Available online at www.sciencedirect.com
ScienceDirect
Acta Biomaterialia 3 (2007) 1024–1032
Acta BIOMATERIALIA
www.elsevier.com/locate/actabiomat

Surface modification of plastic, glass and titanium by photoimmobilization of polyethylene glycol for antibiofouling

Yoshihiro Ito [a,b,*], Hirokazu Hasuda [a], Makoto Sakuragi [a], Saki Tsuzuki [a]

[a] *Kanagawa Academy of Science and Technology, KSP East 309, 3-2-1 Sakado, Takatsu-ku, Kawasaki 213-0012, Japan*
[b] *Nano Medical Engineering Laboratory, RIKEN (The Institute of Physical and Chemical Research), 2-1 Hirosawa, Wako, Saitama, 351-0198, Japan*

Received 21 December 2006; received in revised form 19 May 2007; accepted 21 May 2007
Available online 29 June 2007

Abstract

Photoreactive poly(ethylene glycol) (PEG) was prepared and the polymer was photoimmobilized on organic, inorganic and metal surfaces to reduce their interaction with proteins and cells. The photoreactive PEG was synthesized by co-polymerization of methacrylate-PEG and acryloyl 4-azidobenzene. Surface modification was carried in the presence and the absence of a micropatterned photomask. It was then straightforward to confirm the immobilization using the micropatterning. Using the micropatterning method, immobilization of the photoreactive PEG on plastic (Thermanox™), glass and titanium was confirmed by time-of-flight secondary ion mass spectroscopy and atomic force microscopy observations. The contact angle on an unpatterned surface was measured. Although the original surfaces have different contact angles, the contact angle on PEG-immobilized surfaces was the same on all surfaces. This result demonstrated that the surface was completely covered with PEG by the photoimmobilization. To assess non-specific protein adsorption on the micropatterned surface, horseradish peroxidase (HRP)-conjugated proteins were adsorbed. Reduced protein adsorption was confirmed by vanishingly small staining of HRP substrates on the immobilized regions. COS-7 cells were cultured on the micropatterned surface. The cells did not adhere to the PEG-coated regions. In conclusion, photoreactive PEG was immobilized on various surfaces and tended to reduce interactions with proteins and cells.

Keywords: Bioinert surface; Biomaterials; Non-fouling surface; Photopatterning; Surface modification

1. Introduction

Preventing molecular, cellular and organismic fouling of surfaces is important for the success of medical or biochemical devices. A variety of polymers have been synthesized to reduce protein, cell and bacterial adsorption at interfaces with biological tissues. Among them, one approach that has met with considerable success is surface modification with poly(ethylene glycol) (PEG), a biocompatible polymer that, when immobilized onto surfaces, confers protein and cell resistance [1]. Therefore, attempts at PEG immobilization have been made by many researchers. For example, Rundqvist et al. [2] and Mougin et al. [3] prepared a self-assembled layer of thiol-terminated PEG on gold. Sebra et al. [4], Ko et al. [5], Beyer et al. [6] and Kizilel et al. [7] employed polymerizable PEG monoacrylate. Sun et al. [8] reported ω-PEG carrying alkyne and cyclodiene terminal groups onto an *N*-(ε-maleimidocaproyl) functionalized glass slide via an aqueous Diels–Alder reaction. Shlapak et al. [9], Feng et al. [10] and Patel et al. [11] utilized a PEG-amine for coupling with poly(*N*-hydroxysuccinimidyl methacrylate) films, hydrolyzed poly(methyl methacrylate) or silanized glass slides bearing aldehyde groups, respectively. Bonding of PEG-biotin derivatives onto an avidin surface was performed by Sinclair and Salem [12] and Zhen et al. [13]. PEG-silane was employed for surface modification by Popat et al. [14], Piehler et al. [15], Xu et al. [16], Choi

* Corresponding author. Address: Nano Medical Engineering Laboratory, RIKEN (The Institute of Physical and Chemical Research), 2-1 Hirosawa, Wako, Saitama, 351-0198, Japan. Tel.: +81 48 467 9302; fax: +81 48 467 9300.
E-mail address: y-ito@riken.jp (Y. Ito).

1742-7061/$ - see front matter © 2007 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.actbio.2007.05.010

et al. [17], Xu et al. [18] and Jun et al. [19] immobilized PEG derivatives on poly(acrylonitrile-*co*-maleic acid), polyurethane and Si(1 1 1), respectively. Groll et al. [20–22] prepared isocyanate-terminated star PEG for ultrathin coatings.

Existing immobilization strategies often require the presence of specific surface functional groups and extensive optimization, and they have a limited capacity to be used for modification of a variety of materials. Thus, there exists an ongoing need for versatile immobilization strategies that are capable of robustly anchoring PEG and other antifouling polymers onto a variety of medically relevant material surfaces. For this purpose, Dalsin et al. [23,24] employed a strategy using the adhesive characteristics of 3,4-dihydroxyphenylalanine, an important component of mussel adhesive proteins, to anchor PEG onto surfaces. On the other hand, Weber et al. [25] synthesized 1-aziglycoses, which are glycose-containing diazirines. Because of the low pK_a value and the expected weak nucleophilicity of the hydroxyl groups on the surface of oxidized titanium, 1-aziglycoses modified the titanium. The chemicals generated singlet carbenes that were readily inserted into H–O bonds, leading to the glycosidation of titanium. Photoimmobilization methods are also useful for universal anchoring.

We have employed a photoimmobilization technique, following that used by Matsuda and Sugawara [26], to immobilize growth factors [27], cytokines [28], sulfated hyaluronic acid [29], heparin [30], thermoresponsive polymer [31], β-galactose derivative [32], phosphatidylcholine derivative [33] and pullulan [34], and recently to microarray proteins and cells [35]. This photoimmobilization method is very useful because it is applicable to various materials. In addition, by using photolithography micropatterning, direct comparison between immobilized and non-immobilized surfaces is possible, resulting in the surface patterning of proteins and cells according to the properties of the micropatterned polymers. Although thin hydrogel formation by iniferter-based photopolymerization of dithiocarbamylated PEGs under UV irradiation or photopolymerization of monoacryloyl PEG has been reported by Lee et al. [36], Kwon and Matsuda [37] and Hahn et al. [38], surface modification by photocross-linking has not been reported.

In this study, PEG was chosen as a new material for photocross-linking immobilization on inorganic, organic and metal surfaces, and the subsequent interactions with proteins and cells was investigated. It was expected that the hydrated non-ionic surface would reduce interaction with biocomponents. Photocross-linking immobilization is generally useful in preparing micropatterned surfaces compared with the photopolymerization method, because the former uses a dry process.

2. Materials and methods

2.1. Materials

Polymethacryl–PEG with a molecular weight of about 360 was purchased from Aldrich, and used without further purification. Circular plates of plastic (Thermanox™, which is a polyester), 15 mm in diameter, were purchased from Nunc. Glass plate was purchased from Nikkyo Technos Co. Titanium-coated glass plate was prepared by sputtering of titanium (pressure, 2×10^{-5} Pa; gas, Ar) in Ulvac Inc. The bare and titan-coated glass plate was cleaned by VUV irradiation for 2 min.

2.2. Synthesis of acryloyl 4-azobenzene

Azidoaniline hydrochloride (500 mg, 2.9 mmol) was dissolved in MilliQ water (100 ml) and sodium carbonate (466 mg, 4.4 mmol) was added to the mixture to make the pH of solution 10. Methacryloyl chloride (460 mg, 4.4 mmol) in dioxane (10 ml) was added dropwise to the azidoaniline solution. Subsequently, the solution was allowed to stand for 2 h in the dark. After the reaction, the precipitate formed was recovered by filtration, washed with MilliQ water and dried (425 mg). The yield was 70.8% (57625-92-D). ^{1}H NMR (300 MHz, in $CDCl_3$), *d* (TMS, ppm): 7.56, 7.55 (dd, 2H, Bn-H), 7.50 (Br s, 1H, NH), 7.01, 6.98 (dd, 2H, Bn-H), 5.79, 5.48 (m, 2H, $=CH_2$), 2.06 (s, 3H CH_3).

2.3. Preparation of photoreactive PEG

Polymethacryl–PEG (3.4 g, 9.4 mmol) was mixed with acryloyl-4-azobenzene (100 mg, 0.5 mmol) and azobisisobutyronitrile (27.9 mg, 0.17 mmol) in ethanol (15 ml); the solution was bubbled with nitrogen gas for 20 min and then allowed to stand for 18 h at 60 °C. After removing the ethanol, the product was dialyzed with water and freeze dried. The yield was 2.7 g (78%). This copolymer is referred to as photoreactive PEG (Fig. 1).

2.4. Gel permeation chromatography and spectroscopic measurements

The photoreactive PEG was assayed by gel permeation chromatography (GPC) using TOSOH α-M column at 20 °C. Pure water (MilliQ water, pH 7.3) was used as an elution solvent. Detection was by using the refractive index. UV measurement was performed using a JASCO V-550 spectrophotometer.

2.5. Surface modification

Micropatterning was performed as follows. An aqueous solution of photoreactive PEG (0.1 wt.%) was cast on a substrate and air dried at room temperature. Subsequently, the plate was irradiated with UV light from a UV lamp (UV Spot Light Source L5662, Hamamatsu Photonics) at a distance of 5 cm for 10 s (16 mW cm^{-2}) with or without a photomask (Toppan Printing Co.). The plate was then repeatedly washed with distilled water.

The swelling ratio was determined as follows. The photoreactive PEG was cast on Thermanox™ and dried.

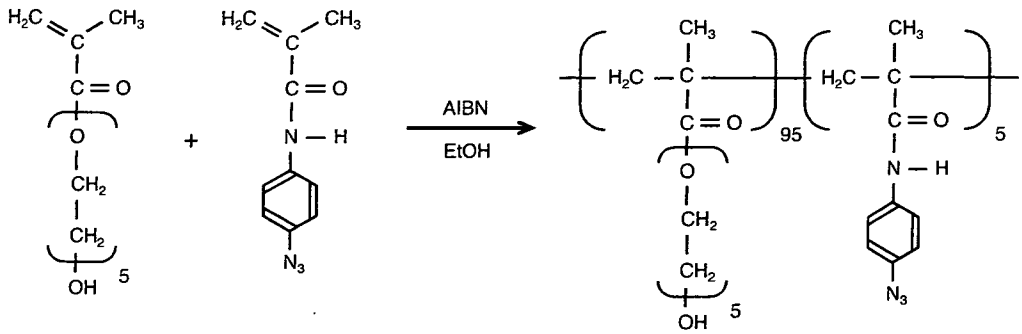

Fig. 1. Synthetic scheme for producing photoreactive PEG.

Subsequently the dried plate was photoirradiated. The plate was then incubated in water and the weight change measured.

2.6. Surface characterization

Contact angle measurement was performed as previously reported [30]. The unpatterned sample was placed on the stage of a CA-W Automatic Contact Angle Meter (Kyowa Interface Co. Ltd.) and a drop of water (0.1 μl) was put on the sample surface. The contact angle of the drop on the surface was measured at room temperature. At least 10 angles were measured at different areas and averaged.

Measurement of time-of-flight secondary ion mass spectrometry (TOF-SIMS) was carried out by using a TFS-2000 (Physical Electronics). The primary ion was $^{69}Ga^{+}$; the accelerating voltage of the ion gun was 25 kV, the pulse width 12 ns, the pulse frequency 8.3 kHz, the range of mass 0–1000 amu and the resolution of time 1.1 ns ch^{-1}.

Atomic force microscopic (AFM) observation was performed using a Nanoscope IV (Digital Instruments Inc.). The micropatterned sample was set in a cell holder into which water could be injected. After observation of the dry sample, distilled water was injected into the sample cell and the same position was observed. The measurement was performed using the tapping mode with a nominal force constant of 0.09 N m^{-1}.

2.7. Protein adsorption

Polyclonal rabbit anti-mouse antibodies conjugated with horseradish peroxidase (HRP-IgG) were purchased from Dako Cytomation and used as representative proteins. The plates were soaked in the protein solution (0.5 mol ml^{-1} diluted in phosphate-buffered saline (PBS)) for 30 min at 37 °C, then washed sequentially with PBS and distilled water. Adsorbed proteins were detected by HRP activity. A 3,3,5,5-tetramethyl benzidine (TMB) peroxidase substrate kit was purchased from Vector Laboratories and used to stain the plates. The blue staining of TMB was observed by phase-contrast microscopy.

HRP-conjugated bovine serum albumin (HRP–BSA, purchased from Rockland) was also used for an adsorption experiment. A 3 μl aliquot of HRP–BSA (50 and 100 ng ml^{-1}) was added to the samples and allowed to stand for 1 h. After washing with PBS, the chemiluminescence from the sample surface was measured using an ECL Advance Western blotting detection kit purchased from Amersham Bioscience. Calibration was performed using HRP–BSA solutions of known concentrations.

2.8. Cell culture

COS-7 cells with epithelial cell morphology growing as monolayers and African green monkey kidney derived from CV-1, a simian cell line (*Cercopithecus aethiops*), were purchased from the Riken Cell Bank and cultured in Dulbecco's modified Eagle's medium (Sigma) containing 10% fetal bovine serum. The cultured cells were recovered by treatment with PBS containing 0.25 wt.% trypsin and 0.9 mM ethylenediaminetetraacetic acid. The recovered cells were washed with the culture medium and finally suspended in this medium (1.8×10^5 cells per well of a 12-well plate). The cell suspension was added to sample plates, which had been sterilized with 70% ethanol. The cells were incubated at 37 °C under 5% v/v of CO_2 for 19 h, and were observed by phase-contrast microscopy.

