Copolymers Including L-Histidine and Hydrophobic Moiety for Preparation of Nonbiofouling Surface

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A new type of copolymer composed of L-histidine (ampholyte) and *n*-butyl methacrylate (hydrophobic moiety) was developed for the preparation of nonbiofouling surfaces. The copolymer adsorbed onto resin surfaces and made the surface very hydrophilic. The hydrophilization effect was higher than that of bovine serum albumin (BSA). When polystyrene surfaces were coated with the copolymer, both the nonspecific adsorption of protein and the adhesion of cells were significantly reduced in comparison with BSA coating. The newly synthesized polymer is a new and useful candidate for the preparation of nonbiofouling surfaces.

Introduction

The preparation of nonfouling surfaces that prevent nonspecific adsorption of proteins and adhesion of cells is important in the development of therapeutic and diagnostic devices. Typically, surface modification with polyethylene glycol (PEG), PEGylation, has been performed for this purpose.[1] PEG is a nontoxic, nonimmunogenic, uncharged polymer that is soluble in water. The hydrophilicity, high mobility, large excluded volume, and steric hindrance effects of PEG contributed to surface-immobilized PEGs ability to resist cell adhesion and protein adsorption.[2−4]

In addition, biomimetic approaches using cell-surface-mimicking polymers have been investigated. One approach is to design interface materials based on the cell surface glycocalyx, which is a complex coating of highly glycosylated molecules that dominate the interface between a cell and its environment.[5,6] Another approach is based on cell surface lipids.[7−18] Nakabayashi and Ishihara have developed a useful polymer using this mimicking method.[7−12] They prepared a phospholipid polymer with a 2-methacryloyloxyethyl phosphatidylcholine (MPC) moiety and demonstrated that the polymer adsorbed onto materials surfaces to reduce interaction with various types of proteins and cells. Kitano et al.[19,20] reported that water-soluble neutral polymers do not disturb the structure of water significantly, whereas the electrostriction effect of polyelectrolytes is quite effective on the structure of water. In contrast, zwitterionic monomer residues do not disturb the hydrogen bonding between water molecules.

Recently, Zhang et al. demonstrated that grafting or adsorption of sulfobetaine- or carboxybetaine-based polymers significantly reduced protein adsorption onto surfaces.[21−25] They reported that the surfaces were capable of resisting nonspecific protein adsorption to a level comparable with well-packed oligo(ethylene glycol).[24,26] Recent studies attribute the nonfouling properties of oligo(ethylene glycol) to its strong hydration capability and well-packed structure.[27−29] Whereas hydrophilic and neutral oligo- or poly(ethylene glycol) form a hydration layer via hydrogen bonds, zwitterions form a hydration layer via electrostatic interactions.[18] It is expected that zwitterions are capable of binding significant quantities of water and are therefore potentially excellent candidates for nonfouling materials. Georgiev et al.[30] proposed an original theory for the explanation of the unique polyzwitterion nonbiofouling properties.

Assuming that ampholyte polymers, a special class of polyelectrolytes that contain both positive and negative charges along the macromolecular chain, do not disturb water structure, thus leading to a nonbiofouling surface,[31] other types of polyampholyte will possibly be candidates for nonbiofouling polymers. Considering that bovine serum albumin (BSA) is usually used as a nonbiofouling agent, amino acid-based polyampholytes may be useful agents. Some amino acid-based polyampholytes have been used in biomedical applications.[32−34] We have also already reported the biomedical applications of some amino acid-based polyampholytes and hydrogels.[35,36]

Here we designed an amino acid-based polyampholyte (a protein-mimicking polymer) that adsorbed onto a hydrophobic surface as the result of incorporation of a hydrophobic moiety into the polyampholyte. The polyampholyte consists of a weak acid (carboxylic acid) and a weak base (ammonium group) and, therefore, is different from other polyampholytes that have been previously reported, which are composed of strong acids, e.g., phosphoric acid[7−20] and sulfonic acid,[21−23,25,37] and strong bases, e.g., quaternary ammonium groups, for construction of a nonfouling surface. It was found that surfaces coated with such weak polyampholytes were very hydrophilic and efficiently inhibited adsorption of proteins and cells.

