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ORIGINAL ARTICLE

A low-fouling polymer surface prepared by controlled segregation of poly(ethylene oxide) and its functionalization with biomolecules

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A polyethylene terephthalate polymer surface was modified by dip coating in a polymer mixture composed of methacrylate-based terpolymers containing both perfluoroalkyl (R_f) groups and poly(ethylene oxide) (PEO) side chains, vinylidenefluoride-tetrafluoroethylene copolymers ($(CH_2CF_2)_{80}(CF_2CF_2)_{20}$) and poly(methyl methacrylate). The terpolymers contained R_f groups with various numbers of carbons. After the dip coating, both the R_f groups and the PEO chains predominantly segregated on the top surface. The modified surfaces were characterized with X-ray photoelectron spectroscopy, protein adsorption, thrombogenesis and cell adhesion. The results indicate that because of the surface-segregated PEO chains, the surfaces exhibited resistance to nonspecific protein adsorption, antithrombogenicity and resistance to cell adhesion. In addition, covalent conjugation of biotin with the PEO termini enabled selective immobilization of streptavidin from a mixed protein solution on the dip-coated surface.
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INTRODUCTION

Specific detection of biomolecules, such as antibodies and enzymes, has commonly involved immobilization and conjugation on substrate surfaces. Because antibodies bind to antigens very selectively, antigen–antibody association is widely used in medical diagnostics and biosensing. Preparation of specialized surfaces is essential for biosensors and biochips.[1–4] However, nonspecific protein adsorption (fouling) on these surfaces can be a significant problem. Thus considerable effort has been expended on the creation of non-fouling surfaces. For example, in a microplate-based immunoassay, amphiphilic animal proteins are widely used as blocking agents to mask a hydrophobic polymeric surface and prevent protein fouling. However, a polymeric surface blocked by bovine serum albumin (BSA), for example, does not completely prevent nonspecific protein adsorption, and BSA blocking sometimes interferes with antibody–antigen recognition.[5]

Poly(ethylene oxide) (PEO) is a flexible, hydrophilic, electrostatically neutral and biocompatible polymer. A PEO-modified surface is non-toxic and resists nonspecific protein adsorption.[6–9] Several methods have been reported to segregate PEO chains on surfaces, such as copolymerization of PEO macromonomers and covalent grafting of PEO.[10,11] Growth of PEO on a hydrophobic surface was performed using an argon plasma, where the PEO surface density was controlled by the exposure time.[12] The PEO-coated surface exhibited hydrophilicity and low protein adsorption.

Previously, we prepared methacrylate-based terpolymers containing both perfluoroalkyl (R_f) groups and PEO chains as surface modifiers. Surface-segregation of the PEO chains was achieved via simple dip coating. The modified surfaces were covered with R_f groups and PEO chains in a balance of the surface-free energy, the free volume effect of PEO chains and its interaction to a matrix resin.[13] The modified surface simultaneously exhibited significant hydrophobicity and adhesive strength.

Here we synthesized methacrylate-based terpolymers that contained both PEO side chains and R_f groups. Modified polyethylene terephthalate (PET) surfaces were prepared by dip coating in the terpolymer solutions. Protein adsorption, antithrombogenic tests and cell adhesion confirmed the low-fouling properties of the terpolymer-modified surfaces. In addition, we performed specific covalent immobilization of a functional biomolecule by using the PEO chain termini as reactive sites.

EXPERIMENTAL PROCEDURE

Materials

Methyl methacrylate (MMA, Nacalai Tesque, Inc., Kyoto, Japan) was distilled under reduced pressure before use. 2,2′-Azobisisobutyronitrile (AIBN, Nacalai Tesque, Inc.) was recrystallized from methanol. The fluorine-containing monomer 2-(perfluoroalkyl) ethyl acrylate (PFEA-4 and -6, DIC Ltd., Tokyo, Japan; PFEA-8, Clariant Corp., Tokyo, Japan), where 4, 6 and 8 are the number of carbons in the perfluoroalkyl groups, was used as received. PEO-containing macromonomers (PEO-OH: BLEMMER PE-350D, NOF Co., Tokyo, Japan) were used as received. Poly (methyl methacrylate) (PMMA) (Acrypet VH, Mitsubishi Rayon Co. Ltd., $M_n = 41\,100$, Tokyo, Japan) was purified by re-precipitation by pouring a PMMA/methyl ethyl ketone (MEK, Nacalai Tesque, Inc.) solution into methanol, which was then used as a matrix resin for dip

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coating. Another matrix resin, vinylidenefluoride-tetrafluoroethylene copolymer ($(CH_2CF_2)_{80}(CF_2CF_2)_{20}$, $M_n = 69{,}300$, Kynar SL, Atfina Chemicals Inc., Philadelphia, PA, USA) (P(2F-4F)), was used without further purification.

