Synthesis of a Photoimmobilizable Histidine Polymer for Surface Modification

Makoto Sakuragi,[1] Saki Tsuzuki,[1] Hirokazu Hasuda,[2] Akira Wada,[1] Kenji Matoba,[1,3] Izumi Kubo,[3] Yoshihiro Ito[1,2]

[1]*Nano Medical Engineering Laboratory, Riken, 2-1 Hirosawa, Wako-Shi, Saitama 351-0198, Japan*
[2]*Regenerative Medical Bioreactor Project, Kanagawa Academy of Science and Technology, KSP East 309, 3-2-1 Sakado, Takatsu-ku, Kawasaki-shi, Kanagawa 213-0012, Japan*
[3]*Department of Bioinformatics, Graduate School of Engineering, Soka University, 1-236 Tangi-cho, Hachioji-shi, Tokyo 192-8577, Japan*

Received 5 April 2008; accepted 4 July 2008
DOI 10.1002/app.29427
Published online 29 December 2008 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: A novel photoreactive polymer with histidine polar groups was synthesized through the copolymerization of two types of methacrylic acid, one carrying histidine groups and the other carrying azidoaniline groups. The polymer was photoimmobilized on polyester disks for surface modification. The effect of the surface modification on the hydrophilic and biofouling properties was investigated. Static contact angle measurements showed that the polymeric surface was modified to be comparatively hydrophilic in the polymer-immobilized region. Micropattern immobilization was carried out with a photolithographic method. Atomic force microscopy measurements showed that the polymer was formed on the disks in response to ultraviolet irradiation. Protein adsorption was reduced on the polymer-immobilized regions, and in those regions, spreading and adhesion of mammalian cells were reduced in comparison with that in nonimmobilized regions. In conclusion, a novel histidine-containing polymer was photoreactively immobilized on a conventional polymer surface, and it had reduced interaction with proteins and cells.
 J Appl Polym Sci 112: 315–319, 2009

Key words: biocompatibility; biological applications of polymers; biomaterials; lithography; surfaces

INTRODUCTION

Surface modification is very important for controlling the biofouling properties of materials, and various methods have been devised for surface modification. Among these, photoimmobilization is a very useful technique because it is applicable to various types of materials.[1,2] Ultraviolet (UV) irradiation causes radical reactions of photoreactive groups, such as azido groups, and excited azido groups can form covalent bonds with a surrounding material. These features allow the micropatterning of a polymer with photolithography.

So far, we have devised several types of photoreactive polymers, such as photoreactive gelatin for growth factor immobilization,[3] sulfated hyaluronic acid,[4] heparin,[5] a polymer containing saccharides in its side chains [poly(ethylene glycol)],[6] and a thermoresponsive polymer.[7] These polymers can be immobilized on various polymeric materials through covalent bonding. In addition, when polymers are immobilized in a micropattern, it is easy to visualize the effect of the surface modification by a comparison of the properties of the adjacent immobilized and nonimmobilized regions.

Recently, three major methods have been used to reduce the interaction of surfaces with biocomponents, including proteins and cells. The first method is the preparation of amphiphilic surfaces with poly (ethylene glycol).[6,8,9] The second method is the preparation of superhydrophilic or superhydrophobic surfaces,[10] and the third method is the preparation of zwitterion-carrying surfaces, such as phosphatidylcholine[11] and sulfobetaine.[12–14] Recently, we found that a weak zwitterion surface consisting of a histidine residue also has antibiofouling properties.[15] In this study, a novel polymer with azido groups and histidine polar groups was prepared to create an antibiofouling, photoreactive coating material.

EXPERIMENTAL

Synthesis of the photoreactive histidine polymer

The polymer synthesis route is shown in Figure 1. First, methacryloyl-L-histidine and 4-azidophenyl methacrylamide were prepared, as previously

Correspondence to: Y. Ito (y-ito@riken.jp).
Contract grant sponsor: Japanese Ministry of Health, Labour, and Welfare.