3. Results and discussion

3.1. Synthesis of photoreactive PEG

GPC chromatograms of photoreactive PEG are shown in Fig. 2. A significant increase of molecular weight was observed for the photoreactive PEG. Its molecular weight was estimated to be around 8100, after calibration with polyethylene glycol standards.

The UV spectrum of photoreactive PEG is shown in Fig. 3. Absorption at 276 nm, attributable to the azidophenyl group, was observed. The absorption was red shifted by 20 nm from the corresponding absorption of 4-azidoaniline. This shift may be due to electron delocalization of the azidophenyl group caused by amide bond formation. In previous studies, the peaks of photoreac-

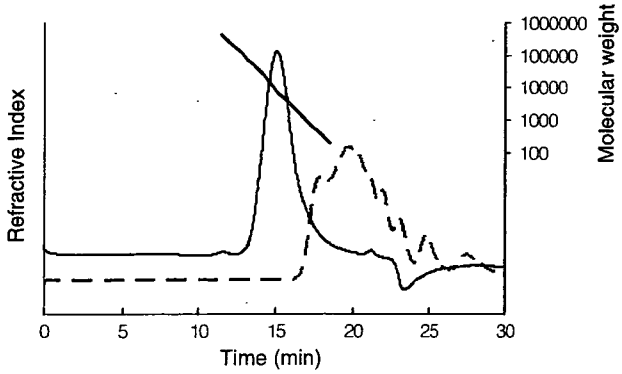

Fig. 2. Gel permeation chromatography of polymethacryl–PEG (broken line) and photoreactive PEG (solid line).

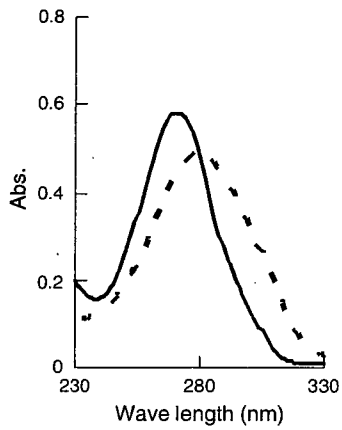

Fig. 3. UV spectrum of 4-azidoaniline hydrochloride (9.2 μg ml^{-1}, broken line) and photoreactive PEG (0.1 wt.% (the content of azidophenyl groups is 10.5 μg ml^{-1}), solid line).

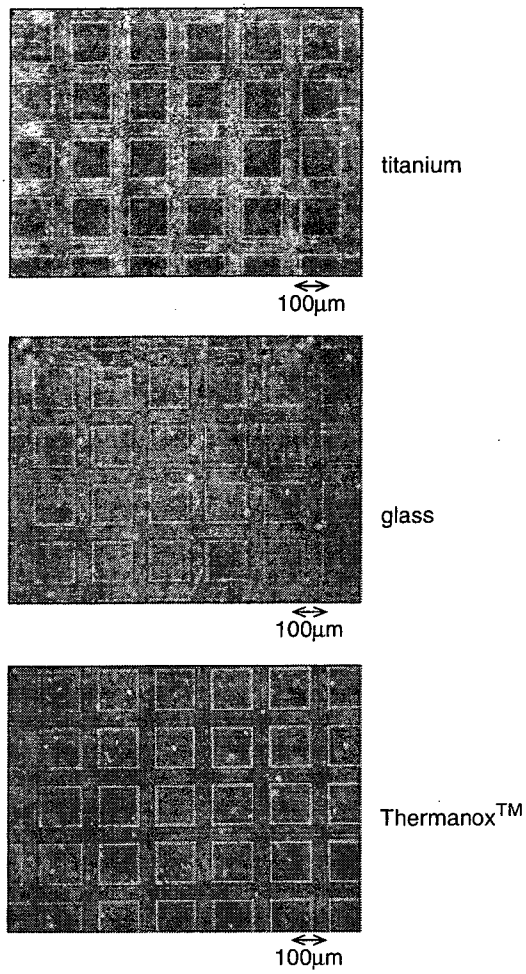

Fig. 4. Phase-contrast micrographs of micropatterned titanium, glass and Thermanox™ surfaces.

tive gelatin, hyaluronic acid, and heparin were also red shifted from azidobenzoic acid [28–30]. However, the shifted wavelength was the highest among the photoreactive biopolymers.

Assuming that the molecular absorption coefficient of the azidophenyl group at 276 nm was the same as that of azidoaniline at 256 nm, the content of azidophenyl groups in the photoreactive PEG was calculated to be 3.3 wt.%.

3.2. Photoimmobilization

Photoreactive PEG was coated onto the glass, titanium and plastic plates, and the coated surface was UV-irradiated in the presence of a photomask (Fig. 4). The wet polymer contained 76 ± 2% water. The micropatterns on three types of surfaces were identical to that of the photomask. This demonstrated that the surfaces could be modified by photoreactive PEG. The cast photoreactive PEG formed intra- and intermolecular networks and bonded with the surfaces because of the presence of these radical groups.

The micropattern was also confirmed by TOF-SIMS. In the spectrum of positive secondary ion, peaks of $^{31}CH_3O^+$, $^{45}C_2H_5O^+$, $^{59}C_3H_7O^-$ and $^{89}C_4H_9O_2^+$, which were attributed to organic materials, were observed. In the spectrum of negative secondary ions, peaks of $^{43}C_2H_3O^-$, $^{55}C_3H_3O^-$, $^{59}C_2H_5O_2^-$ and $^{85}C_4H_5O_2^-$, which were attributed to organic materials, were observed. These fragments were observed in the lattices on titanium, glass and plastic surfaces as the $C_2H_5O^+$ ion image of Fig. 5. No signal of organic materials between the lattices of titanium surfaces was detected. Because Thermanox™ is a polyester, there are some signals of $C_2H_5O^+$. However, the intensity from immobilized PEG was higher than on non-immobilized areas. On glass surfaces, the contrast was between titanium and Thermanox™. On the other hand, there were no signals of PEG detected from the areas non-irradiated on titan, glass and Thermanox™, by Ti^+, $Si_2O_5H^-$ and $C_7H_4O^+$, respectively. This result shows the formation of defect-free PEG layers.

In addition, the thickness of the micropattern-immobilized PEG was investigated by AFM, as shown in Fig. 6.

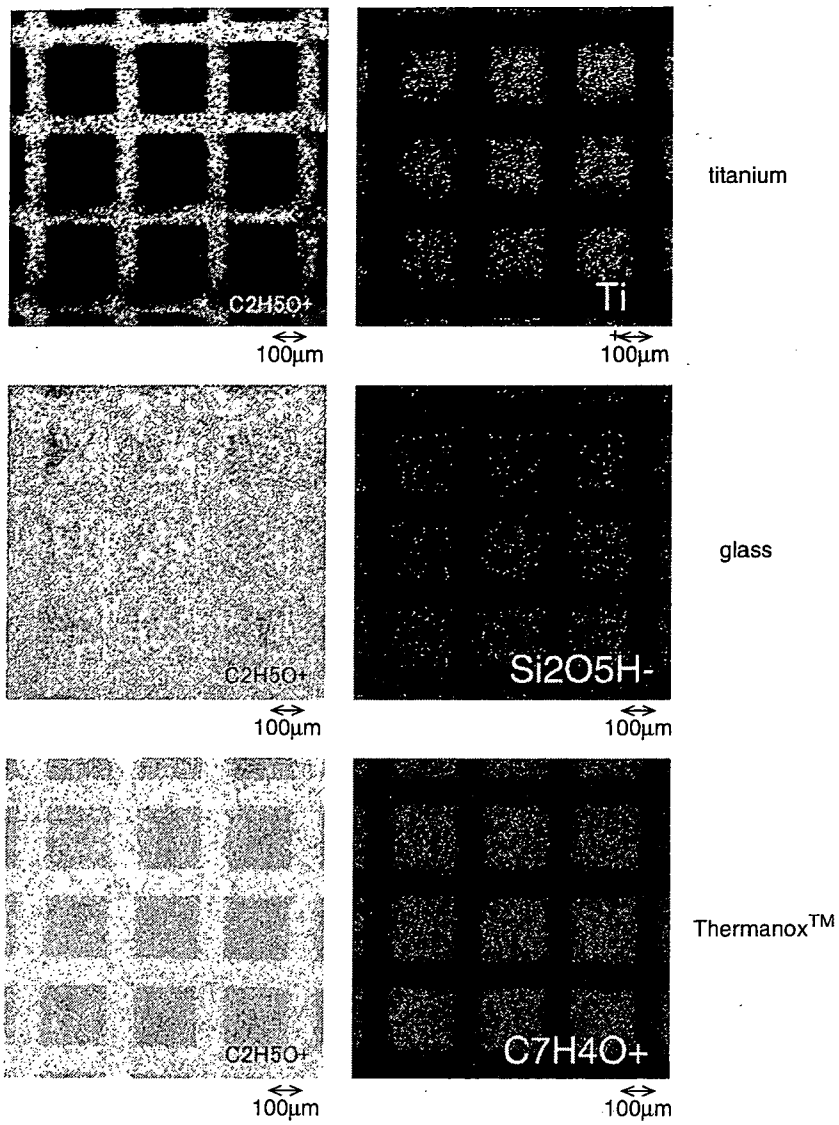

Fig. 5. TOF-SIMS image of PEG-micropatterned titanium, glass and Thermanox™.

The thickness (several hundred nanometers) was similar to that formed by the same amount of other photoreactive polymers that we prepared [33,39].

The surface properties of the immobilized photoreactive PEG without using a photomask were investigated by contact angle measurements, as shown in Table 1. Although the original polymer or modified glass had different contact angles, the photoreactive PEG-immobilized surfaces had almost the same contact angle. These results indicated that the surfaces were completely covered using the photoimmobilization method.

Photoimmobilization of photoreactive polymers on plastic materials has been previously reported [26–31, 33–35]. The phenyazide groups in the photoreactive polymers were photolyzed to generate highly reactive nitrene, which spontaneously formed intra- and intermolecularly bonds with neighboring organic materials.

Weber et al. [25] synthesized 1-aziglycoses, which are glycose-containing diazirines. Because of the low pK_a value and the expected weak nucleophilicity of the hydroxyl groups on the surface of oxidized titanium, 1-aziglycoses were used to modify the titanium. Although they did not show any evidence for a direct bond between the glucose and titanium, it was considered that the chemicals generated singlet carbenes that were readily inserted into H–O bonds, leading to the glycosidation of titanium. Considering that the concentration of photoreactive groups is very low and that bonds will be formed only at the surface of the substrate, it is very difficult to detect the bonds. Recently Adden et al. [40] developed a bifunctional copolymer of (4-vinylbenzyl)phosphoric acid diethylester and *N*-acryloxysuccinimide that is bound to a titanium surface. However, they detected no direct chemical bond between the phosphoric acid and titanium by XPS.

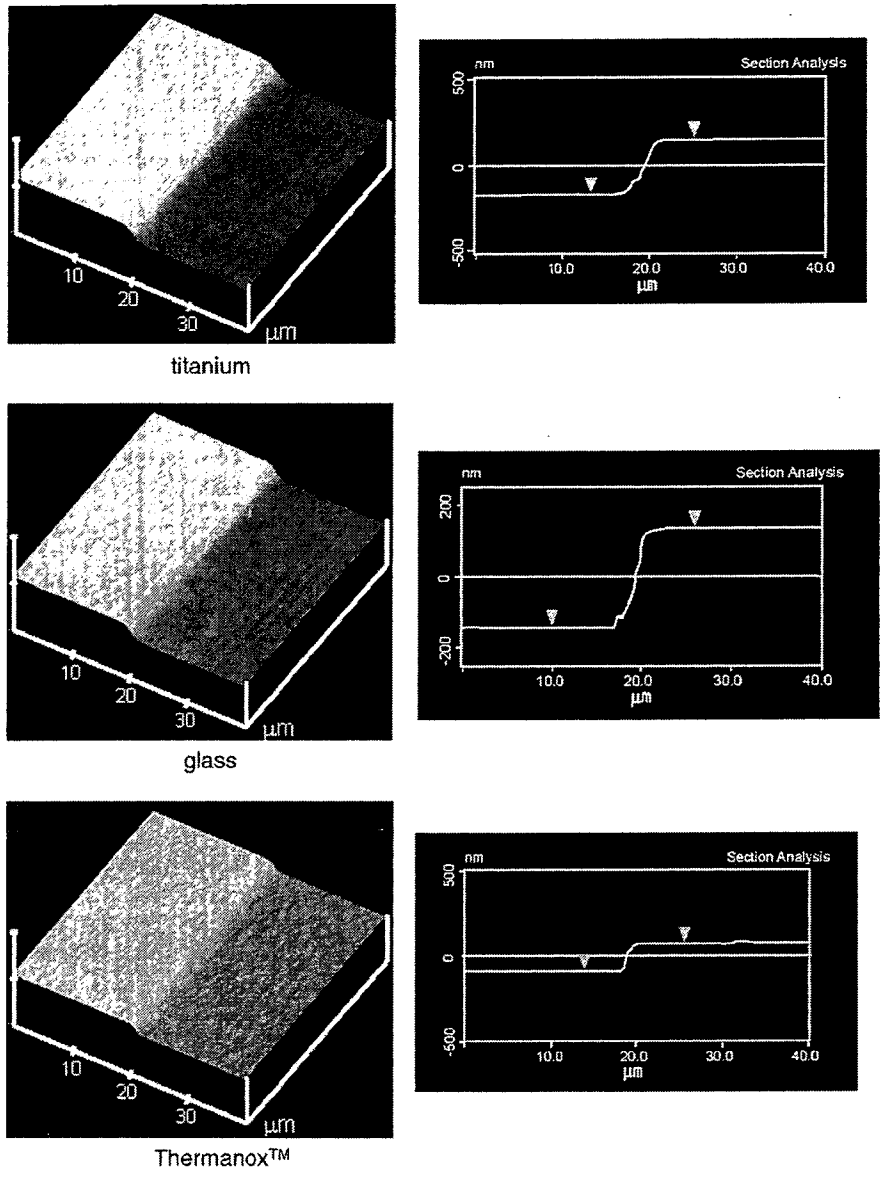

Fig. 6. AFM images of PEG micropatterned on titanium, glass and Themanox™.