Materials and Methods

Materials. L-Histidine (98%), methacryloyl chloride (97%), and 2,2′-azoisobutyronitrile (AIBN, 98%) were purchased from Wako Pure

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Chemical Inc. (Osaka, Japan). AIBN was recrystallized from methanol. *n*-Butyl methacrylate was purchased from Kanto Chem. Inc. (Tokyo, Japan) and distilled under reduced pressure. All other chemical reagents were used as received. Bovine serum albumin was purchased from Sigma (St. Louis, MO). Lipidure was kindly provided by Nippon Oil (Tokyo, Japan).

Synthesis of *N*-Methacryloyl-L-histidine (MHis). *N*-Methacryloyl-L-histidine (MHis) was prepared according to the method previously reported by Okamoto.[38] L-Histidine (10 g, 64 mmol) was dissolved in 2 N NaOH (40 mL), and the aqueous solution was cooled in an ice bath. Methacryloyl chloride (7.3 mL, 76 mmol, 1.2 eq.) was dissolved in 20 mL of dioxane. The dioxane solution was added to the aqueous solution of L-histidine dropwise under a nitrogen atmosphere. During the addition, the reaction mixture was kept under 5 °C by external ice-bath cooling. After mixing, the solution was allowed to stand for 1 h at room temperature. After the reaction, the dioxane was evaporated and 6 N HCl was added until the solution reached pH 2. Unreacted chemicals and byproducts were removed by ether extraction. Subsequently, the pH of the aqueous solution was adjusted to 5 using 2 N NaOH, and the product was extracted with ethanol. By this process, L-histidine and NaCl were removed. The ethanol was removed and mixed with an excess of acetone to precipitate the product. The product was dissolved in ethanol and precipitated in acetone. The product was vacuum-dried overnight and MHis was obtained. ^{1}H NMR (400 MHz, D_2O): δ = 1.75 (s, 3H, C*H*$_3$C(=CH$_2$)H-), 2.95−3.23 (m, 2H, -C*H*$_2$-imidazole), 4.41−4.46 (q, H, -NHC*H*(COOH)CH$_2$-), 5.31−5.52 (m, 2H -C*H*$_2$C(CH$_3$)-), 7.12 (s, 1H imidazole, -C=C*H*N=), 8.44 (s, 1H imidazole, -N=C*H*NH-).

Polymerization. Poly(*N*-methacryloyl-L-histidine) (PMHis), poly(*n*-butyl methacrylate) (PBMA), and poly(*N*-methacryloyl-L-histidine-*co*-*n*-butyl methacrylate) (P(MHis/BMA)) copolymer were synthesized by conventional free-radical polymerization. PMHis was obtained as follows. The mixture of MHis and/or BMA (the total monomer was adjusted to 0.5 mmol) in ethanol (20 mL) containing AIBN (0.05 mmol) was purged with N_2 gas and then allowed to react under N_2 atmosphere at 70 °C for 20 h. The polymer obtained was purified using seamless cellophane dialysis tubing (MWCO 3500) in distilled water or ethanol for 2 days and then lyophilized to give a white powder. All of the obtained polymers were dissolved in a methanol/0.1 N $NaOH_{aq}$ (9/1 vol) mixture. A 0.5 wt % solution was used for polymer coating.

Polymer Characterization. Size exclusion chromatography (SEC) measurements were carried out using a TSK gel column (TSKgel α-M, TOSOH, Tokyo, Japan) and an internal refractive index (RI) detector. For PMHis, 0.1 M Tris buffer (pH 8.0, containing 0.2 M NaCl) was used as the eluent at a flow rate of 0.6 mL/min at 25 °C. For PBMA and P(MHis/BMA), DMF containing 10 mM lithium bromide was used as the eluent at a flow rate of 0.6 mL/min at 25 °C. Commercially available poly(ethylene oxide) or polystyrene were used for the calibration of PMHis and P(MHis/BMA) or PBMA chromatography, respectively. ^{1}H NMR spectra were monitored using a JEOL EX400 (Akishima, Japan) spectrometer at 400 MHz in D_2O or a D_2O/CD_3OD mixture. FT-IR spectra were monitored using a Shimadzu FTIR-8400S (Kyoto, Japan) equipped with an ATR attachment (Durasampl II, SensIR Tech., Danbury, CT).