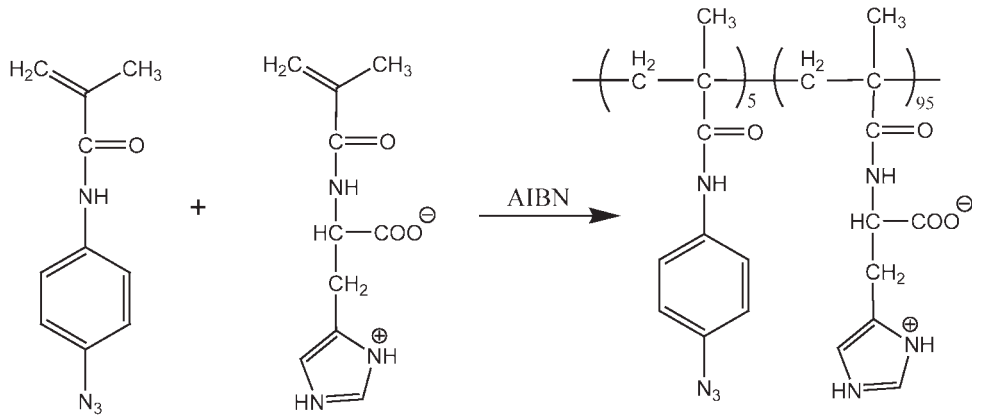

Figure 1 Synthetic scheme for a photoreactive histidine-containing polymer.

reported.[6,15] Subsequently, methacryloyl-L-histidine (2.1 g) and 4-azidophenyl methacrylamide (100 g) were mixed in a ratio of 95 : 5 (mol/mol) in ethanol (30 mL). Then, 2,2′-azobisisobutyronitrile (AIBN; 6.4 g) was added to the mixture. Nitrogen gas was bubbled in the mixture for a period of 20 min, and the mixture was then incubated in a water bath at 60°C for a period of 24 h. Then, the mixture was dialyzed with a dialysis cassette (Pierce, Rockford, IL) against water for 3 days with a semipermeable membrane (Spectra/Por 6 no. 132592, Spectrum Laboratories, Inc., Compton, CA). The resultant solution was freeze-dried. Elemental analysis indicated that the azidophenyl groups in the purified photo-immobilizable methacryloyl-L-histidine polymer (photo-MHis) composed 6% of the side chains.

Micropatterning

The micropatterning method is illustrated in Figure 2. Commercial polyester plate cell culture disks were used (Thermanox, Nunc, Roskilde, Denmark). Photo-MHis (20 mg/mL) was dissolved in a 0.1*N* hydrochloric acid solution, and 2 μL of the solution was mixed with 18 μL of methanol immediately before casting. The mixture was cast onto a Thermanox plate and air-dried at room temperature in the dark. Subsequently, the plate was covered with a photomask manufactured by Toppan Printing Co., Ltd. (Tokyo, Japan), and was UV-irradiated with a UV lamp (model L5662 UV spot light source, Hamamatsu Photonics, Hamamatsu, Japan) at a distance of 5 cm for a period of 10 s (90 mW/cm^2). The photomask was not employed when a nonpatterned surface was prepared for the measurement of contact angles. The plate was then repeatedly washed with distilled water and Dulbecco's formula modified phosphate-buffered saline (PBS) at 37°C.

Measurement of the contact angle

A nonpatterned sample was placed in the holder of an automatic contact angle meter (Kyowa Interface Science Co., Ltd., Saitama, Japan), and a drop of water (1.0 μL) was placed on the sample surface. The contact angle of the drop on the surface was measured at room temperature. At least 10 contact angles in different areas were measured and averaged. A *t* test was used for statistical analysis, and the *P* value was calculated. If the *P* value was below the threshold chosen for statistical significance (in this case, at the 0.01 level), then the null hypothesis, which usually states that the two groups do not differ, was rejected in favor of an alternative hypothesis, which typically states that the groups do differ.

Atomic force microscopy (AFM)

AFM measurements were performed with a model SPA-400 AFM (Seiko Instruments, Inc., Chiba, Japan) with an SI-DF20 cantilever attached. Micropatterned polymer-photoimmobilized substrates were prepared as described previously. The surface shapes and thicknesses of the polymer were measured in the AFM dynamic force mode (tapping mode). The scan rate was in the range of 0.5–0.8 Hz, and the scan angle was set to −90°. The images were analyzed with a Seiko model SPI3800N AFM (Seiko Instruments).