Table 1
Contact angles on surfaces

Surface	Contact angle (°)
Titanium	0[a]
Titanium with photoreactively immobilized PEG	39.2 ± 1.3
Glass	6.4 ± 1.2
Glass with photoreactively immobilized PEG	40.9 ± 0.7
Thermanox™	78.8 ± 0.7
Thermanox™ with photoreactively immobilized PEG	41.0 ± 2.7

[a] The value was reported in a previous report [44].

Therefore, we performed micropatterning. If a micropattern was formed, we considered that photoimmobilization occurred. Without photoirradiation, no PEG was found by TOF-SIMS and AFM on the surface where there was no irradiation. It was considered that aryl azide derivatives form short-lived nitrenes that react extremely rapidly with the surrounding chemical environment [41]. Recent evidence, however, indicates that photolyzed intermediates of aryl azides can undergo ring expansion to create nucleophile-reactive dehydroazepines [41].

3.3. Protein adsorption

To assess the non-specific adsorption of protein on immobilized PEG surfaces, micropatterned plates of titanium-coated glass, glass and plastics were soaked in HRP-conjugated antibody solution. Adsorbed proteins

were detected by their HRP activity. Blue staining by HRP substrates was clearly observed on the non-PEG immobilized region of each surface and the staining was vanishingly small on the PEG photoimmobilized regions, as shown in Fig. 7.

Protein adsorption was investigated using another protein, albumin (BSA), which is smaller than the antibody and the amount of adsorbed protein was quantitatively determined. As shown in Fig. 8a, the adsorption was significantly reduced by the immobilization of PEG. The reduction effect is quantitatively evaluated and shown in Fig. 8b.

3.4. Cell adhesion

Adhesion of COS-7 cells on the micropatterned surfaces is shown in Fig. 9. Taking into consideration previous reports, an incubation time of 19 h was considered sufficient to allow comparison of the immobilized PEG and uncoated surfaces [42,43]. No cell adhesion was observed on the PEG-immobilized regions. It is known that COS-7 cells have epithelial cell properties and adhere to various materials [42,43]. However, immobilized PEG inhibited the adhesion of these very sticky cells. As the PEG-immobilized surface formed a hydrophilic diffused layer, it was thus considered to reduce cell adhesion, as do conventional hydrogel surfaces, which also reduce interactions with cells [33,34]. Employment of photoreactive PEG is therefore considered a new method for the preparation of bioinert surfaces, as it can reduce interactions with biological proteins and cells.

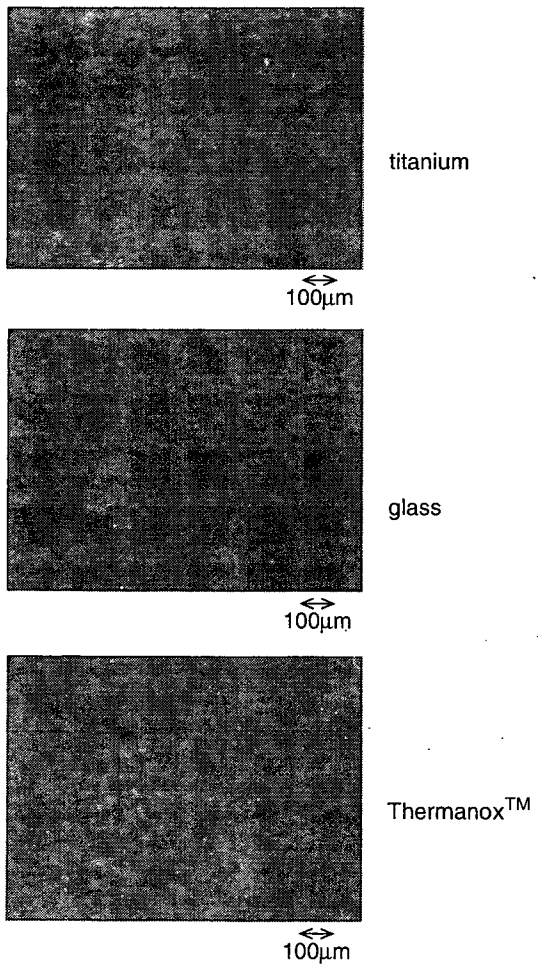

Fig. 7. Protein adsorption using HRP-IgG on PEG-micropatterned titanium, glass, and Thermanox™.

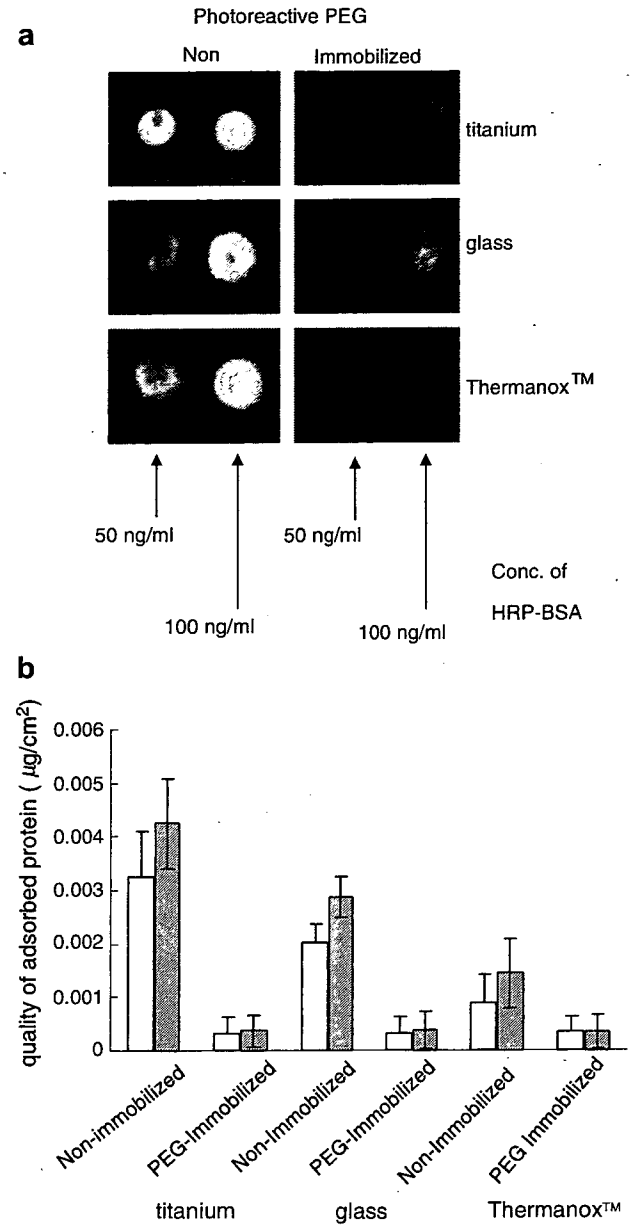

Fig. 8. (a) Chemiluminescence of HRP–BSA on non-immobilized and PEG-photoimmobilized titanium, glass, and Thermonox™. (b) The amount of HRP–BSA on non-immobilized and PEG-photoimmobilized titanium, glass, and Thermonox™. Open and closed columns indicate 50 and 100 ng ml^{-1} of protein concentrations, respectively.

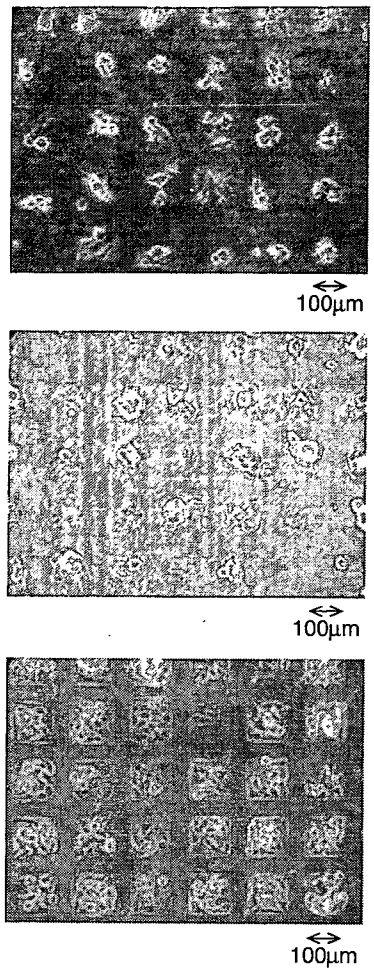

Fig. 9. Adhesion of COS-7 cells on micropattern photoimmobilized PEG on titanium, glass and Thermanox™.

4. Conclusions

This study demonstrated photoimmobilization of PEG onto various types of surfaces, including metal, glass and plastics. The immobilization technique is very useful for surface modification because of its convenience. In addition, micropatterning was achieved by the immobilization technique. The modified surface significantly reduced the interaction with proteins. In addition, the modified surface stably reduced cell adhesion in culture.

References

[1] Ratner BD, Hoffman AS. Nonfouling surfaces. In: Ratner BD, Hoffman AS, Schoen FJ, Lemons JE, editors. Biomaterials science. 2nd ed. San Diego, CA: Elsevier Academic Press; 2004. p. 197–201.
[2] Rundqvist J, Hoh JH, Haviland DB. Directed immobilization of protein-coated nanospheres to nanometer-scale patterns fabricated by electron beam lithography of poly(ethylene glycol) self-assembled monolayers. Langmuir 2006;22:5100–7.
[3] Mougin K, Ham AS, Lawrence MB, Fernandez EJ, Hillier AC. Construction of a tethered poly(ethylene glycol) surface gradient for studies of cell adhesion kinetics. Langmuir 2005;21:4809–12.
[4] Sebra RP, Masters KS, Cheung CY, Bowman CN, Anseth KS. Detection of antigens in biologically complex fluids with photografted whole antibodies. Anal Chem 2006;78:3144–51.
[5] Ko YG, Kim YH, Park KD, Lee HJ, Lee WK, Park HD, et al. Immobilization of poly(ethylene glycol) or its sulfonate onto polymer surfaces by ozone oxidation. Biomaterials 2001;22:2115–23.
[6] Beyer M, Felgenhauer T, Bischoff FR, Breitling F, Stadler V. A novel glass slide-based peptide array support with high functionality resisting non-specific protein adsorption. Biomaterials 2006;27:3505–14.
[7] Kızılel S, Sawardecker E, Teymour F, Pérez-Luna VH. Sequential formation of covalently bonded hydrogel multilayers through surface initiated photopolymerization. Biomaterials 2006;27:1209–15.
[8] Sun XL, Stabler CL, Cazalis CS, Chaikof EL. Carbohydrate and protein immobilization onto solid surfaces by sequential Diels–Alder and azide–alkyne cycloadditions. Bioconj Chem 2006;17:52–7.
[9] Schlapak R, Pammer P, Armitage D, Zhu R, Hinterdorfer P, Vaupel M, et al. Glass surfaces grafted with high-density poly(ethylene glycol) as substrates for DNA oligonucleotide microarrays. Langmuir 2006;22:277–85.
[10] Feng CL, Zhang Z, Forch R, Knoll W, Vancso GJ, Schonherr H. Reactive thin polymer films as platforms for the immobilization of biomolecules. Biomacromolecules 2005;6:3243–51.
[11] Patel S, Thakar RG, Wong J, McLeod SD, Li S. Control of cell adhesion on poly(methyl methacrylate). Biomaterials 2006;27:2890–7.
[12] Sinclair J, Salem AK. Rapid localized cell trapping on biodegradable polymers using cell surface derivatization and microfluidic networking. Biomaterials 2006;27:2090–4.
[13] Zhen G, Eggli V, Voros J, Zammaretti P, Textor M, Glockshuber R, et al. Immobilization of the enzyme beta-lactamase on biotin-derivatized poly(L-lysine)-*g*-poly(ethylene glycol)-coated sensor chips: a study on oriented attachment and surface activity by enzyme kinetics and in situ optical sensing. Langmuir 2004;20:10464–73.
[14] Popat KC, Mor G, Grimes CA, Desai TA. Surface modification of nanoporous alumina surfaces with poly(ethylene glycol). Langmuir 2004;20:8035–41.
[15] Piehler J, Brecht A, Valiokas R, Liedberg B, Gauglitz G. A high-density poly(ethylene glycol) polymer brush for immobilization on glass-type surfaces. Biosens Bioelectron 2000;15:473–81.
[16] Xu ZK, Nie FQ, Qu C, Wan LS, Wu J, Yao K. Tethering poly(ethylene glycol)s to improve the surface biocompatibility of poly(acrylonitrile-*co*-maleic acid) asymmetric membranes. Biomaterials 2005;26:589–98.
[17] Choi I, Kang SK, Lee J, Kim Y, Yi J. In situ observation of biomolecules patterned on a PEG-modified Si surface by scanning probe lithography. Biomaterials 2006;27:4655–60.
[18] Xu H, Kaar JL, Russell AJ, Wagner WR. Characterizing the modification of surface proteins with poly(ethylene glycol) to interrupt platelet adhesion. Biomaterials 2006;27:3125–35.
[19] Jun Y, Cha T, Guo A, Zhu XY. Patterning protein molecules on poly(ethylene glycol) coated Si(1 1 1). Biomaterials 2004;25:3503–9.
[20] Groll J, Ameringer T, Spatz JP, Moeller M. Ultrathin coatings from isocyanate terminated star PEG prepolymers: layer formation and characterization. Langmuir 2005;21:1991–9.
[21] Groll J, Haubensak W, Ameringer T, Moeller M. Ultrathin coatings from isocyanate terminated star PEG prepolymers: patterning of proteins on the layers. Langmuir 2005;21:3076–83.
[22] Groll J, Ademovic Z, Ameringer T, Klee D, Moeller M. Comparison of coatings from reactive star shaped PEG-stat-PPG prepolymers and grafted linear PEG for biological and medical applications. Biomacromolecules 2005;6:956–62.
[23] Dalsin JL, Lin L, Tosatti S, Vörös J, Textor M, Messersmith PB. Protein resistance of titanium oxide surfaces modified by biologically inspired mPEG-DOPA. Langmuir 2005;21:640–6.
[24] Dalsin JL, Hu B-H, Lee BP, Messersmith PB. Mussel adhesive protein mimetic polymers for the preparation of nonfouling surfaces. J Am Chem Soc 2003;125:4253–8.