Adsorption of Polymers onto Surfaces. To investigate the adsorption of polymers onto polystyrene surfaces, 0.1% (wt/v) polymer or BSA solution was added to 1.0 g of polystyrene beads (200−400 mesh) purchased from Tokyo Chem. Ind. Co., Ltd. (Tokyo, Japan) in test tubes. After vigorous shaking with a vortex mixer, the test tubes were centrifuged (1000 rpm, 5 min, r.t.) and the absorbance of the supernatant at 210 nm was measured. The amount of adsorbed polymer was estimated from a calibration curve.

Contact Angle Measurement. The static contact angles of air bubbles on the surfaces of polymer-coated substrates were measured with a contact angle meter DM 500 (Kyowa Interface Science, Saitama, Japan) at room temperature by the air-in-water method, which followed a captive bubble technique in which a sample film was immersed in water and a small air bubble was placed onto the film from the surface using a curved needle. The polymer films were prepared as follows. The polymer solution was cast onto a nontreated polystyrene substrate and dried in air for 3 h. The substrate was rinsed with PBS solution and immersed in distilled water just prior to use. An air bubble (1 μL) was attached to the immersed substrate, and the contact angle was measured at least 5 times to give a reliable average value.

Protein Adsorption. Protein adsorption was measured by two methods. One was the measurement of decreases in protein solution concentrations following adsorption of proteins onto the surfaces. The other was direct observation of the protein adsorbed onto the plates.

For the former measurement, a Protein Detector ELISA kit (HRP/ABTS system) from Kirkegaard & Perry Lab., Inc. (Gaithersburg, MD) was used for the quantitative evaluation of nonspecifically adsorbed proteins on the polymer coated surface. In brief, the wells of nontreated 96-well plates were filled with each polymer solution and then emptied immediately, and the polymer-coated wells were air-dried. In the case of BSA, the wells were filled with 1.0% BSA solution and allowed to stand for 1 h. The coated wells were washed with 200 μL of PBS solution at least three times to completely remove nonadsorbed polymer, and then 100 μL of horseradish peroxidase-labeled anti-mouse immunoglobulin (HRP-IgG) solution (0.2 μg/mL) was added and allowed to stand for 1 h to adsorb onto the well surface. The wells were washed at least three times with 200 μL of wash solution containing 0.02% Tween, and then 50 μL of peroxidase substrate (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid, ABTS) reaction solution was added and allowed to react until color developed sufficiently. Then 50 μL of stop solution was added, and the absorbance of an aliquot of the solution was measured at 415 nm using a microplate reader (Bio-Rad model 680, Bio-Rad Laboratories, Tokyo, Japan).

For the latter measurement, a chemical luminescent imaging assay was employed. A total of 3 μL of a solution of horseradish peroxidase-linked bovine serum albumin in PBS (500 ng/mL, HRP−BSA, Rockland) was added to the noncoated or polymer-coated plates and allowed to stand for 15 min. Subsequently, the surfaces were washed twice with PBS for 3 min each time. The chemical luminescence reaction was performed with 10 μL of ECL advance solution (GE Healthcare) for 3 min at 20 °C. The reaction area was surrounded with liquid blocker (Daido Sangyo, Japan) to prevent the reaction solution from running over. The chemical luminescent images were measured using a Light Capture system (ATTO Corporation, Japan). Calibration was performed using HRP−BSA of known concentrations and ECL advance solution.

Cell Culture. Mouse osteoblast cells (MC3T3-E1) purchased from the RIKEN Cell Bank (Tsukuba, Ibaraki, Japan) were cultured on culture dishes (Corning Co., Ltd., Corning, NY) containing medium composed of minimum essential medium (MEM-α, Kohjin Bio Co. Ltd., Sakado, Japan) supplemented with 10% fetal bovine serum (FBS, BioWest, Nuaille, France) in a fully humidified atmosphere with a volume fraction of 5% CO_2 at 37 °C.

For the investigation of cell adhesion, 100 μL of each polymer solution was precoated onto each well of nontreated 12-well plates (IWAKI, Tokyo, Japan). They were then dried in air and rinsed twice with PBS. The cells were harvested with a 0.25% trypsin solution containing 0.5 mM EDTA. The recovered cells were then washed with culture medium and suspended in the medium. The cell suspensions were seeded at 4×10^3 cells/cm^2 onto polymer-precoated wells and allowed to stand for 5 h in a fully humidified atmosphere with a volume fraction of 5% CO_2 at 37 °C. After incubation, the number of adherent cells in a certain area was counted by microscopy.

