toward the interior. Thus, significant time at reduced temperature is required to recover an intact cell sheet completely. Rapid recovery of cell sheets is considered important both to maintaining the biological function and viability of recovered cell sheets, as well as for practical assembly of tissue structures.

The rate limiting step to cell sheet recovery is the hydration of the underlying PIPAAm grafted surface. To accelerate the hydration of the hydrophobic PIPAAm segments bound to the cell sheet, use of a highly water permeable substrate to interface between the cell sheets and the polymer surface is optimal. As shown here, a grafted porous membrane substrate permits rapid 2-dimensional cell sheet manipulation by facilitating rapid water movement to the interface between cell sheets and membrane surfaces, producing cell sheet release.

2. MATERIALS AND METHODS

2.1 Materials

N-Isopropylacrylamide (IPAAm) was kindly given by Kohjin (Tokyo, Japan). Tissue culture grade polystyrene dishes (TCPS, Falcon 3001) and Cell Culture Insert™ (Falcon 3090) with a microporous poly(ethylene terephthalate) membrane (pore size = 0.45 μm) were purchased from Becton Dickinson Labware (Oxnard, CA, USA). Trypsin-EDTA solution, streptomycin, penicillin, and fungizone were bought from Gibco BRL (Grand Island, NY, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Iwaki (Chiba, Japan).

2.2 Preparation of PIPAAm-grafted porous membranes

The PIPAAm-grafted porous membranes used for single cell and cell sheet recovery were prepared as previously reported.[13] The TCPS dishes were cut to match the porous membrane size and PIPAAm-grafted TCPS dishes were prepared by the same method to compare to PIPAAm-grafted porous membranes.

2.3 Surface characterization

The amount of PIPAAm grafted onto TCPS dishes and porous membranes was determined by ATR-FTIR (JASCO Valor-III, Tokyo, Japan). The control substrates, polystyrene and porous poly(ethylene terephthalate) membranes, have strong absorption bands attributed to aromatic groups and ester bonds at 1600 and 1720 cm^{-1}, respectively. As PIPAAm was grafted onto each substrate, an amide absorption band appeared in the region of 1650 cm^{-1}. The peak intensity ratio ($I_{1650/1600}$ or $I_{1650/1720}$) was used to determine the amount of PIPAAm grafted on each surface using a calibration curve of known PIPAAm amount cast on TCPS from solution. PIPAAm-grafted porous membranes were also analyzed using ESCA (ESCA-200, Scienta, Uppsala, Sweden). Survey spectra were acquired of a take-off angle of 90° (sampling depth of ~90 Å) and surface elemental compositions were calculated from integrated peak areas for each element.

2.4 Water contact angle measurements

Four samples of each PIPAAm-grafted and -ungrafted TCPS dish and porous membrane were cut in size (1.5 x 0.8 cm) to measure water contact angles. Water contact angles were determined by the sessile drop method at 20 and 37°C with a FACE contact angle meter (Image processing type CA-X, Kyowa Interface Science, Saitama, Japan). All the samples were measured five times, and the averages were calculated. Water contact angle at 20 and 37°C were presented as a mean value and standard deviation.

2.5 Cell culture

Primary bovine aortic endothelial cells (BAECs) were obtained as previously described using dispase for cell dissociation, and cultured on TCPS dishes in DMEM supplemented with 10% FBS, 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL fungizone at 37°C in a humidified atmosphere with 5% CO_2. BAECs were recovered from ungrafted TCPS dishes by treatment with 0.25% trypsin-0.26 mM EDTA in PBS and subcultured on PIPAAm-grafted TCPS discs in TCPS dishes or on porous membranes. Cell morphology was photographed under phase-contrast microscopy (ET300, Nikon, Tokyo, Japan).