[25] Weber M, Vasella A, Textor M, Spencer ND. Glycosydene carbenes: Part 271. Glucosidation of titanium dioxide with 1-aziglucoses: preparation and characterization of modified titanium-dioxide E surface. Helv Chim Acta 1998;81:1359–72.
[26] Sugawara T, Matsuda T. Photochemical protein fixation on polymer surfaces via derivatized phenyl azido group. Langmuir 1995;11:2272–6.
[27] Chen G, Ito Y. Gradient micropattern immobilization of EGF to investigate the effect of artificial juxtacrine stimulation. Biomaterials 2001;22:2453–7.
[28] Ito Y, Hasuda H, Yamauchi T, Komatsu N, Ikebuchi K. Immobilization of erythropoietin to culture erythropoietin-dependent human leukemia cell line. Biomaterials 2004;25:2293–8.
[29] Chen G, Ito Y, Imanishi Y, Magnani A, Lamponi S, Barbucci R. Photoimmobilization of sulfated hyaluronic acid for antithrombogenicity. Bioconjugate Chem 1997;8:730–4.
[30] Ito Y, Hayashi M, Imanishi Y. Gradient micropattern immobilization of heparin and its interaction with cells. J Biomater Sci Polym Edn 2001;12:367–78.
[31] Liu H, Ito Y. Gradient micropattern immobilization of a thermoresponsive polymer to investigate its effect on cell behavior. J Biomed Mater Res 2003;67A:1424–9.
[32] Kang I-K, Kim GJ, Kwon OH, Ito Y. Co-culture of hepatocytes and fibroblasts by micropatterned immobilization of beta-galactose derivatives. Biomaterials 2004;25:4225–32.
[33] Konno T, Hasuda H, Ishihara K, Ito Y. Photo-immobilization of a phospholipid polymer for surface modification. Biomaterials 2005;26:1381–8.
[34] Hasuda H, Kwon OH, Kang I-K, Ito Y. Synthesis of photoreactive pullulan for surface modification. Biomaterials 2005;26:2401–6.
[35] Ito Y. Photoimmobilization for microarrays. Biotechnol Prog 2006;22:924–32.
[36] Lee BP, Huang K, Nunalee FN, Shull KR, Messersmith PB. Synthesis of 3,4-dihydroxyphenylalanine (DOPA) containing monomers and their co-polymerization with PEG-diacrylate to form hydrogels. J Biomater Sci Polym Edn 2004;15:449–64.
[37] Kwon IK, Matsuda T. Photo-iniferter-based thermoresponsive block copolymers composed of poly(ethylene glycol) and poly(*N*-isopropylacrylamide) and chondrocyte immobilization. Biomaterials 2006;27:986–95.
[38] Hahn MS, Taite LJ, Moon JJ, Rowland MC, Ruffino KA, West JL. Photolithographic patterning of polyethylene glycol hydrogels. Biomaterials 2006;27:2519–24.
[39] Chen G, Imanishi Y, Ito Y. pH-Sensitive thin hydrogel microfabricated by photolithography. Langmuir 1998;14:6610–2.
[40] Adden N, Gamble LJ, Castner DG, Hoffman A, Cross G, Menzel H. Synthesis and characterization of biocompatible polymer interlayers on titanium implant materials. Biomacromolecules 2006;7:2552–9.
[41] Hermanson GH. Bioconjugate techniques. Academic Press; 1996.
[42] Ito Y, Nogawa M. Preparation of a protein micro-array using a photo-reactive polymer for a cell-adhesion assay. Biomaterials 2003;24:3021–6.
[43] Ito Y, Nogawa M, Takeda M, Shibuya T. Photo-reactive polyvinylalcohol for photo-immobilized microarray. Biomaterials 2005;26:211–6.
[44] Heydari M, Hasuda H, Sakuragi M, Yoshida Y, Suzuki K, Ito Y. Modification of the titan surface with photoreactive gelatin to regulate cell attachment. J Biomed Mater Res; in press. doi:10.1002/jbm.a.31368.

Grid Pattern of Nanothick Microgel Network

Guoping Chen,*,† Naoki Kawazoe,† Yujiang Fan,†,§ Yoshihiro Ito,‡ and Tetsuya Tateishi†

Biomaterials Center, National Institute for Materials Science, 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan and Nano Medical Engineering Laboratory, Institute of Physical and Chemical Research, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

Received March 31, 2007

A novel grid pattern of two kinds of nanothick microgels was developed by alternate patterning using photolithography. At first, 100-μm-wide nanothick PAAm microgel stripes were grafted on a polystyrene surface by UV irradiation of the photoreactive azidobenzoyl-derivatized polyallylamine-coated surface through a photomask with 100-μm-wide stripes. Then, a second set of 100-μm-wide nanothick PAAc microgel stripes were grafted across the PAAm-grated polystyrene surface by UV irradiation of the photoreactive azidophenyl-derivatized poly(acrylic acid)-coated surface through a photomask placed perpendicularly to the first set of PAAm microgel stripes. The PAAc microgel stripe pattern was formed over the PAAm microgel stripe pattern. The cross angle of the two microgel stripes could be controlled by adjusting the position of the photomask when the second microgel pattern was prepared. Swelling and shrinking of the microgels were investigated by scanning probe microscopy (SPM) in an aqueous solution. SPM observation indicated that the thickness of the gel network was 100 to 500 nm. The regions containing PAAm, PAAc, and the PAAc-PAAm overlapping microgels showed different swelling and shrinking properties when the pH was changed. The PAAm microgel swelled at low pH and shrank at high pH whereas the PAAc microgel swelled at high pH and shrank at low pH. However, the PAAc-PAAm overlapping microgel did not change as significantly as did the two microgels, indicating that the swelling and shrinking of the two gels was partially offset. The pH-induced structural change was repeatedly reversible. The novel grid pattern of nanothick microgels will find applications in various fields such as smart actuators, artificial muscles, sensors, and drug delivery systems as well as in tissue engineering and so forth.

Introduction

Intelligent hydrogel systems have many potential applications because they undergo huge changes in volume in response to external stimuli such as changes in solvent composition,[1] pH,[2] temperature,[3] ion concentration,[4] electric field,[5] and light irradiation.[6] Some systems respond to a combination of two or more stimuli.[7,8] They have been used in actuators,[9] sensors,[10] drug delivery systems,[11] bioseparations,[12] biomedicine,[13] cell culture, and tissue engineering.[14,15] Many studies have focused on the response and structure of hydrogels. Their sensitivity can be controlled by molecular design technologies.[16−18] To accelerate the swelling and shrinking of hydrogels, efforts have been made to introduce porosity into hydrogels[19] or to reduce the size of hydrogels such as synthesizing microgels.[20,21] However, there are no reports on controlling the network pattern of different microhydrogels. In this study, an alternate patterning technique was developed to influence the network pattern of microgels. Photolithography was used to prepare a novel grid pattern of two different nanothick microgels. The pH responses of the microgel networks were observed by scanning probe microscopy (SPM). The grid patterns consisted of poly(acrylic acid), polyallylamine, and overlapping poly(acrylic acid)-on-polyallylamine microgel areas that demonstrated different pH responses.

Experimental Section

Synthesis of Azidophenyl-Derivatized Poly(acrylic acid). Azidophenyl-derivatized poly(acrylic acid) conjugate was synthesized by coupling poly(acrylic acid) with 4-azidoaniline. Poly(acrylic acid) (MW 450 000, 1.0 mmol per monomer unit), 4-azidoaniline hydrochloride (0.1 mmol), and 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide (water-soluble carbodiimide, WSC, 6.0 mmol) were dissolved in deionized water (110 mL). The pH of the solution was adjusted to 7.0 by adding NaOH and HCl. After being stirred at 4 °C for 48 h, the reaction solution was dialyzed against MilliQ water through a seamless cellulose tube (cutoff molecular weight 12 000) until the absence of azidoaniline in the washing solution was confirmed by ultraviolet spectroscopy. The dialyzed polymer was freeze dried. The azidophenyl-derivatized poly(acrylic acid) was

* To whom correspondence may be addressed. E-mail: guoping.chen@nims.go.jp. Tel: +81-29-860-4496. Fax: +81-29-860-4715.
† National Institute for Materials Science.
‡ Institute of Physical and Chemical Research.
§ Current affiliation: National Engineering Research Center for Biomaterials, Sichuan University, 29 Wangjiang Road, Chengdu 610064, China.

(1) Tanaka, T.; Fillmore, D.; Sun, S. T.; Nishio, I.; Swislow, G.; Shah, A. *Phys. Rev. Lett.* **1980**, *45*, 1636−1639.
(2) Zhao, B.; Moore, J. S. *Langmuir* **2001**, *17*, 4748−4763.
(3) Wang, C.; Stewart, R. J.; Kopecek, J. *Nature* **1999**, *397*, 417−420.
(4) Holz, J. H.; Holz, J. S.; Munro, C. H.; Asher, S. A. *Anal. Chem.* **1998**, *70*, 780−781.
(5) Osada, Y.; Okuzaki, H.; Hori, H. *Nature* **1992**, *355*, 242−243.
(6) Suzuki, A.; Tanaka, T. *Nature* **1992**, *346*, 345−346.
(7) Sahoo, S. K.; De, T. K.; Ghosh, P. K.; Maitra, A. *J. Colloid Interface Sci.* **1998**, *206*, 361−368.
(8) Kim, S. J.; Spinks, G. M.; Prosser, S.; Whitten, P. G.; Wallace, G. G.; Kim, S. I. *Nat. Mater.* **2006**, *5*, 48−51.
(9) Beebe, D. J.; Moore, S. J.; Bauer, J. M.; Yu, Q.; Liu, H. *Nature* **2000**, *404*, 588−590.
(10) Byrne, M. E.; Park, K.; Peppas, N. A. *Adv. Drug Delivery Rev.* **2002**, *54*, 149−161.
(11) Guiseppi-Elie, A.; Brahim, S. I.; Narinesingh, D. *Adv. Mater.* **2002**, *14*, 743−746.
(12) Galaev, I. Y.; Mattiasson, B. *TIBTECH* **1999**, *17*, 335−340.
(13) Hoffman, A. S. *Adv. Drug Delivery Rev.* **2002**, *43*, 3−12.
(14) Alsberg, E.; Anderson, K. W.; Albeiruti, A.; Rowley, J. A.; Mooney, D. J. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 12025−12030.
(15) Stabenfeldt, S. E.; Garcia, A. J.; LaPlaca, M. C. *J. Biomed. Mater. Res., Part A* **2006**, *77*, 718−725.
(16) Yoshida, R.; Uchida, K.; Kaneko, Y.; Sakai, K.; Kikuchi, A.; Sakurai, Y.; Okano, T. *Nature* **1995**, *374*, 240−242.
(17) Chen, G.; Hoffman, A. S. *Nature* **1995**, *373*, 49−51.
(18) Yoshida, R.; Takahashi, T.; Yamaguchi, T.; Ichijo, H. *Adv. Mater.* **1997**, *9*, 175−178.
(19) Barbucci, R.; Leone, G.; Vecchiullo, A. *J. Biomater. Sci., Polym. Ed.* **2004**, *15*, 607−619.
(20) Wang, J.; Gan, D.; Lyon, L. A.; El-Sayed, M. A. *J. Am. Chem. Soc.* **2001**, *123*, 11284−11289.
(21) Chen, G.; Imanishi, Y.; Ito, Y. *Macromolecules* **1998**, *31*, 4379−4381.

10.1021/la700931u CCC: $37.00
Published on Web 04/26/2007

referred to as AzPhPAAc. The number of azidophenyl groups in the polymer was determined by ^{1}H NMR from the peak intensities of the azidophenyl protons at 7.0 ppm and those of the methylene and methylidyne protons of the polymer main chain at 1.3 and 2.5 ppm.

Preparation of Azidophenyl-Derivatized Polyallylamine. *N*-(4-Azidobenzoyloxy) succinimide was at first synthesized. A solution of DCC (13.3 g, 64.6 mmol) in tetrahydrofuran (THF 50 mL) was added dropwise to a solution of *N*-hydroxysuccinimide (7.43 g, 64.6 mmol) and 4-azidobenzoic acid (9.57 g, 58.7 mmol) in 150 mL of THF in an ice bath under stirring. After 3 h, the reaction mixture was slowly warmed to room temperature, and stirring was continued overnight. The white solid that formed was filtered off, and the solvent was removed under reduced pressure. The remaining yellow residue was crystallized from isopropyl alcohol/isopropyl ether. Then the azidophenyl-derivatized polyallylamine conjugate was synthesized by coupling polyallylamine with *N*-(4-azidobenzoyloxy) succinimide. An aqueous solution (pH 7.0, 10 mL) containing polyallylamine (MW 60 000, 30 mg) was added to the DMF solution (20 mL) of *N*-(4-azidobenzoyloxy) succinimide (8.4 mg) under stirring in ice. After reaction at 4 °C for 24 h under stirring, the solution was ultrafiltered (Millipore MoleCutII, filtration off below 10 kDa) and washed with two 5-mL portions of 1/2 DMF/H_2O and then with 5 mL of MilliQ water. The number of azidophenyl groups in the polymer was determined by ^{1}H NMR from the peak intensities of the azidophenyl protons at 7.0 ppm and those of the methylene and methylidyne protons of the polymer main chain at 1.3 and 2.5 ppm.