BSA and fibrinogen were purchased from Sigma Co. (St Louis, MO, USA). Eagle's minimal essential medium (MEM) containing kanamycin was purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and biotin hydrazide were purchased from Indofine Chemical Company Inc. (Hillsborough, NJ, USA) and Dojindo Molecular Technologies, Inc. (Kumamoto, Japan), respectively. AlexaFluor 488-labeled streptavidin and AlexaFluor 647-labeled BSA (0.1 mg ml^{-1}, Life Technologies Co., Carlsbad, CA, USA) were used as fluorescence-labeled proteins. Other chemicals were purchased from Nacalai Tesque, Inc.

Fresh whole blood supplied from a healthy human donor was collected at the medical center for student health at Kobe University. All samples were obtained in accordance with ethical committee regulations.

Sample preparation

The terpolymer structures are shown in Figure 1. The detailed synthesis of each terpolymer P(MMA/PFEA-n/PEO-OH), $n = 4$, 6, or 8) was described previously.[13] Briefly, the terpolymers were synthesized by free-radical polymerization using MMA, 2-(perfluoroalkyl) ethyl acrylate, PEO-containing macromonomers and an AIBN initiator (5% w/w vs monomers) in ethyl acetate at 75 °C for 7 h. The monomer concentration was 30% w/w. After polymerization, an excess of hexane was added to the reactant solution at room temperature to precipitate the terpolymer, which was re-precipitated from an acetone solution of P(MMA/PFEA-n/PEO-OH) using distilled water, followed by drying at 40 °C. The actual monomer compositions of these terpolymers were determined to be MMA:PFEA-n:PEO-OH = 1000:69:90 ($n = 4$), 1000:47:114 ($n = 6$) and 1000:27:110 ($n = 8$) from the elemental analysis.

PMMA, P(2F-4F) and a terpolymer (PMMA/P(2F-4F)/terpolymer = 5/4/1, weight ratio) were co-dissolved into MEK/methyl isobutyl ketone (7/3 weight ratio) to yield a 10% w/w clear solution. After being stirred overnight, the solution was dip coated on a PET film, followed by annealing at 140 °C for 1 h. This polymer-coated surface (thickness = 5–15 μm) is hereafter referred to as the 'modified surface.'

Characterization of fouling

Sample films were immersed in a BSA solution (1 mg ml^{-1}, in phosphate-buffered saline (PBS)) for 2.5 h at room temperature and were then rinsed three times in distilled water to remove weakly adsorbed BSA. The amount of remaining adsorbed BSA was determined by the micro bicinchoninic acid method,[14] in which the absorbance change at 562 nm was measured using an ultraviolet-visible spectrophotometer (U-2000; Hitachi Ltd, Tokyo, Japan). Eight samples were tested with an unmodified PET film as a reference. The adsorption of fibrinogen was also evaluated by the micro bicinchoninic acid method.

For X-ray photoelectron spectroscopy (XPS), films were immersed in each protein solution for 24 h at room temperature and rinsed three times in distilled water. The films were then dried at room temperature overnight.

Square pieces (1×1 cm^2) of bare and modified PET films were immersed in fresh human whole blood for 20 min and rinsed in PBS solution three times. Then the films were immersed in a glutaraldehyde solution at 37 °C for 24 h to fix the adsorbed blood, followed by drying at room temperature for 24 h.

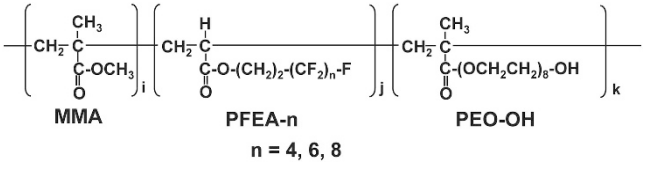


Figure 1 Chemical structures of P(MMA/PFEA-n/PEO-OH). The ratios of i:j:k were 1000:69:90 for $n = 4$, 1000:47:114 for $n = 6$ and 1000:27:110 for $n = 6$.

For cell adhesion studies, the films were sterilized by immersion for 5 h in Eagle's MEM containing 60 mg ml^{-1} kanamycin. L929 mouse fibroblast cells were cultured on the modified PET films in Eagle's MEM supplemented with 10% fetal bovine serum, 1% v/v L-glutamine and 2% v/v sodium hydrogen carbonate at 37 °C in a humidified atmosphere with 5% CO_2. The pH of the medium was adjusted to 7.4, and L929 cells were seeded on the sterilized films at a population density of 0.5×10^5 cells ml^{-1}. After culturing, the films were gently rinsed twice with approximately 1.5 ml of PBS to remove weakly adhered cells. The films were imaged with a phase-contrast microscope (PM-10AK, Olympus Co., Tokyo, Japan), and the cell density was determined by counting the number of cells per unit area. The experiments were performed in triplicate, and the data were averaged.