To investigate the cytotoxicity of the polymers, the cells were cultured for 2 days and the cell number evaluated using a Cell Counting Kit (WST-1 method, Dojindo Lab., Kumamoto, Japan).[39] Briefly, after the MC3T3-E1 cells reached confluence, they were trypsinized and seeded at 1×10^4 cells/cm^2 into 96-well microplates (Corning Co., Ltd.) and then incubated for 2 days in a humidified atmosphere containing 5% CO_2 at 37 °C. After removing the cultured medium,

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Table 1. Molecular Weights and Composition of Prepared Copolymers

abbreviation	MHis composition in feed (mol %)	M_w (M_w/M_n)	MHis composition in copolymer (mol %)
PMHis	100	9.2×10^4 (2.54)	100
P(MHis/BMA)_7:3	70		72.0
P(MHis/BMA)_5:5	50	2.3×10^4 (2.33)	50.1
P(MHis/BMA)_3:7	30	1.6×10^4 (2.22)	28.7
P(MHis/BMA)_1:9	10	3.5×10^4 (1.90)	8.6
PBMA	0	3.2×10^4 (1.76)	0

100 μL of MHis, PMHis, and P(MHis/BMA) solution (or suspension) in culture medium supplemented with 10% (v/v) FBS was added to each well and allowed to stand in a fully humidified atmosphere with a volume fraction of 5% CO_2 at 37 °C. The MHis concentration of P(MHis/BMA) indicates the MHis monomer concentration in the polymer. After 24 h incubation, 10 μL of WST-1 reagent was added to each well and incubated for 2 h at 37 °C, and then 10 μL of 0.1 N HCl aqueous solution was added to each well to stop the reaction. To remove insoluble copolymer, the plate was centrifuged (1000 rpm, 5 min), and then 50 μL of the supernatant was transferred to another plate. The absorbance of an aliquot of the solution was measured at 450 nm, with reference to the absorbance at 655 nm, using a microplate reader (Bio-Rad model 680).

Results and Discussion

Polymer Properties. The molecular weights and the chemical compositions of copolymers were measured by SEC and elemental analysis, respectively. The copolymer compositions were almost the same as the composition of the feed, and the molecular weights were as expected from the monomer/initiator ratios as shown in Table 1.

The solubilities of the copolymers were different from that of the homopolymer. Although the L-histidine homopolymer is only soluble in water, all of the copolymers including the homopolymer were soluble in a mixture of water and methanol. Because alcohol does not usually affect resin surfaces, it is a good solvent for coating polymers without significant influence on the surface properties of resins. Lipidure coating is also performed with alcohol. Considering these results, a methanol/water cosolvent (pH 12.4) was employed for further experiments.

Coating with Polymers. The polymers were solubilized in a mixture of water and methanol. Polystyrene beads were incubated in these solutions and the amounts of polymer adsorbed were determined as shown in Figure 1. The homopolymer PMHis hardly adsorbed onto the polystyrene beads. However, with increases in the *n*-butyl methacrylate composition, the amount of adsorbed polymer increased. These results indicate that the hydrophobic component of *n*-butyl methacrylate contributed to the adsorption of the polymers through their hydrophobicity. In the reported design of Lipidure, it was noted that *n*-butyl methacrylate was employed for enhancement of adsorption.[10] Chang et al.[23] reported diblock copolymer containing poly(sulfobetaine methacrylate) with poly(propylene oxide) as a hydrophobic moiety for coating material.

Table 2 shows the contact angles of air bubbles in water. The higher value of θ indicates the higher hydrophilicity. Polymer adsorption significantly enhanced the hydrophilicity of the polystyrene surfaces. The enhancement effect of all copolymers was higher than that of BSA and was independent