Alternate Pattern Grafting of Polyallylamine and Poly(acrylic acid) Microgels. A polystyrene plate (2 cm × 2 cm) was cut from a tissue culture polystyrene flask. The azidophenyl-derivatized polyallylamine was dissolved in water (200 μg/mL). The solution (100 μL) was placed on the polystyrene plate and air dried at room temperature in the dark. The plate was covered with a patterned photomask having a 100-μm-wide stripe network and irradiated with ultraviolet light at an intensity of 10^5 μJ/cm^2 from a distance of 15 cm for 60 s. After irradiation, the plate was immersed in dilute hydrochloric acid (pH 3.0) and sonicated to completely remove the unreacted polymer in the unirradiated areas. After complete washing, the plate was dried in air. A polyallylamine microgel having the same pattern as that of the photomask was grafted onto the polystyrene plate surface.

Subsequently, the azidophenyl-derivatized poly(acrylic acid) was dissolved in water (500 μg/mL), and the solution (100 μL) was placed on the polystyrene plate patterned with the polyallylamine microgel and air dried at room temperature. The plate was covered with the same photomask that was turned 90° from its position for the PAAm microgel. The plate was irradiated with ultraviolet light at an intensity of 10^5 μJ/cm^2 from a distance of 15 cm for 60 s. The irradiated plate was immersed in and rinsed with an alkaline solution (pH 10) and then sonicated to remove unreacted azidophenyl-derivatized poly(acrylic acid). A PAAc microgel having the same pattern as that of the photomask was grafted onto the polystyrene plate surface and was laid perpendicularly to the PAAm microgel stripes. The grid pattern of PAAm and PAAc microgel networks was prepared. A similar grid pattern of PAAc and PAAm microgel networks was also fabricated using the same process by changing the preparation order of the two microgels.

Observation by SPM. An SPA400 (SII NanoTechnology Inc.) equipped with an Olympus rectangular cantilever (OMCL-RC800PB-1) having a spring constant of 0.11 N/m in contact mode was used for the measurements. All of the SPM measurements were made at room temperature (23 °C). The polystyrene plate grafted with the grid pattern of the PAAm and PAAc microgel network was first immersed in an aqueous HCl solution of pH 1.66, and a 150 μm × 150 μm area of the network was observed by SPM. The solution was then changed to a phosphate buffer (pH 7.4) or aqueous NaOH solution (pH 10.89), and the same measurements were performed.

Results and Discussion

Two photoreactive polymers, azidophenyl-derivatized poly(acrylic acid) (AzPhPAAc) and azidophenyl-derivatized polyallylamine (AzPhPAAm), were synthesized by coupling poly(acrylic acid) (PAAc) and polyallylamine (PAAm) with azidoaniline or azidobenzoic acid, respectively. The percentages of carboxylic groups in the PAAc and the amino groups in the PAAm coupled with the azidophenyl groups were 6.2 and 8.6%, respectively.

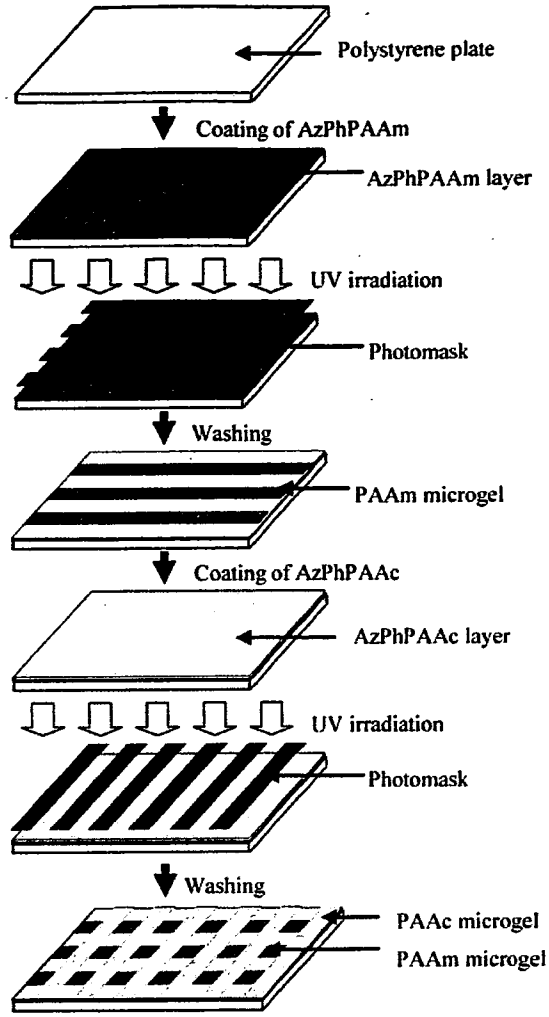

Figure 1. Photolithographic fabrication scheme of the grid pattern of microgel networks.

The preparation scheme of the grid pattern of microgels is shown in Figure 1. An aqueous solution of AzPhPAAm or AzPhPAAc was eluted on a polystyrene plate and air dried in the dark. The cast plate was photoirradiated in the presence of a photomask having a 100-μm-wide stripe pattern. AzPhPAAm or AzPhPAAc in the irradiated areas should be intermolecularly and intramolecularly crosslinked and grafted to the polystyrene surface. AzPhPAAm or AzPhPAAc in the other areas should not be crosslinked and could be removed by washing with an acidic or alkaline solution. After washing, a stripe network of a PAAm or PAAc microgel having the same pattern as that of the photomask was synthesized. Subsequently, a second microgel stripe pattern was grafted onto the first microgel. An aqueous solution of AzPhPAAc or AzPhPAAm was eluted onto the polystyrene plate grafted with the PAAm or PAAc microgel stripe pattern. The cast plate was photoirradiated in the presence of the same photomask that was turned 90° from the first pattern graft position. AzPhPAAc or AzPhPAAm in the irradiated areas should be intermolecularly and intramolecularly crosslinked and

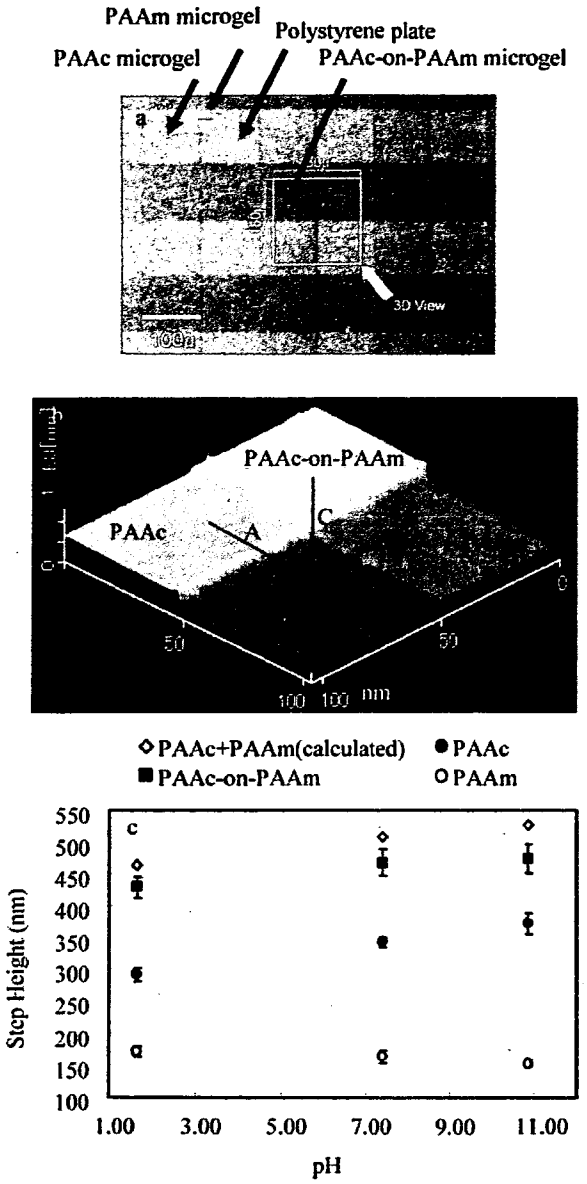

Figure 2. (a) Optical micrograph and (b) 3D topographic image of scanning probe microscopy (SPM) of a grid pattern of the PAAc-on-PAAm microgel network and (c) changes in the heights of the areas of PAAm, PAAc, and PAAc-PAAm microgels with changes in pH. The average value and the standard deviation for $n = 5$. The calculated height is the sum of the heights of PAAm and PAAc microgels.

grafted to the polystyrene surface and the first set of microgel stripes. AzPhPAAc or AzPhPAAm in the other areas should not be crosslinked and could be removed by washing with an acidic or alkaline solution. After washing, a grid pattern of a PAAc-on-PAAm or PAAm-on-PAAc microgel network was synthesized.

The grid pattern of PAAc-on-PAAm microgels was confirmed by optical microscopy (Figure 2a). The width of the microgel stripes and interstices was 100 μm. The swelling and shrinking behaviors of the PAAc-on-PAAm microgels were observed by SPM using contact mode. A 150 μm × 150 μm area was analyzed by changing the pH of the surrounding aqueous solution from 1.5 to 11.0. The terrace structure of the grid pattern of the PAAc-on-PAAm microgel at pH 10.89 was confirmed by a 3D image (Figure 2b). The areas of bare polystyrene, PAAc microgel, PAAm microgel, and PAAc-PAAm microgel had different heights and comprised the terrace structure. The step heights of the four areas were measured by scanning the adjacent areas along lines A−C. The thickness of the microgels was 140 to 500 nm and changed with the pH (Figure 2c). The height of the polystyrene area remained the same because polystyrene does not respond to changes in pH. The areas containing the PAAc, PAAm, and PAAc-PAAm microgels showed different swelling and shrinking properties when the pH was changed. The PAAc microgel swelled at high pH and shrank at low pH. The PAAm microgel swelled at low pH and shrank at high pH. However, the thickness of the joint areas did not change as significantly as those of the PAAc and PAAm microgel areas, indicating that the swelling and shrinking effects of the two microgels offset each other in the joint region. The pH-induced structure change was repeatedly reversible.

The pH responses of the PAAc, PAAm, and PAAc-PAAm microgels were demonstrated by SPM. Their swelling and shrinking can be interpreted as the result of the ionization and deionization of the carboxylic and amino groups in the PAAc, PAAm, and PAAc-PAAm microgel networks. For the PAAc area, the carboxy groups of the PAAc microgel network became ionized and the network swelled at pH 10.89 as a result of the repulsive interaction between negatively charged carboxylic groups. As the pH of the solution decreased, the ionized carboxylic groups became deionized, and hydrogen bonds between the carboxylic groups formed. As a result, the network shrank. However, for the PAAm area the situation was completely the opposite. The amino groups of the PAAm microgel network became ionized and the network swelled at pH 1.66 as a result of the repulsive interaction between positively charged carboxylic groups. As the pH of the solution increased, the ionized amino groups became deionized, and hydrogen bonds between the amino groups formed. Subsequently, the PAAm microgel area shrank. For the PAAc-PAAm microgel area, the response to pH is the sum as the effects from both the PAAc and PAAm microgels. At low pH, the amino groups of the PAAm microgel were ionized and swelled, and the carboxylic groups of the PAAc microgel deionized and shrank. At high pH, the carboxylic groups of the PAAc microgel were ionized and swelled, and the amino groups of the PAAm microgel became deionized and shrank. The opposite response of the PAAc and PAAm microgels partially counteracted the swelling and shrinking effects of each other in the joint area. However, the height of the joint area was not the simple sum of the heights of the PAAc and PAAm areas. It was lower in the range of 30 to 50 nm than the sum of the heights of the PAAc and PAAm areas. The reason might be that the interface of the PAAm and PAAc microgels in the joint area may not be a simple lamination of the two microgel sheets. The photoreactive polymer solution might penetrate the first microgel network in the joint area during the second patterning process. Therefore, the upper microgel might penetrate the microgel lying beneath it. The negatively charged carboxylic groups and positively charged amino groups might form ion complexes that result in the formation of a compact structure at the very thin interface and may not be a response to pH change. The compact interface structure thus decreased the total height of the overlapped area.

In recent years, microfabrication and micromachining techniques and nanotechnology have been widely used in various fields such as the integrated circuit industry, molecular electronics, biosensing, tissue engineering, and so forth. It would be interesting to apply polymer gels in such fields because polymer gels undergo abrupt changes in volume by swelling and shrinking in response to external stimuli. However, it has been difficult to control the

structure of microgels and to synthesize microgel networks. In the present study, photolithography was used to prepare a grid pattern of pH-sensitive nanothick microgels. The grid pattern constituted of four grids that had different responses to changes in pH. The nanothick microgels showed a rapid, reversible response to changes in pH. By using this technique, it would be possible to prepare nanothick microgel networks with both positional and functional patterns by using different stimuli-responsive polymers. These kinds of nanothick microgel networks would find wide applications in devices such as smart actuators, artificial muscles, sensors, and drug delivery systems as well as in tissue engineering and so forth.