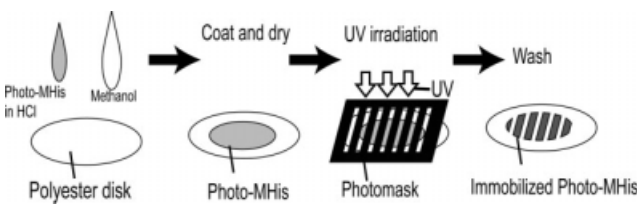

Figure 2 Schematic illustration of the micropatterning procedure.

Interaction with proteins

Fluorescein isothiocyanate (FITC) labeled bovine serum albumin (BSA) and horseradish peroxidase (HRP) labeled immunoglobulin G (IgG) were purchased from Sigma Chemicals (St. Louis, MO) and DakoCytomation (Glostrup, Denmark), respectively. The sample plates were incubated in PBS containing FITC-labeled albumin (0.2 mg/mL) or HRP-labeled IgG (0.02 mg/mL) at 37°C for a period of 30 min. HRP-labeled IgG was stained with a 3,3′,5,5′-tetramethylbenzidine stain kit (Vector Laboratories, Inc., Burlingame, CA). After being washed with PBS, the sample was observed with fluorescence and phase-contrast microscopes.

Cell culture

STO cells (fibroblast-like cells originating from mouse embryos) were purchased from Riken Cell Bank (Tsukuba, Japan) and were cultured in suspension minimum essential medium (Sigma Chemicals) with 10% fetal bovine serum (Hyclone, Logan, UT), 1% glutamate (Sigma Chemicals), and penicillin-streptomycin (Sigma Chemicals) with 1/100 dilution. The cell suspension was added to the sample plate, which was sterilized with 70% ethanol. The cells were incubated at 37°C under 5% (v/v) CO_2. After 3 h of incubation, the cells were observed with a phase-contrast microscope. Ten areas of adhered cells and rounded shape cells were counted and averaged. The *t* test was also performed for statistical analysis.

RESULTS AND DISCUSSION

Synthesis of the photoreactive histidine polymer

The UV absorption spectrum of photo-MHis is shown in Figure 3. In the UV spectra of the photoreactive polymer and azidoaniline, absorption peaks that were assignable to the azidophenyl group were observed at 270 and 276 nm, respectively. The spectra showed that the polymer contained azidophenyl groups, as we designed. The molecular weight of the photo-MHis was 3×10^3, as measured by gel permeation chromatography (Fig. 4).

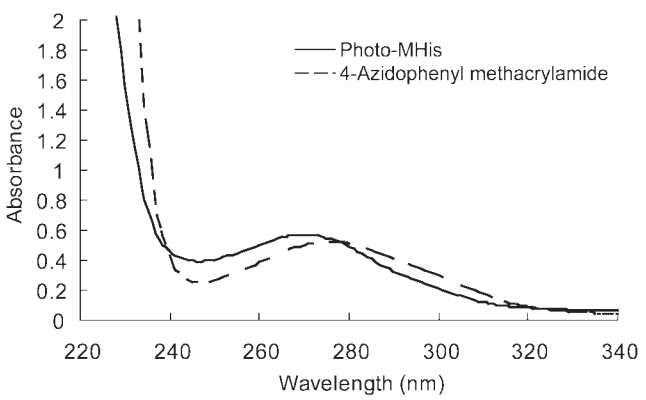

Figure 3 UV absorption spectra of 4-azidophenyl methacrylamide and photo-MHis.

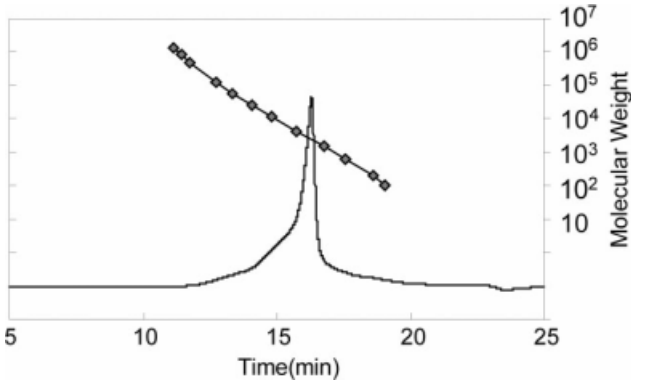

Figure 4 Gel permeation chromatography measurements for molecular weight distribution analysis. The calibration line was obtained with a poly(ethylene glycol) kit (no. 2080-0200), which was purchased from Polymer Laboratories, a part of Varian, Inc.