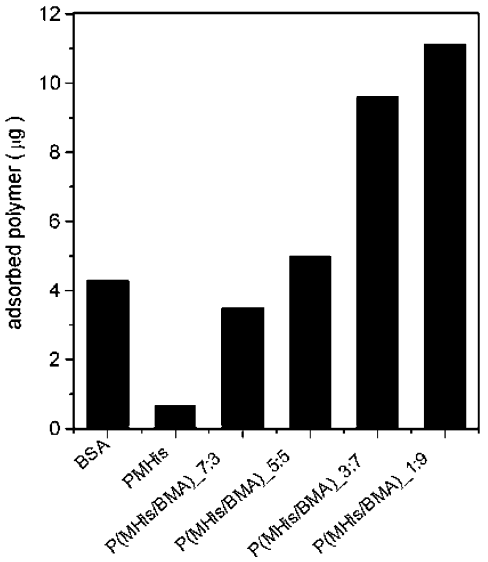
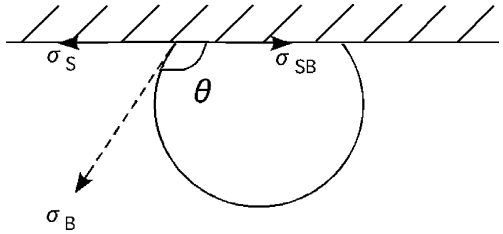


Figure 1. Amounts of polymers adsorbed onto polystyrene beads (1 g). As PBMA does not have UV absorption, its adsorption was not measured. $n = 3$.

Table 2. Contact Angles of the Polymer-Coated Substrate



polymers for coating	coating solvent	contact angle θ (deg)
none		117.6 ± 5.5
PMHis	methanol/0.1 N NaOH(aq) (9:1)	138.9 ± 2.7
P(MHis/BMA)_7:3	methanol/0.1 N NaOH(aq) (9:1)	165.0 ± 1.1
P(MHis/BMA)_5:5	methanol/0.1 N NaOH(aq) (9:1)	162.8 ± 1.2
P(MHis/BMA)_3:7	methanol/0.1 N NaOH(aq) (9:1)	163.6 ± 0.7
P(MHis/BMA)_1:9	methanol/0.1 N NaOH(aq) (9:1)	163.6 ± 1.8
PBMA	ethanol	124.4 ± 2.8
BSA	phosphate-buffered solution	151.5 ± 7.3
Lipidure	ethanol/water (1:1)	159.7 ± 2.7

of the MHis composition, although it was difficult to directly compare the effects of the coating materials because of the use of different coating solvents. This result indicates that the L-histidine residues significantly contributed to the hydrophilicity of the copolymers. The low effect of the homopolymer PMHis is considered to be due to low adsorption onto the surface.

Nonfouling Properties. Figure 2A shows HRP-IgG adsorption onto the polymer-coated resin. With copolymer coating, the adsorption of HRP-IgG was significantly reduced. In particular, the copolymer containing 50% histidine almost completely inhibited nonspecific adsorption of IgG. The lower nonfouling effect of PMHis was considered to be the low coverage of the surface and resulted low hydrophilicity. To investigate this result in detail, the enzyme reaction time was increased and the effect was enhanced as shown in Figure 2B. The reduction effect was higher than for BSA or Lipidure, which are usually employed for reduction of nonspecific adsorption of proteins in enzyme-linked immunosorbent assays (ELISA).

Figure 3 shows the direct observation by chemical luminescence of adsorbed HRP−BSA on polystyrene plates. The protein

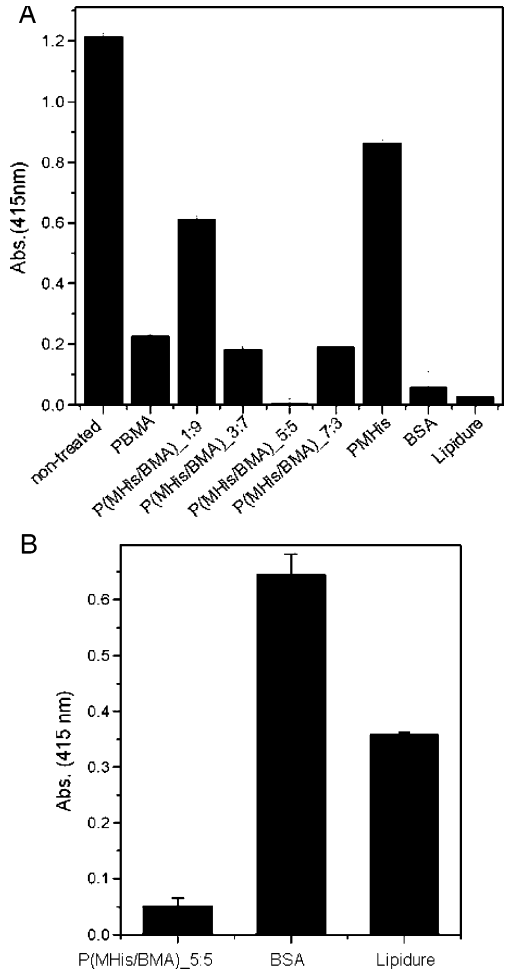


Figure 2. Adsorption of HRP-IgG onto non- or polymer-coated polystyrene plates. Incubation times were 5 (A) and 120 min (B). $n = 3$.