Acknowledgment. This work was supported in part by the New Energy and Industrial Technology Development Organization of Japan and in part by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

LA700931U

Available online at www.sciencedirect.com
ScienceDirect
Biomaterials 29 (2008) 23–32
Biomaterials
www.elsevier.com/locate/biomaterials

Chondrogenic differentiation of human mesenchymal stem cells on photoreactive polymer-modified surfaces

Likun Guo[a,b], Naoki Kawazoe[a], Yujiang Fan[a,b], Yoshihiro Ito[c], Junzo Tanaka[a,1], Tetsuya Tateishi[a], Xingdong Zhang[b], Guoping Chen[a,*]

[a]*Biomaterials Center, National Institute for Materials Science, 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan*
[b]*National Engineering Research Center for Biomaterials, Sichuan University, Chengdu, China*
[c]*Nano Medical Engineering Laboratory, Institute of Physical and Chemical Research, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan*

Received 9 May 2007; accepted 27 August 2007

Abstract

Human mesenchymal stem cells (MSCs) were cultured on polystyrene surfaces modified with photoreactive azidophenyl-derivatives of three different chargeable polymers, poly(acrylic acid) (PAAc), polyallylamine (PAAm), and poly(ethylene glycol) (PEG). The MSCs adhered and spread both on a PAAm-modified surface and on PAAc-modified and polystyrene (control) surfaces. However, the cells adhered more easily to the PAAm-modified surface. The MSCs did not attach to the PEG-modified surface and aggregated to form pellets immediately after cell seeding. The cells proliferated on the PAAc-, PAAm-modified and control surfaces with culture time, formed a monolayer, and aggregated to form pellets. The cells in the pellets that formed on the PAAm- and PEG-modified surfaces after 2 weeks culture had a round morphology and the extracellular matrices were positively stained by safranin O and toluidine blue, while those that formed on the PAAc-modified and control surfaces had a spindle, fibroblast-like morphology and were not positively stained by safranin O and toluidine blue. The pellets that formed on the PAAm- and PEG-modified surfaces contained significantly higher levels of sulfated glycosaminoglycans than did those that formed on the PAAc-modified and control surfaces. Type II collagen and cartilage proteoglycan were immunohistologically detected in the pellets that formed on PAAm- and PEG-modified surfaces, but not those that formed on the PAAc-modified and control surfaces. The MSCs cultured on the PAAm- and PEG-modified surfaces expressed a high level of cartilaginous genes encoding type II collagen and aggrecan, while the MSCs cultured on the PAAc-modified and control surfaces did not express these genes. These results suggest that the PAAm-modified surface supported cell adhesion and proliferation and also promoted chondrogenic differentiation of the MSCs. The PAAc-modified and polystyrene surfaces supported cell adhesion and proliferation, but not chondrogenic differentiation. The PEG-modified surfaces did not support cell adhesion, but did promote chondrogenic differentiation. The adhesion, proliferation, and differentiation of the MSCs could be controlled by surface chemistry.

Keywords: Mesenchymal stem cells; Chondrogenic differentiation; Surface modification; Surface grafting; Surface property

1. Introduction

The surface properties of biomaterials and scaffolds such as chemical composition, nano- or microstructured morphology, wettability, and electrostatic property are very important for cell behaviors such as cell attachment, proliferation, extracellular matrix (ECM) secretion, and differentiation [1–5]. To elucidate the effects of surface properties, especially surface chemistry on cell functions, various methods have been reported to present the surfaces with different functional groups.

Poly(ethylene glycol) (PEG)-terephthalate–poly(butylene terephthalate) (PEGT/PBT) block copolymer substrates with various PEG lengths and mole fractions have been used to study the surface properties such as wettability, swelling, biodegradation rate and mechanical properties on

*Corresponding author. Tel.: +81 29 860 4496; fax: +81 29 860 4715.
E-mail address: Guoping.CHEN@nims.go.jp (G. Chen).
[1]Present address: Department of Metallurgy and Ceramics Science, Tokyo Institute of Technology, 2-12-1 Ookayama, Meguro-ku, Tokyo 152-8550, Japan.

0142-9612/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2007.08.043

protein adsorption and chondrocyte functions [6–8]. The block copolymer substrates with high PEG molecular weight and PEGT ratio exhibited a low fibronectin to vitronectin adsorption ratio and resulted in poor cell attachment, but showed an increased ability to maintain the primary human articular chondrocyte phenotype. In contrast, those with low PEG molecular weight and PEGT ratio showed preferential surface adsorption of fibronectin compared to vitronectin and enabled cell attachment, but resulted in chondrocyte dedifferentiation to a fibroblastic phenotype. A balance of hydrophilic and hydrophobic segments is needed for chondrocyte attachment and maintenance of the chondrogenic phenotype.

Self-assembled monolayers (SAMs) of alkanethiols on gold are another useful model system to systematically investigate the effects of surface chemistry on cell functions. Many researchers have used this method to study the functional surface groups on the adsorption and conformational change of proteins and the resulting effects on cell adhesion, spread, alignment, and proliferation [9–14]. Methyl, hydroxyl, carboxyl, and amino groups have been presented on gold surfaces by SAMs to study their effects on cell functions in different culture conditions. The effects depend not only on surface composition, but also on culture condition and cell type.

Silane-modified glasses presenting methyl ($-CH_3$), hydroxyl (–OH), carboxyl (–COOH), amino ($-NH_2$), and silane (–SH) have also been used. Curran et al. [15,16] reported the effects of surfaces presenting these functional groups on the differentiation of human mesenchymal stem cells (MSCs). The glass control and $-CH_3$ surfaces maintained the multipotent phenotype of MSCs, the $-NH_2$- and –SH-modified surfaces promoted osteogenesis, and the –OH- and –COOH-modified surfaces promoted chondrogenesis.

The above-mentioned methods of surface modification can create a well-defined surface chemistry, but the use of copolymers is limited to specific copolymers such as PEGT/PBT. The SAM method and silane-modification are only applicable to gold and glass substrates. Matsuda and Ito have developed a method of photochemical modification that can be used to introduce functional groups to the surfaces of any organic substrate [17–19]. The introduced groups are covalently bound to the surface and remain stable during long-term cell culture. In this study, we used this method for the surface modification of polystyrene cell culture plates. Three kinds of surfaces with different functional groups/electronic properties were designed and prepared by the photochemical method, namely a NH_2/positively charged surface, which was modified by photoreactive polyallylamine (PAAm), a COOH/negatively charged surface, which was modified by photoreactive poly(acrylic acid) (PAAc), and a neutral surface, which was modified by photoreactive PEG. The functional groups were grafted on the surface of polystyrene cell culture plates and their effects on the adhesion, proliferation, and chondrogenic differentiation of human MSCs were investigated.

2. Materials and methods

2.1. Synthesis of azidophenyl-derivatized poly(acrylic acid)

Azidophenyl-derivatized PAAc conjugate was synthesized by coupling PAAc with 4-azidoaniline, as shown in Fig. 1a. PAAc (Sigma-Aldrich Inc., M_w = 450,000, 1.0 mmol on monomer unit), 4-azidoaniline hydrochloride (Sigma-Aldrich Inc., 0.1 mmol), and 1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride (WSC, Wako Pure Chemical Industries Ltd., 6.0 mmol) were dissolved in deionized water (110 mL). The pH of the solution was adjusted to 7.0 by adding NaOH or HCl. After being stirred at 4 °C for 48 h, the reaction solution was dialyzed against MilliQ water through a seamless cellulose tube (cutoff M_w, 12,000) until the absence of azidoaniline in the washing solution was confirmed by ultraviolet spectroscopy. The dialyzed polymer was freeze-dried. The azidophenyl-derivatized PAAc was referred to as AzPhPAAc. The amounts of the azidophenyl groups in the polymer were determined by ^{1}H nuclear magnetic resonance (^{1}H-NMR) from the peak intensities of the azidophenyl protons at 7.0 ppm, and those of the methylene and methine protons of the polymer main chain at 1.3 and 2.5 ppm, respectively.

2.2. Synthesis of azidophenyl-derivatized polyallylamine

Azidophenyl-derivatized PAAm conjugate was synthesized by coupling PAAm with 4-azidobenzoic acid (Fig. 1b and c). *N*-(4-azidobenzoyloxy) succinimide was first synthesized (Fig. 1b). A solution of dicyclohexylcarbodiimide (13.3 g, 64.6 mmol) in tetrahydrofuran (THF 50 mL) was added dropwise to a solution of *N*-hydroxysuccinimide (Wako Pure Chemical Industries Ltd., 7.43 g, 64.6 mmol) and 4-azidobenzoic acid (Tokyo Kasei Kogyo Co. Ltd., 9.57 g, 58.7 mmol) in 150 mL THF in an ice bath under stirring. After 3 h, the reaction mixture was slowly warmed to room temperature (RT) and stirring was continued overnight. The white solid that formed was filtered off, and the solvent was removed under reduced pressure. The remaining yellow residue was crystallized from isopropyl alcohol/isopropyl ether. The azidophenyl-derivatized PAAm conjugate was then synthesized by coupling PAAm with *N*-(4-azidobenzoyloxy) succinimide (Fig. 1c). An aqueous solution (pH 7.0, 10 mL) containing PAAm (Sigma-Aldrich Inc., M_w 60,000, 30 mg) was added to the DMF solution (20 mL) of *N*-(4-azidobenzoyloxy) succinimide (8.4 mg) under stirring in ice. After being stirred at 4 °C for 24 h, the solution was ultrafiltered (Millipore MoleCut, filtration cut-off below 10 kDa), washed twice with 5 mL DMF/H_2O (1/2) solution, then with 5 mL MilliQ water until the absence of *N*-(4-azidobenzoyloxy) succinimide in the washing solution was confirmed by ultraviolet spectroscopy. The azidophenyl-derivatized PAAm was referred to as AzPhPAAm. The amounts of the azidophenyl groups in the polymer were determined by ^{1}H-NMR from the peak intensities of the azidophenyl protons at 7.0 ppm and those of the methylene and methine protons of the polymer main chain at 1.3 and 2.5 ppm, respectively.

2.3. Synthesis of azidophenyl-derived poly(ethylene glycol)

Azidophenyl-derived PEG was synthesized by the reaction of bis-amino PEG (NOF Corporation, Japan, PEG, M_w 5106, 100 mg) and *N*-(4-azidobenzoyloxy) succinimide (81 mg) in chloroform under stirring overnight (Fig. 1d). The obtained product was purified from chloroform/dehydrated diethyl ether three times until the absence of *N*-(4-azidobenzoyloxy) succinimide in the reprecipitation solution was confirmed by ultraviolet spectroscopy. The azidophenyl-derivatized PEG was referred to as AzPhPEG. The amounts of the azidophenyl groups in AzPhPEG were determined by ^{1}H-NMR.

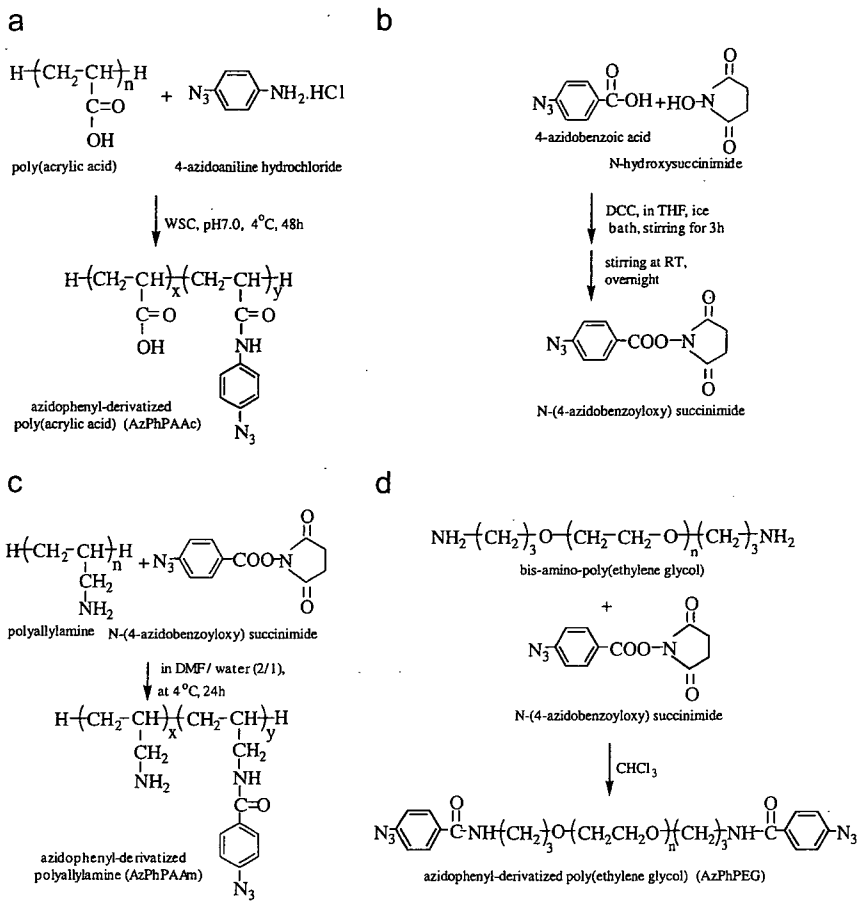

Fig. 1. Synthetic scheme of azidophenyl-derivatized poly(acrylic acid) (AzPhPAAc) (a), *N*-(4-azidobenzoyloxy) succinimide (b), azidophenyl-derivatized polyallylamine (AzPhPAAm) (c) and azidophenyl-derivatized poly(ethylene glycol) (AzPhPEG) (d).

2.4. Surface grafting of polyallylamine, poly(acrylic acid), and poly(ethylene glycol), measurement of contact angle and scanning probe microscopy (SPM) observation

AzPhPAAm, AzPhPAAc, and AzPhPEG were dissolved in water (1 mg/mL). The solutions were placed in the wells of 6-well polystyrene cell culture plates (40 μL/well) and air-dried at RT in the dark. The plates were irradiated with ultraviolet light at an intensity of 10^5 μJ/cm^2 from a distance of 15 cm for 60 s. After irradiation, the irradiated plates were immersed in diluted hydrochloric acid (pH 4), alkaline solution (pH 10), and MilliQ water, respectively and then sonicated to completely remove any unreacted polymers. After washing, the plates were sterilized with 70% ethanol aqueous solution and used for cell culture.