Photoimmobilization

The photo-MHis was coated on the plates, and the coated surface was UV-irradiated with a photomask [Fig. 5(a)]. The surface pattern was the same as that of the photomask. The micropatterned surface was observed with phase-contrast microscopy [Fig. 5(b)]. It was thought that the photolyzed aryl azide intermediates could undergo ring expansion to create nucleophile-reactive dehydroazepines that could react with the surrounding chemical environment to form covalent bonds.[1,2]

AFM measurements were carried out to assess the height and geometry of the polymer photoimmobilized

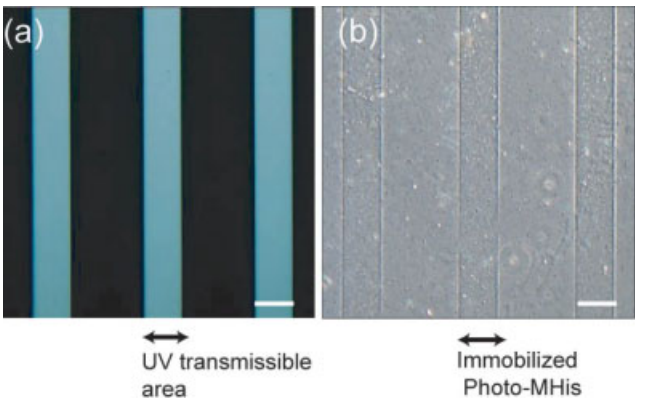

Figure 5 Phase-contrast micrographs of (a) the photomask and (b) the micropatterned surface of the polyester plate (scale bar = 200 μm). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

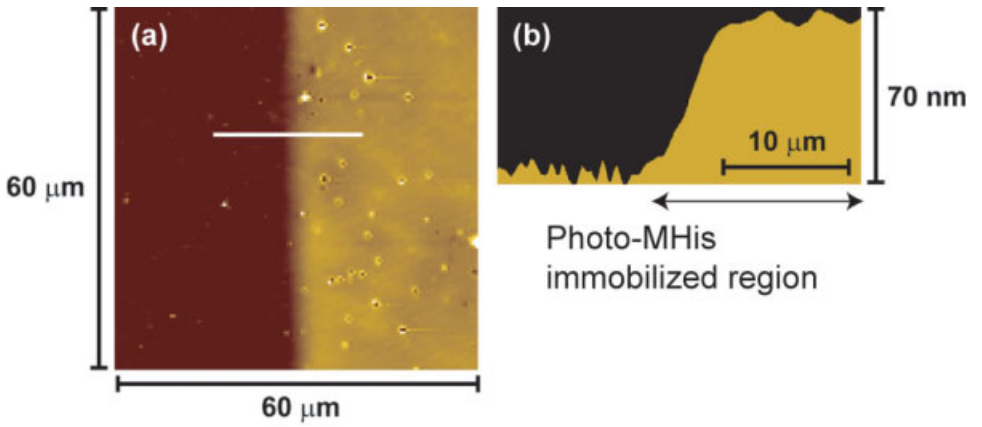

Figure 6 AFM images of (a) photo-MHis micropatterned on a polyester surface and (b) height profile data of the white line shown in image a. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

on the substrate surface. Figure 6 shows an AFM image of photo-MHis on Thermanox, which reveals the formation of microscale boundaries from the photomasking. This feature indicates that photo-MHis was properly immobilized and micropatterned on the substrate's surface. In addition, the observation of a nanoscale height of the polymer (ca. 70 nm) demonstrated that the photo-MHis was crosslinked with itself and also with the substrate molecules through the photoirradiation treatment. In the polymer-immobilized region, a number of microscale pores were observed that were not observed with other types of polymers. [3–7] The formation of these pores may be due to air bubbles from the drying procedure.