X-ray photoelectron spectroscopy

To investigate the surface composition of the films, XPS was performed with an ESCA-850 (Shimadzu, Kyoto, Japan). Spectra generated from Mg $K\alpha$ radiation at 8 kV and 30 mA were collected at a 90° take-off angle between the sample and the analyzer. The pressure in the instrumental chamber was $< 1.0 \times 10^{-5}$ Pa. No radiation damage was observed.

Dynamic contact angle measurements

To estimate the surface wettability, dynamic contact angles were measured. The dynamic contact angle of water was measured at room temperature. The advancing contact angle (θ_a) and receding contact angle (θ_r) were measured while the droplet enlarged (< 2 mm diameter) and reduced in size, respectively. An average of 15 readings was used for the contact angle determination.

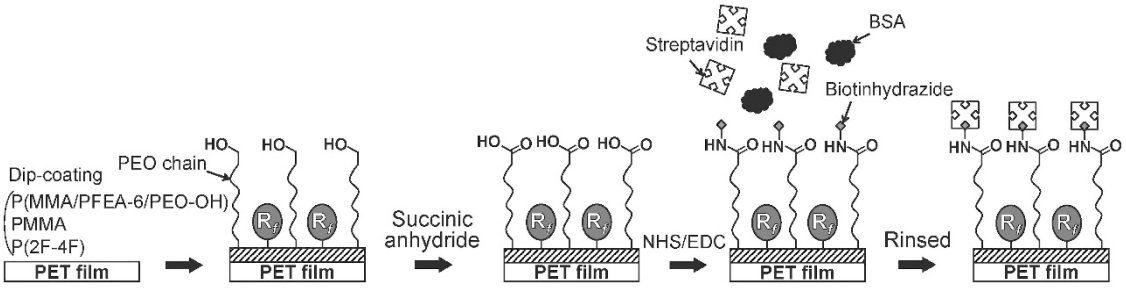
Immobilization of fluorescent compounds on modified surfaces

PET films (0.7×1.5 cm) modified with a terpolymer P(MMA/PFEA-6/PEO-OH) were characterized for streptavidin immobilization. (Hereafter, the P(MMA/PFEA-6/PEO-OH) modified surfaces are denoted as 'PEO-OH'.) To convert the PEO hydroxy groups to carboxy groups, the modified film was immersed in distilled water containing succinic anhydride (20% w/w) and a drop of perchloric acid catalyst. After 24 h at room temperature, the film was rinsed with distilled water and dried overnight at room temperature. The modified surface was immersed in water containing NHS (100 mM) and EDC (400 mM) for 20 min at room temperature to convert the carboxy groups to reactive NHS esters. Subsequently, the film was immersed in a solution of biotin hydrazide (10 mM in ethanol/water = 2/8 v/v) for 1 h at room temperature. To remove unreacted NHS esters, the films were immersed in a sodium borate buffer (pH 9.0) for 2 h at room temperature.[15] The films were then transferred to PBS (pH 7.4) containing AlexaFluor 488-labeled streptavidin (0.1 mg ml^{-1}) or AlexaFluor 647-labeled BSA (0.1 mg ml^{-1}), followed by consecutive rinsing in PBS and distilled water, respectively. The fluorescently labeled streptavidin was excited at 460 nm and imaged at 515 nm, whereas the labeled BSA was excited at 630 nm and imaged at 670 nm using an Image Quant LAS 4000 (GE Healthcare, Buckinghamshire, UK). A schematic of the dip-coating procedure for preparing the modified surfaces is shown in Scheme 1.

RESULTS AND DISCUSSION

Surface segregation of PEO chains

Previously, we reported the surface segregation of perfluoroalkyl (R_f) groups and PEO chains in the methacrylate-based terpolymer when the terpolymer was dip coated on a substrate with P(2F-4F) and PMMA.[13] Figure 2a shows the XPS C_{1s} spectra of the terpolymer-modified surface. In this case, the surface modification was performed by dip coating a mixture of P(MMA/PFEA-8/PEO-OH), P(2F-4F) and PMMA. The C_{1s} spectrum assignment agreed well with previous reports.[16,17] The curve resolving was performed using a set of Gaussian peaks in the Fityk software (http://www.unipress.waw.pl/fityk/) under the following conditions: the area of the CH_2-(CF_2) peak was equal to that of the CF_2-(CH_2) and the area of the C-O-(C = O) peak was equal to that of the C = O peak. Figure 2b shows the C-O atomic percent



Scheme 1 Preparation of a low-fouling surface using dip coating and immobilization of streptavidin. R_f represents a perfluoroalkyl group.