The contact angles of a sessile water drop on the surfaces of polymer-grafted polystyrene were measured by an Automatic Contact Angle Meter (Kyowa Interface Science Co., Ltd.). Six samples of each surface were used for the measurements. The data were expressed as the average ± standard deviation of the six samples.

For SPM observation, the AzPhPAAm, AzPhPAAc, and AzPhPEG were pattern-grafted on polystyrene discs using a patterned photomask. The AzPhPAAm, AzPhPAAc, and AzPhPEG were coated onto the polystyrene discs (4 cm^2, cut from 6-well polystyrene cell culture plates) at the same densities as those for surface grafting described above. The discs were covered with a patterned photomask having a 200 μm-wide stripe network and irradiated with ultraviolet light under the same conditions as that described above. After complete washing, the pattern-grafted surfaces were dried and observed by an SPA400 (SII NanoTechnology Inc.) equipped with an Olympus rectangular cantilever (SI-DF20) having a spring constant of 15 N/m in non-contact mode. The SPM measurements were made at RT. The thicknesses of the grafted polymers were measured from the SPM images. Nine spots from the topographic images of each kind of grafted pattern were used to calculate the mean height and standard derivatives. The data were expressed as the average ± standard deviation of the nine spots.

2.5. Cell culture

Human bone marrow-derived MSCs were obtained from Osiris (Worthington Biochemical, Lakewood, NJ) at passage 2. The cells were seeded in T-75 culture flasks using the proliferation medium from Osiris. The proliferation medium contained 440 mL MSC basal medium, 50 mL mesenchymal cell growth supplement, 10 mL 200 mM L-glutamine, and 0.5 mL penicillin/streptomycin mixture. The cells were further subcultured once after reaching confluence and used at passage 4. The cells were collected by treatment with trypsin/EDTA solution, washed once with DMEM serum-free medium, and suspended in DMEM serum-free medium at a density of 7.0×10^5 cells/mL. The cell solution was added to each well of the PAAm-, PAAc-, PEG-modified, and non-modified six-well cell culture plates (1 mL/well). Each well was supplemented with 5 mL chondrogenic induction medium and cultured for another 2 weeks under static conditions. The chondrogenic induction medium consisted of serum-free DMEM containing 4500 mg/L glucose, 584 mg/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1 mM non-essential amino acids, 0.4 mM proline, 50 mg/L ascorbic acid, 10^{-7} M dexamethasone, and 10 ng/mL TGF-β3 (Sigma-Aldrich, St. Louis, MO, USA). The TGF-β3 was thawed and supplemented immediately before use. The medium was

changed three times per week and was done carefully to avoid removing the cell pellets that formed during culture. The cell pellets that formed after 2 weeks culture were harvested for histological examination and gene expression analysis.

2.6. Biochemical analysis

The pellets that formed after 2 weeks culture in the chondrogenic induction medium were used for biochemical analysis of DNA and sulfated glycosaminoglycans (GAGs). The pellets were washed with pure water, frozen, and freeze-dried. The dried pellets were weighed and digested with 0.5 mL of papain. The amount of DNA in the papain digests was measured with fluorescent dye, Hoechst 33258 (Sigma-Aldrich, St. Louis, MO, USA), and a spectrofluorometer (JASCO, Tokyo, Japan) [20]. The sulfated GAG content was determined using a sulfated GAG assay kit, BlyscanTM (Biocolor Ltd., Newtownabbey, Northern Ireland) and an ultraviolet–visible spectrophotometer (JASCO, Tokyo, Japan) at 656 nm [21]. The amounts of DNA and GAG were divided by the weights of the pellets to determine their contents in each milligram of pellet. The GAG contents were also divided by the DNA amount to compare their contents per μg DNA. Every three pellets under each condition were used for the measurement to calculate the means and standard deviations. The data were expressed as the average ± standard deviation.

2.7. Cell proliferation

Cell proliferation was measured using the WST-1 assay (Roche Diagnostics, Indianapolis, IN, USA). This is a colorimetric assay for the quantification of cell viability and proliferation that is based on the cleavage of a tetrazolium salt (WST-1) by mitochondrial dehydrogenases in viable cells. Increased enzyme activity leads to an increase in the amount of formazan dye, which is measured with a spectrophotometer. 96-well cell culture plates were used. The three kinds of photoreactive polymers were grafted on the surfaces of the wells in the same manner as the grafting procedure described above by changing the volume of the eluted aqueous solution to 1.1 μL/well. After grafting, the plates were sterilized with 70% ethanol aqueous solution and used for cell culture. The MSC solution in DMEM serum-free medium at a density of 5.0×10^4 cells/mL was added to each well (100 μL/well) and cultured for 3 h or 1 day. After each incubation period, the culture medium was aspirated and 100 μL of DMEM supplemented with 10% FBS was added along with 10 μL of Cell Proliferation Reagent WST-1. For all time points, a standard curve was developed by plating 0.5×10^3, 2.0×10^3, 5.0×10^3, 1.0×10^4, and 1.5×10^4 cells in 100 μL of DMEM serum medium and 10 μL of Cell Proliferation Reagent WST-1. The plates were then incubated for an additional 4 h at 37 °C. After incubation, the absorbance of the samples against the background control on a microtiter plate reader (Bio-Rad Benchmark PlusTM Microplate Spectrophotometer) was obtained at a wavelength of 440 nm with a reference wavelength of 650 nm. Six wells under each condition were used for the measurement to calculate the means and standard deviations.

2.8. RNA isolation and real-time PCR

The MSCs cultured in the wells in the chondrogenic induction medium for 2 weeks were washed with PBS. The cell pellets were taken from the culture plates and frozen in liquid nitrogen. The frozen pellets were crushed into powder by an electric crusher. The powder from each sample was dissolved in 1 mL of Isogen reagent (Nippon Gene, Toyama, Japan) and RNA was isolated. DNase-treated RNA was treated with RQ1 RNase-free DNase (Promega) prior to being converted to cDNA by AMV Reverse Transcription (Takara Bio). Real-time PCR was amplified for GAPDH, types I, II, and X collagen, sox9, and aggrecan. The reaction was performed with 1 μL cDNA, 300 and 150 nM each PCR primer and PCR probe, and TaqMan Universal PCR Master Mix (Applied Biosystems). Reactions were cycled using a Bio-Rad iCycler for 40 cycles. The data were analyzed using Bio-Rad iCycler software. The level of expression of each target gene was normalized to GAPDH. The cell culture and gene expression analysis were performed twice under the same conditions. The primer and probe sequences (Applied Biosystems) followed those of Martin et al. [22] and Schaefer et al. [23]. These sequences were:

GAPDH:(F): 5′-ATGGGGAAGGTGAAGGTCG-3′;
(R): 5′-TAAAAGCAGCCCTGGTGACC-3′;
(probe): 5′-CGCCCAATACGACCAAATCCGTTGAC-3′.
Type I collagen:(F): 5′-CAGCCGCTTCACCTACAGC-3′;
(R): 5′-TTTTGTATTCAATCACTGTCTTGCC-3′;
(probe): 5′-CCGGTGTGACTCGTGCAGCCATC-3′.
Type II collagen:(F): 5′-GGCAATAGCAGGTTCACGTACA-3′;
(R): 5′-CGATAACAGTCTTGCCCCACTT-3′;
(probe): 5′-CCGGTATGTTTCGTGCAGCCATCCT-3′.
Type X collagen :(F): 5′-CAAGGCACCATCTCCAGGAA-3′;
(R): 5′-AAAGGGTATTTGTGGCAGCATATT-3′;
(probe): 5′- TCCCAGCACGCAGAATCCATCTGA -3′.
Sox9 :(F): 5′- CACACAGCTCACTCGACCTTG -3′;
(R): 5′-TTCGGTTATTTTTAGGATCATCTCG-3′;
(probe): 5′-CCCACGAAGGGCGACGATGG-3′.
Aggrecan :(F): 5′- TCGAGGACAGCGAGGCC-3′;
(R): 5′- TCGAGGGTGTAGCGTGTAGAGA-3′;
(probe): 5′-ATGGAACACGATGCCTTTCACCACGA-3′.

2.9. Histological and immunohistological staining

The pellets that formed during cell culture in the chondrogenic differentiation medium were fixed in neutral buffered formalin, embedded in paraffin, and sectioned. The pellet sections were stained with hematoxylin and eosin (H&E) for the nucleus and stained with safranin-O/fast green and toluidine blue to visualize the extracellular GAGs.

The type I collagen, type II collagen, and cartilage proteoglycan were immunohistologically stained using rabbit anti-human type I collagen antibody (Sanbio b.v., Uden, Netherlands), mouse anti-human type II collagen monoclonal antibody (Neomarkers, Fremont, CA), and mouse anti-human cartilage proteoglycan monoclonal antibody that recognizes the short peptides substituted with keratin sulfate side chains and within the core protein of proteoglycans in articular cartilage (Chemicon International, Temecula, CA) and a Dako LSAB Kit, Peroxidase (Dako, Carpinteria, CA) according to the instructions accompanying the kit. Briefly, the deparaffinized sections were incubated with proteinase K enzyme in Tris-buffered saline (1:50 working dilution, pH 7.4, TBS) at RT for 5 min and blocked with peroxidase blocking solution for 10 min and 10% goat serum solution for 30 min. The sections were then incubated with anti-type I collagen (1:400 working dilution), anti-type II collagen (1:200 working dilution), and anti-proteoglycan antibodies (1:1200 working dilution) for 30 min. The biotinylated secondary antibody (anti-rabbit or mouse immunoglobulins) was applied for 30 min followed by incubation with horseradish peroxide-conjugated streptavidin for 10 min. The sections were then incubated with 3-amino-9-ethylcarbazole as the color substrate for 10 min to visualize the bound antibodies. The nuclei were counterstained with hematoxylin. All incubations were at RT.

3. Results

3.1. Surface grafting and characterization

Photoreactive AzPhPAAc was synthesized by coupling PAAc with 4-azidoaniline (Fig. 1a). Photoreactive AzPhPAAm and AzPhPEG were synthesized by coupling PAAm and bis-amino PEG with *N*-(4-azidobenzoyloxy) succinimide, respectively (Fig. 1b–d). The synthesized

AzPhPAAc, AzPhPAAm, and AzPhPEG were purified by dialysis, ultrafiltration, and reprecipitation, respectively. The introduction of the photoreactive azido groups in AzPhPAAm, AzPhPAAc, and AzPhPEG was confirmed by the appearance of two peaks at about 7 ppm in ^{1}H-NMR that were derived from the four protons in the azidophenyl groups. The percentages of the carboxylic groups in the PAAc and the amino groups in the PAAm and bis-amino PEG coupled with the azidophenyl groups were 6.2%, 8.6%, and 100.0%, respectively.

An aqueous solution of AzPhPAAm, AzPhPAAc, or AzPhPEG was eluted in the wells of a 6-well cell culture polystyrene plate and air-dried in the dark. The cast plate was irradiated with ultraviolet light to graft the AzPhPAAm, AzPhPAAc, or AzPhPEG to the polystyrene surface. The contact angles of the polystyrene surfaces modified with PAAc, PAAm, or PEG decreased from 75.7 ± 2.4 degrees to 35.6 ± 3.6, 58.1 ± 4.4, or 53.3 ± 4.3 degrees, respectively. This result indicates that the surface became hydrophilic after surface modification.

The photoreactive polymers were pattern-grafted on polystyrene plates to observe the homogeneity and height of the grafted polymers. The patterning was performed under the same conditions as that of normal surface grafting, but with a photomask. SPM observation of the dried surfaces showed that the photoreactive polymers were homogenously grafted onto the surface. The grafted thicknesses of the dried AzPhPAAm, AzPhPAAc, and AzPhPEG layers were 61.8 ± 6.1, 142.3 ± 6.7, and 59.6 ± 10.2 nm, respectively. The AzPhPEG-grafted surface was rougher than were the AzPhPAAm- and AzPhPAAc-grafted surfaces. Observation of the grafted surfaces under a phase-contrast microscope also showed no evidence of defects of the grafted surfaces. These results indicate that the polystyrene surfaces were homogenously grafted by the photoreactive polymers.

3.2. Cell adhesion, proliferation, differentiation, and biochemical analysis

After culture passage 4, the MSCs were cultured on the modified surfaces and 6-well polystyrene cell culture plates (control) in a chondrogenic induction medium consisting of serum-free DMEM supplemented with dexamethasone and TGF-β3. The cells adhered to the PAAm-modified surface and spread after 30 min culture, spread more after 3 h, and proliferated to confluence after 3 days (Fig. 2). After reaching confluence, the cells gradually aggregated and detached to form pellets. The MSCs on the PAAc-modified and control surfaces were very similar. The cells adhered to the PAAc-modified and control surfaces and spread slightly after 30 min culture. They spread more after 3 h and proliferated to confluence after 3 days. The confluent cells also aggregated and formed pellets, but at a rate slower than those on the PAAm-modified surface. However, the MSCs did not attach to the PEG-modified surface at all and began to aggregate immediately after cell seeding. After 24 h, the cells aggregated into pellets. After 2 weeks, the cells on all surfaces formed pellets. The pellets that formed on the PAAm- and PEG-modified surfaces were larger than were those that formed on the PAAc-modified and control surfaces (Fig. 3).