The surface properties of the immobilized photo-MHis without the use of a photomask were investigated with contact-angle measurements. The contact angle on the photo-MHis immobilized region was lower than that on the nonimmobilized region (Fig. 7; $P < 0.01$). This change in the contact angle was assumed to be due to the histidine polar groups.

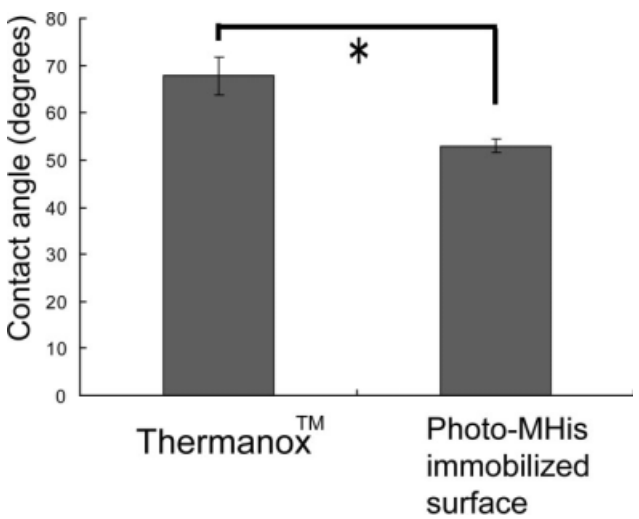

Figure 7 Static contact angle of a polymer-immobilized region and a nonimmobilized region of a polyester disk (*$P < 0.01$).

Interaction with biological components

The sample plates were immersed in the protein solutions, and the samples were stained with labeled proteins (Fig. 8). HRP–IgG and FITC–BSA predominantly adsorbed onto the nonimmobilized surface. The dye and FITC fluorescence intensity of the adsorbed proteins was significantly higher than that of photo-MHis alone. Previously, we reported that the adsorption of a polymer carrying histidine residues inhibited the adsorption of proteins and was accompanied by an increase in hydrophilicity.[15] This study confirms the data in previous reports.

STO cells seeded on the micropatterned surface are shown in Figure 9(a). The numbers of adhered cells and round-shaped cells were counted after a 3-h incubation period. No significant defect in cell

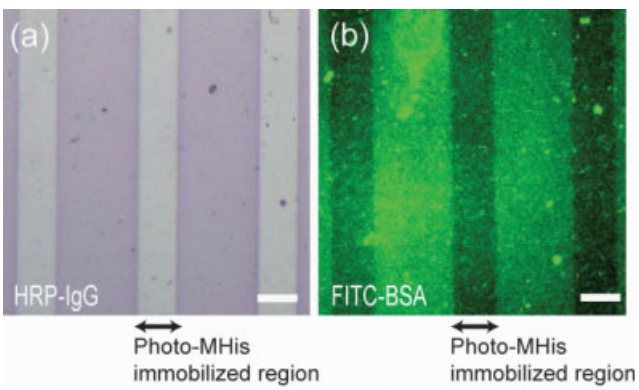

Figure 8 Fluorescence and phase-contrast micrographs of proteins adsorbed onto a photo-MHis micropatterned polyester surface: (a) HRP–IgG and (b) FITC–BSA (scale bar = 200 μm). HRP–IgG was stained with a blue dye. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