2.6 Detachment of single endothelial cells from PIPAAm-grafted surfaces

Detachment of single endothelial cells was achieved using low temperature treatment after incubation at 37°C for 2 hr. BAECs were plated on each surface at a density of 3 x 10^4 cells/cm^2 and cultured for 2 hr to allow attachment and spreading on each grafted surface. For low temperature treatment, spread cells were transferred to a CO_2 incubator equipped with a cooling unit fixed at 20°C. After 5, 15, 25, 35, and 50 min incubation, cell morphology was observed using a phase-contrast microscope and photographed. Both rounded and spread cells in the photographs were counted, and the percentage of rounded cells to the total cells counted were presented as a mean value (n=4) and standard deviation.

2.7 Detachment of confluent endothelial cell sheet from PIPAAm-grafted surfaces

BAECs were plated onto each surface at a density of 1.3 times confluency (1.3 x 10^6 cells/dish) and cultured at 37°C. After 24 hr incubation, unattached cells were removed by medium exchange. Cell sheets were cultured for 8 days after reaching confluency, and each plate was transferred to the CO_2 incubator fixed at 20°C, and periodically removed to acquire photographs during detachment. The photographs were scanned into a computer system for analysis. Software (NIH Image ver. 1.61) was used to measure the area of each detached cell sheet. Areas of detached cell sheets relative to *in situ* confluently cultured cell sheet area were calculated and averaged from four photographs of each sample.

3. RESULTS AND DISCUSSION

3.1 Surface characterization

Results of elemental analysis determined by ESCA, and amounts of PIPAAm grafted on both TCPS surfaces and porous membranes are presented in Table I.

Table I. Amount of grafted PIPAAm and surface chemical composition of the PIPAAm-grafted and -ungrafted surfaces determined by ATR-FTIR and ESCA, respectively

Substrate	Grafted PIPAAm (μg/cm^2)	Atom (%)		
		C	O	N
TCPS dish (control)		87.85	10.85	1.30
PIPAAm-TCPS dish	1.6 ± 0.2	85.77	7.78	6.45
Porous membrane (control)		73.17	25.93	0.90
PIPAAm-porous membrane	1.4 ± 0.2	73.69	21.27	5.04

Increased atomic percent nitrogen was observed on both PIPAAm-grafted TCPS dishes and porous membranes after electron beam irradiation. Because each ungrafted control TCPS or porous membrane surface does not contain nitrogen in their chemical structures, these results directly support PIPAAm grafting onto each surface by electron beam irradiation.

Table II shows water contact angle data from each surface using the sessile drop method at 20°C and 37°C. PIPAAm-grafted surfaces exhibited decreasing contact angles by lowering temperature from 37 to 20°C, while control surfaces had negligible contact angle changes with changing temperature. This result indicates that PIPAAm surfaces, hydrophobic at the

higher temperature, became markedly more hydrophilic in response to temperature reduction due to spontaneous hydration of surface-grafted PIPAAm.[10-12]

Table II. Contact angles of the PIPAAm-grafted tissue culture polystyrene (TCPS) dish and porous membrane estimated by different methods

Substrate	Sessile drop method (°)	
	20°C	37°C
Porous membrane (control)	55.2 ±2.2	55.1 ±3.1
PIPAAm-porous membrane	45.4 ±3.2	56.0 ±1.3
TCPS dish (control)	66.2 ±0.8	66.4 ±3.0
PIPAAm-TCPS dish	54.0 ±1.5	66.0 ±2.2

By lowering temperature, such a hydrophobic/hydrophilic polymer change and polymer matrix swelling induce the detachment of cultured cells from PIPAAm-grafted surfaces.[10]

3.2 Single cell detachment

After short culture time, seeded BAECs were well attached and spread on both PIPAAm-grafted and ungrafted surfaces. After 1 hr incubation at 37°C, more than 90 percent of seeded cells were attached and spread on both surfaces, despite the porosity of the membrane surface. Cells cannot sense distances longer than the average length of a pseudopodium.[14] In this study, porous membranes had a pore size of 0.45 μm in diameter, much smaller than a single cell.