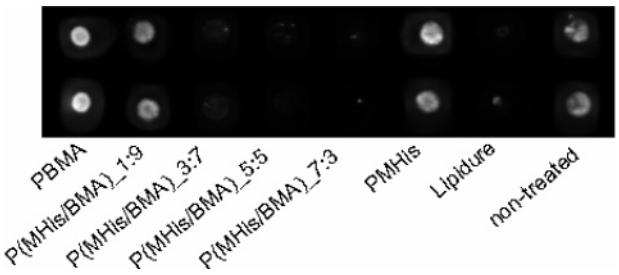


Figure 3. Chemical luminescence images of HRP−BSA on non- or polymer-coated polystyrene plates.

was highly adsorbed on unmodified, PBMA-coated, and PMHis-coated plates. However, on the copolymer containing a high content of MHis, the adsorption was significantly less. The protein adsorption was quantitatively evaluated and is shown in Figure 4. Although it is very difficult to directly compare the results in Figures 2 and 4, because of the differences in proteins and experimental conditions, it was concluded that the copolymer containing the higher content of MHis apparently reduced protein adsorption, comparable to using Lipidure or BSA.

Figure 5 shows photos of cells adhered on various surfaces. The ratio of round-shaped cells to spread ones was higher on the copolymer-coated surfaces than on the nontreated or BSA-coated surfaces. In the comparison with Lipidure-coated surfaces, no spreading cells were found on the P(MHis/BMA)_5:5-coated surfaces. These round cells were easily washed away.

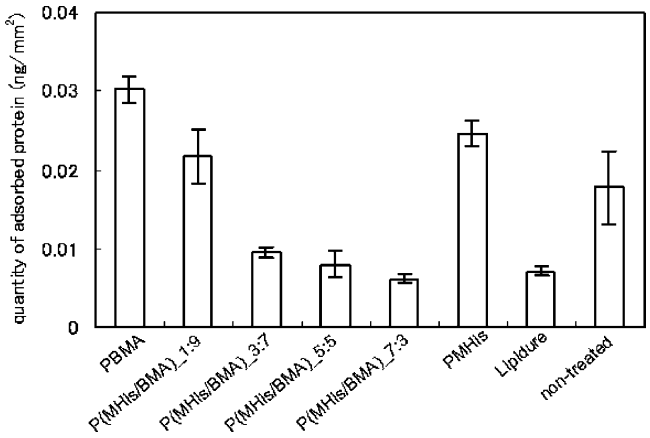


Figure 4. Amount of HRP-BSA adsorbed on non- and polymer-coated polystyrene plates. $n = 3$.

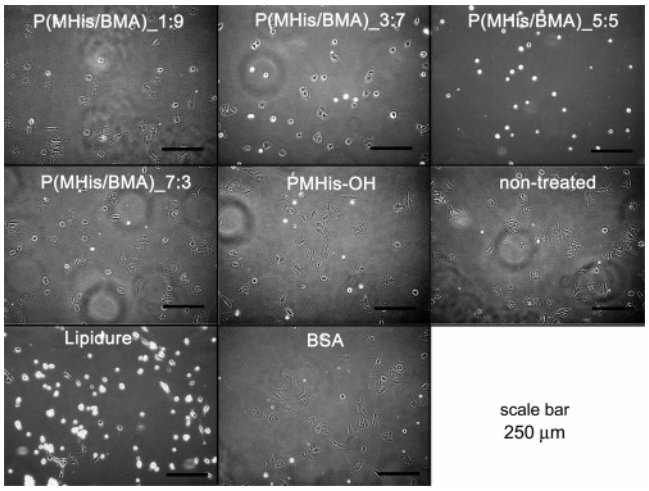


Figure 5. Phase contrast micrographs of adherent cells on polymer-coated polystyrene plates for 5 h.