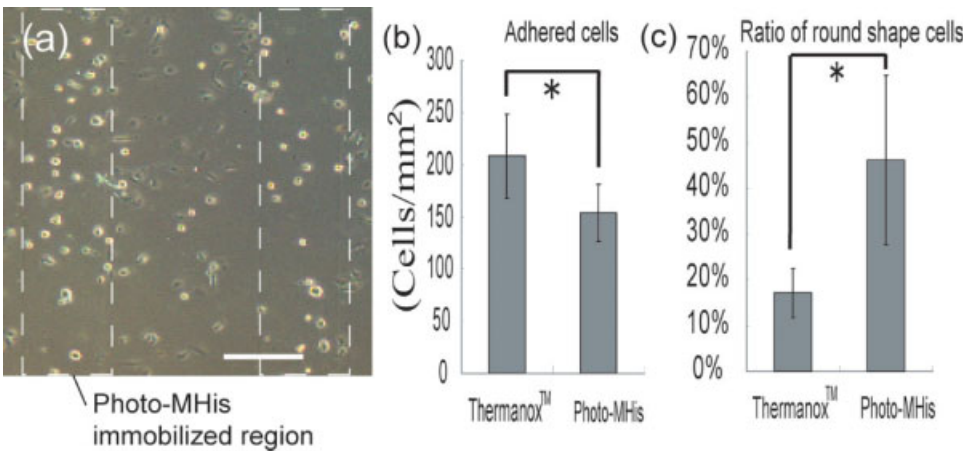

Figure 9 Cell adhesion on a polymer-immobilized region: (a) adhered STO cells on immobilized and nonimmobilized regions (polyester surface) of photo-MHis (observed with phase-contrast microscopy), (b) cell density on the polymer-immobilized and nonimmobilized regions, and (c) ratio of round-shaped cells in both regions (*$P < 0.01$; scale bar = 200 μm). The boxes denote the photo-MHis immobilized areas. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

viability or proliferation was observed in this culture period, even after a 3-day incubation period on the micropatterned plates (data not shown). When the polymer was immobilized on the polyester plates, the number of cells that adhered to the polymer-immobilized region was less than the number of cells that adhered to the nonimmobilized region [Fig. 9(b); $P < 0.01$]. The ratio of the round-shaped cells to the adhered but not spread cells was higher in the polymer-immobilized region [Fig. 9(c); $P < 0.01$]. This demonstrates that polymer immobilization reduces cell adhesion and spreading. The photoreactive polymer had a mild inhibitory effect on cell adhesion [it decreased to 75 ± 13% on the nontreated resin surface; Fig. 9(b)].

CONCLUSIONS

This study has demonstrated the photoimmobilization of a histidine-containing polymer and has visualized the interactions with biocomponents such as proteins and cells. Our photoimmobilization technique is applicable to surface modification, and the zwitterion polymers reduce the interactions with proteins and cells.

References

1. Mizutani, M.; Arnold, C. S.; Matsuda, T. Biomacromolecules 2002, 3, 668.
2. Ito, Y. Biotechnol Prog 2006, 22, 924.
3. Makino, H.; Hasuda, H.; Ito, Y. J Biosci Bioeng 2004, 98, 374.
4. Ito, Y. J Inorg Biochem 2000, 79, 77.
5. Ito, Y.; Hayashi, M.; Imanishi, Y. J Biomater Sci Polym Ed 2001, 12, 367.
6. Ito, Y.; Hasuda, H.; Sakuragi, M.; Tsuzuki, S. Acta Biomater 2007, 3, 1024.
7. Liu, H.; Ito, Y. J Biomed Mater Res A 2003, 67, 1424.
8. Ratner, B. D.; Hoffman, A. S.; Schoen, F. J.; Lemons, J. E. Biomaterials Science, 2nd ed.; Elsevier Academic: San Diego, 2004; p 197.
9. Zhang, Z.; Ma, H.; Hausner, D. B.; Chilkoti, A.; Beebe, T. P., Jr. Biomacromolecules 2005, 6, 3388.
10. Tamada, Y.; Ikada, Y. In Polymers in Medicine II; Chiellini, E.; Giusti, P.; Migliaresi, C.; Nicolis, L., Eds.; Plenum: New York, 1984; p 101.
11. Konno, T.; Hasuda, H.; Ishihara, K.; Ito, Y. Biomaterials 2005, 26, 1381.
12. Zhang, Z.; Chen, S.; Chang, Y.; Jiang, S. J Phys Chem B 2006, 110, 10799.
13. Zhang, Z.; Chao, T.; Chen, S.; Jiang, S. Langmuir 2006, 22, 10072.
14. Chang, Y.; Chen, S.; Zhang, Z.; Jiang, S. Langmuir 2006, 22, 2222.
15. Ishii, T.; Wada, A.; Tsuzuki, S.; Casolaro, M.; Ito, Y. Biomacromolecules 2007, 8, 3340.