The DNA and sulfated GAGs contents in each pellet after 2 weeks cultures were analyzed with Hoechst 33258 dye and BlyscanTM GAG assay, respectively. The DNA contents in the pellets formed on the PAAm-, PAAc-, PEG-modified, and control surfaces were 8.71 ± 0.34, 14.82 ± 0.36, 7.08 ± 0.41, 12.42 ± 0.41 μg/mg, respectively. The GAG contents in the pellets formed on the PAAm-, PAAc-, PEG-modified, and control surfaces were 37.71 ± 1.13, 13.98 ± 0.62, 31.43 ± 0.75, 5.60 ± 0.76 μg/mg, respectively. The ratio of GAG to DNA in the pellets formed on the PAAm-, PAAc-, PEG-modified, and control surfaces were 4.33 ± 0.13, 0.94 ± 0.04, 4.44 ± 0.11, 0.45 ± 0.06 μg/μg,

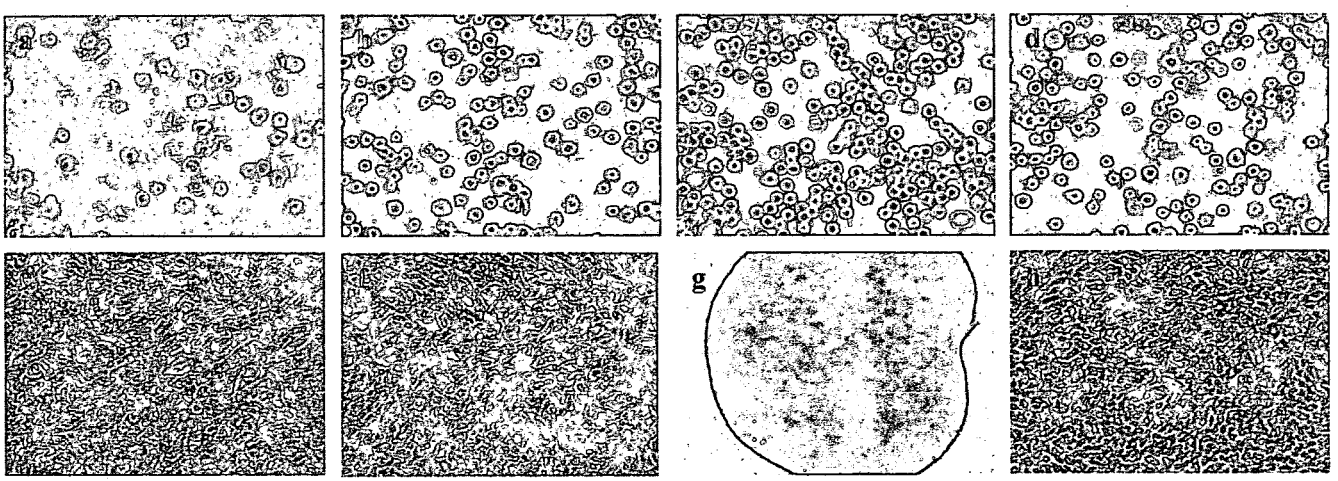

Fig. 2. Phase-contrast micrographs of MSCs cultured on PAAm- (a, e), PAAc- (b, f), PEG-modified (c, g), and control (d, h) surfaces in chondrogenic induction medium for 30 min (a–d) or 3 days (e–h).

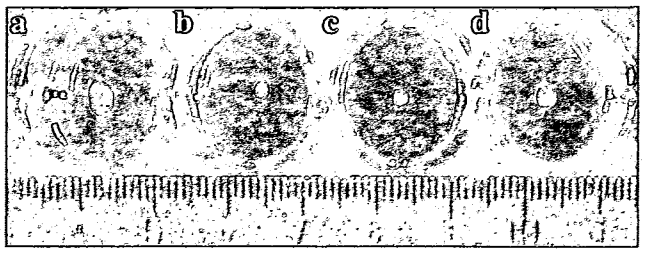

Fig. 3. Pellets that formed on PAAm- (a), PAAc- (b), PEG-modified (d), and control (c) surfaces after cultured in chondrogenic induction medium for 2 weeks.

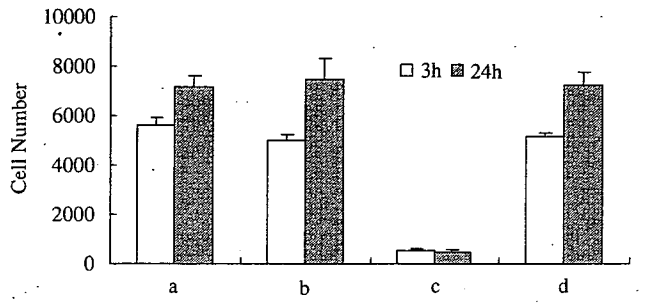

Fig. 4. Proliferation rates of MSCs cultured on PAAm- (a), PAAc- (b), PEG-modified (c), and control (d) surfaces in serum-free DMEM. Data represent the average ± SD of six samples.

respectively. The pellets on the PAAm- and PEG-modified surfaces produced significantly more GAG than did those on the PAAc-modified and control surfaces.

The cells proliferated on the PAAm- and PAAc-modified surfaces at almost the same rate as those on the polystyrene cell culture plates (Fig. 4). Almost no cells were detected on the PEG-modified surface because very few cells attached to the surface.

3.3. Histological and immunohistological examinations

The pellets that formed during cell culture were fixed, embedded, and histologically stained with hematoxylin/eosin, safranin O/fast green, and toluidine blue stains (Fig. 5). Histological examination using H&E stains indicated that the cells cultured on the PAAm- and PEG-grafted surfaces had a round morphology; those on the PAAc and control surfaces had a spindle, fibroblast-like morphology. The bright safranin O-positive stain indicated that GAGs were abundant and homogeneously distributed around the cells cultured on the PAAm- and PEG-modified surfaces. Toluidine blue staining revealed the typical metachromasia (purple color) of articular cartilage, coinciding with the results of safranin O staining. However, the cells on the PAAc-modified and control surfaces were not positively stained by safranin O and toluidine blue.

Immunohistological stainings indicate that the pellets that formed on the PAAm- and PEG-grafted surfaces were positively stained with type I collagen, type II collagen, and cartilage proteoglycan, while the pellets that formed on the PAAc-grafted and control surfaces did not show any obvious positive staining for type II collagen and cartilage proteoglycan (Fig. 6). These results indicate that the PAAm- and PEG-grafted surfaces provided microenvironments for MSCs to change to a round morphology and produce cartilaginous ECMs.

3.4. Real-time PCR

The gene expression of type I collagen, type II collagen, type X collagen, sox9, and aggrecan in the pellets was examined by real-time PCR (Fig. 7). The cell culture was performed twice and gene expression of the cells in the pellets from the two cultures was analyzed. The gene expression pattern of the two cultures showed similar trends. After culture passage 4, the MSCs expressed genes encoding type I collagen, a low level of sox9, and aggrecan; they did not express genes encoding type II and type X collagen. The MSCs cultured on the PAAm- and PEG-modified surfaces expressed all these genes. The genes encoding type II and type X collagen, sox9, and aggrecan were upregulated. The cells cultured on the PAAc- and control surfaces expressed genes encoding type I collagen, a low level of sox9, and almost no genes encoding type II and type X collagens and aggrecan. The cartilaginous genes of type II collagen and aggrecan were expressed only by cells cultured on the PAAm- and PEG-modified surfaces. The gene expression results coincided with the histological, immunohistochemical and biochemical results, which indicated that the PAAm- and PEG-modified surfaces promoted the chondrogenic differentiation of the MSCs, but that the PAAc-modified and control surfaces did not.

4. Discussion

Photochemical modification was used to introduce functional groups to cell culture polystyrene plate surfaces. The modified surfaces showed improved water wettability. This method can be used for the surface modification of any organic substrate. The modified surfaces were stable and changed their states in response to the pH of the aqueous solution. Photochemically modified surfaces have been shown to affect cell functions such as cell adhesion, proliferation, and differentiation. The positively charged PAAm-modified surface supported cell adhesion, proliferation, and differentiation. The negatively charged and control surfaces supported cell adhesion and proliferation, but not differentiation. The neutral PEG-modified surface supported neither cell adhesion nor proliferation, but did promote cell differentiation. The PAAm-modified, PAAc-modified, and control surfaces supported MSCs adhesion, but there were some differences among them. The MSCs adhered more rapidly to the PAAm-modified surface than to the PAAc-modified and control surfaces. This difference might be caused by the different electronic properties of these surfaces. The PAAm-modified surface promotes cell adhesion through the electrostatic attractive interaction

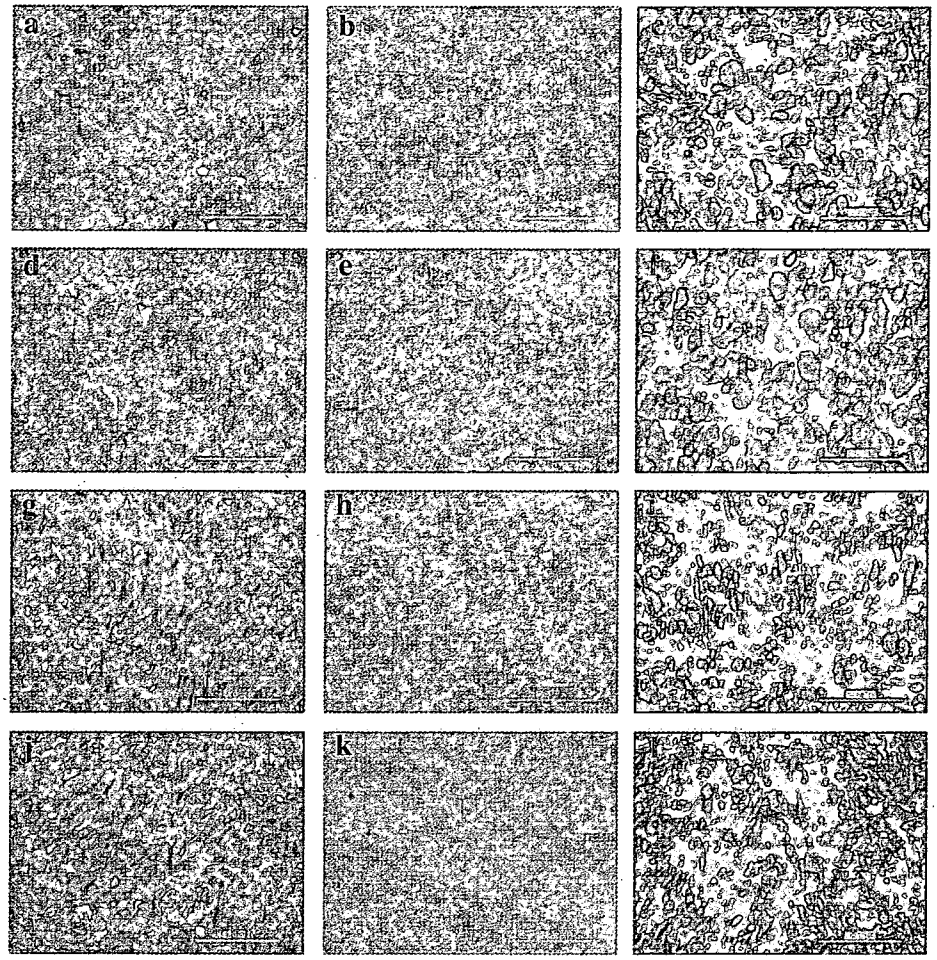

Fig. 5. Hematoxylin/eosin (a, d, g, j), safranin-O/fast green (b, e, h, k), and toluidine blue (c, f, i, l) staining of pellets that formed on PAAm- (a–c), PEG- (d–f), PAAc-modified (g–i), and control (j–l) surfaces after culture in chondrogenic induction medium for 2 weeks. The scale bar is 50 μm.

between the positively charged surface and the negatively charged cells. The negatively charged PAAc-modified and control surfaces do not provide such attractive interaction for cell adhesion. The PEG-modified surface did not have any electrostatic attractive interaction between surface and cells. It also did not support protein adsorption. Therefore, the cells did not adhere to the PEG-modified surface and aggregate directly after cell seeding.

Although the cells cultured on all the surfaces formed pellets, the effects of the PAAm- and PEG-modified surfaces were more evident than were those of the PAAc- and control surfaces. Cells on PEG-modified surface began to aggregate immediately after cell seeding and formed pellets after 24 h. The rapid formation of pellets on the PEG-modified surface indicates that cell–cell interaction is stronger than cell–surface interaction. The cells on the other surfaces also formed pellets after becoming confluent. Shrinkage of the confluent cells may result in the detachment of the cell sheet from the surface and in cell aggregation. The cells in the pellets that formed on the PAAm- and PEG-modified surfaces had a round morphology; expressed cartilaginous genes such as type II collagen, aggrecan, and sox9; were positively stained by safranin O, toluidine blue, anti-type II collagen antibody, and anti-cartilage proteoglycan antibody; and produced more GAG. The PAAm- and PEG-modified surfaces promoted the chondrogenic differentiation of the MSCs. However, the pellets that formed on the PAAc-modified and control surfaces did not display any evidence of chondrogenesis, indicating that these surfaces did not support the chondrogenic differentiation of MSCs. The cells cultured on the PAAm-modified surface in the serum medium also detached and formed pellets, but more slowly than those in the chondrogenic induction medium. However, the cells cultured in the serum medium on the PAAc-modified and control surfaces did not detach and form pellets until 2 weeks culture (data not shown). Culture in the chondrogenic medium facilitated cell detachment from the surface after confluence. A surface grafted with another neutral polymer, poly(vinyl alcohol), showed a similar effect as did that of PEG (data not shown). Although further investigation using other chargeable polymers and longer periods of inductive culture (4 weeks) should be considered, the 2 week culture results using PAAm, PAAc, and PEG clearly