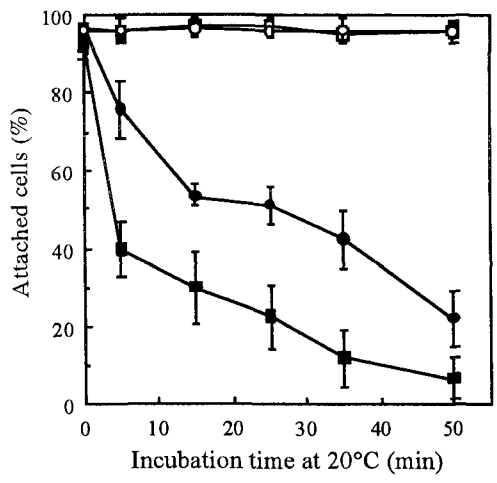

Fig. 1. The percentage of still attached single cells on control TCPS surfaces (□), control porous membrane surfaces (○), PIPAAm-grafted TCPS surfaces (●), and PIPAAm-grafted porous membrane surfaces (■) as a function of incubation time in culture medium at 20°C.

Cells appeared to override these surface discontinuities produced by track-etched pores without difficulty. Fig. 1 shows the percentage of still attached single cells on both PIPAAm-grafted TCPS dishes and porous membranes as a function of lower culture temperature treatment time. Spread cells detached from both surfaces at 20°C, but cells cultured on the grafted porous membrane detached more rapidly than those on grafted TCPS dishes. On PIPAAm-grafted TCPS dishes, water required to hydrate PIPAAm at lower temperature can readily penetrate the culture matrix from only the periphery of each cell to the interface between the cell and grafted PIPAAm chains. On porous membranes, water hydration of PIPAAm is supplied through pores underneath adherent cells as well as from the periphery of each cell. Ready, rapid access of bulk water to PIPAAm grafts through pores beneath attached cells should accelerate single cell detachment.

3.3 Cell sheet detachment

In previous reports,[11,12] we have described the recovery of bovine aortic endothelial cell sheets from PIPAAm-grafted TCPS surfaces by low temperature treatment. However, cell sheets formed on PIPAAm-grafted TCPS surfaces detach slowly and gradually, beginning from sheet edges and moving toward the cell sheet interior, presumably because water penetrates the interface between cell sheets and dish surfaces only from the edge. Time required to recover cell sheets completely from TCPS surfaces grafted with PIPAAm is therefore impractical.

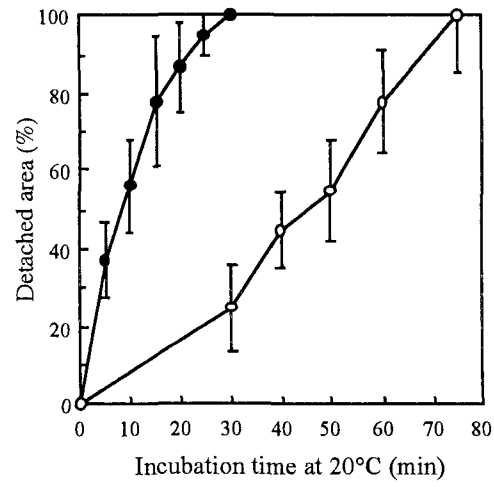

Fig. 2. Average detached areas determined for cell sheets recovered from both PIPAAm-grafted TCPS surfaces (○) and PIPAAm-grafted porous membrane surfaces with culture area of 4.2 cm^2 (●) as a function of incubation time in culture medium at 20°C.

Results shown in Fig. 1 suggest the possibility to achieve rapid 2-dimensional cell sheet detachment

using PIPAAm-grafted porous membranes through which water is supplied both from underneath as well as the periphery of a cell sheet, resulting in rapid hydration of grafted PIPAAm molecules.