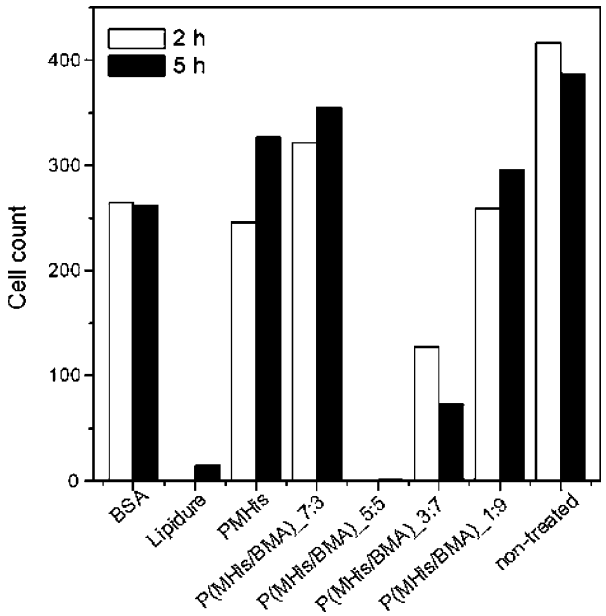


Figure 6. Number of cells adherent on polymer-coated polystyrene plate (cells/mm²). $n = 3$.

Therefore, the copolymer containing 50% histidine almost completely inhibited the adhesion of cells, as shown in Figure 6, an effect comparable to that of Lipidure. No cell adhesion was observed on P(MHis/BMA)_5:5-coated surfaces, even after

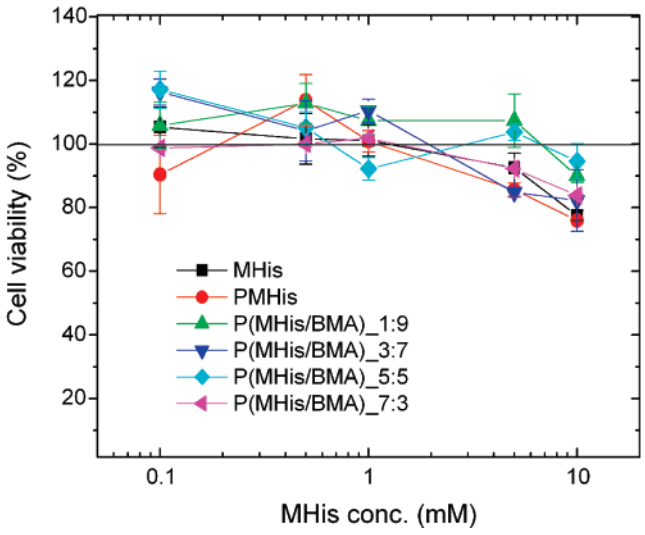


Figure 7. Viability of cells cultured in the presence of polymers for 2 days. $n = 3$.

5 h. The copolymers containing smaller ratios of histidine did not have adequate inhibitory effects.

Cytotoxicity. To evaluate the cytotoxicity of the prepared polymers, cell culture was performed in the presence of the polymers. We have already reported no cytotoxicity for polymers containing histidine residues.[35,36] Here we also found no significant cytotoxicity of these new polymers up to 5 mM, as shown in Figure 7.

In comparison with nonbiofouling polyampholytes carrying phosphatydylcholine residues investigated by other researchers,[7−20] the copolymers that were synthesized in this investigation had weak acid and base groups. However, similar or greater effects were observed for our polymers. As our polymer is based on an amino acid, this mild zwitterion polymer will be important as a new nonbiofouling polymer.

Conclusion

This study demonstrated the synthesis of alcohol-soluble polyzwitterions by the copolymerization of *N*-methacryloyl-L-histidine and a hydrophobic monomer. Coating with the copolymer enhanced hydrophilicity and was efficient for the preparation of nonbiofouling surfaces active against protein and cell adhesion. In particular, the copolymer containing about 50% content of histidine monomer was the most suitable candidate for nonfouling for both proteins and cells. In addition, the copolymer was nontoxic. Therefore, the copolymer will be useful as a new nonbiofouling agent.

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