Fig. 2 shows changes in detached area of cell sheets on both PIPAAm-grafted TCPS and microporous membranes as a function of reduced temperature treatment time. Cell sheets are observed to detach more rapidly from the porous membranes (~30 min.) than TCPS surfaces (~75 min.) with culture area of 4.2 cm^2 under quiescent culture conditions and reduced culture temperature. These results strongly support that accelerated detachment of cell sheets is due to the existence of pores on membrane surface.

Recovered viable, large cell sheets might prove useful for application to tissue restoration, as a transplant material, or for construction of artificial organs. However, time scales required to detach cell sheets from TCPS culture surfaces will scale exponentially with increasing culture size. Extended low temperature treatment necessary for larger sheet recovery might have negative effects on cell functions. Thus, rapid detachment and recovery methods are optimal to maintain cell phenotype and biological functions, especially for large cell sheets. Porous membranes grafted with thermosensitive PIPAAm are shown capable of achieving such rapid 2-dimensional cell sheet detachment.

ACKNOWLEDGEMENTS

The present work was supported by the Japan Society for the Promotion of Science, "Research for the Future" Program (JSPS-RFTF96I00201). The authors appreciate Dr. Y. Iwasaki, Tokyo Medical and Dental University, for his assistance with and discussions about ESCA measurements, and Prof. D. W. Grainger (Colorado State University) for technical discussions.

REFERENCES

1) N. A. Peppas and J., Klier, *J Control. Rel.*, **1 6**, 203-214 (1991).
2) R. Yoshida, K. Sakai, T. Okano, Y. Sakurai, Y. H. Bae, and S. W. Kim, *J Biomater. Sci. Polymer Edn.*, **3**, 155-162 (1991).
3) P. S. Stayton, T. Shimoboji, C. Long, A. Chilkoti, G. Chen, J. M. Harris, and A. S. Hoffman, *Nature* **3 7 8**, 472-474 (1995).
4) S. R. Eisenberg and A. J. Grodzinsky, *J Membrane Sci.*, **1 9**, 173-194 (1984).
5) Y. Ito, N. Sugimura, O. H. Kwon, and Y. Imanishi, *Nat. Biotechnol.*, **1 7**, 73-75 (1999).
6) Y. G. Takei, T. Aoki,, K. Sanui, N. Ogata, T. Okano, and Y. Sakurai, *Bioconjugate Chem.*, **4**, 341-348, (1993).
7) R. Yoshida, K. Sakai, T. Okano,, Y. Sakurai, *Adv. Drug Delivery Rev.* **1 1**, 85-108 (1993).
8) H. A. von Recum, S. W. Kim, A. Kikuchi, M. Okuhara, Y. Sakurai, and T. Okano, *J Biomed. Mater. Res.*, **4 0**, 631-639, (1998).
9) T. Okano and R. Yoshida, "Intelligent Polymeric Materials for drug Delivery," in *Biomedical Application of Polymeric Materials*, Ed. by T. Tsuruta, T. Hayahi, K. Kataoka, K. Ishihara, and Y. Kimura, CRC Press, Boca Raton, FL., (1993) pp. 407-428.
10) N. Yamada, T. Okano, H. Sakai, F. Karikusa, Y. Sawasaki, and Y. Sakurai, *Makromol. Chem. Rapid Commun.*, **1 1**, 571-576 (1990).
11) A. Kikuchi, M. Okuhara, F. Karikusa, Y. Sakurai, and T. Okano, *J Biomater. Sci. Polymer Edn.*, **9**, 1331-1348 (1998).
12) A. Kushida, M. Yamato, C. Konno, A. Kikuchi, Y. Sakurai, and T. Okano, *J Biomed. Mater. Res.*, **4 5**, 355-362 (1999).
13) O. H. Kwon, A. Kikuchi, M. Yamato, Y. Sakurai, and T. Okano, *J Biomed. Mater. Res.*, in press.
14) A. Curtis, C. Wilkinson, *Biomaterials* **1 8**, 1573-1583 (1997).

(Received December 16, 1999; Accepted September 30, 2000)