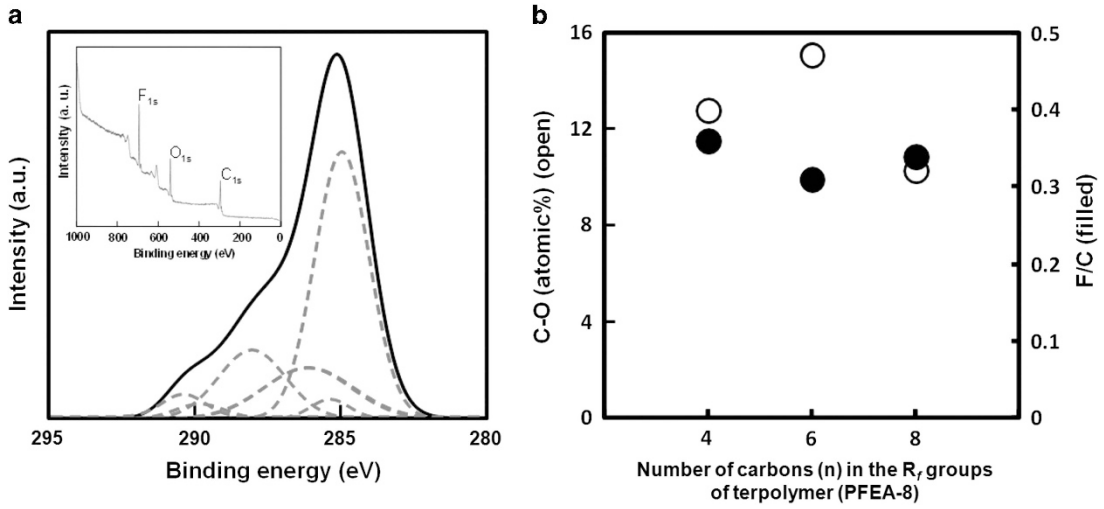


Figure 2 (**a**) Typical XPS C_{1s} narrow spectrum of the terpolymer-modified surface. The surface modification was performed by dip coating a mixture of P(MMA/PFEA-8/PEO-OH), P(2F-4F) and PMMA. Inset represents the XPS wide spectrum. The incident angle of the X-ray was 90°. (**b**) Effect of the number of carbons (n) in the R_f groups of the terpolymers on the C-O atomic percent and on the F/C atomic ratio of the terpolymer-modified surface. The C-O atomic percent and the F/C atomic ratios were determined by XPS measurements.

Table 1 Dynamic contact angles of water on terpolymer-modified surfaces

	PET	*PFEA-4*	*PFEA-6*	*PFEA-8*
θ_a	83.2±1.9	88.8±3.4	93.6±2.8	92.4±2.6
θ_r	45.5±1.5	42.1±4.0	35.4±3.4	36.6±2.9

Abbreviations: PET, polyethylene terephthalate; PFEA, 2-(perfluoroalkyl) ethyl acrylate.
PFEA-4, PFEA-6 and PFEA-8 represent PET films coated with a mixture of P(MMA/PFEA-n/PEO-OH), P(2F-4F) and PMMA.

and F/C atomic ratios of the surfaces modified with terpolymers. The C-O atomic percent was mostly assigned to the ether bonds of the PEO chains, which were found to be present on the modified surface in all cases. In particular, a relatively large amount of PEO chains was found on the PFEA-6-modified surface. Figure 2b also shows the small effect of the number of carbons (n) in the R_f groups of the terpolymer on the F/C atomic ratio on the surface, whereas the amount of segregated PEO chains depended on the number of carbons.

To examine the surface wettability, dynamic contact angle measurements were performed. Table 1 shows dynamic contact angles of water on terpolymer-modified surfaces. In each case, the terpolymer-modified surface showed relatively high θ_a because of the surface-segregated R_f groups as well as relatively low θ_r because of the surface-segregated PEO chains. These results indicate that whether the R_f groups or the PEO chains dominate the surface property can be changed depending on the contact medium.

Resistance to protein adsorption

Figure 3 plots the amounts of BSA and fibrinogen adsorbed on the modified surfaces. These proteins are relatively hydrophobic and are widely used as fouling models. A bare PET surface adsorbed 1.2 $\mu g\,cm^{-2}$ of BSA, which was much greater that that observed for the terpolymer-modified surfaces. For example, a PFEA-8-modified surface adsorbed less than one-third of that amount by the bare PET surface. The terpolymer-modified surfaces were also highly effective in reducing fibrinogen adsorption. For example, PFEA-4 modification reduced fibrinogen adsorption to 0.30 $\mu g\,cm^{-2}$ compared with the adsorption of 1.1 $\mu g\,cm^{-2}$ observed on bare PET. The resistance to protein adsorption varied with the number of carbons in the terpolymer R_f groups. As reported previously, dip coating the terpolymer along with PMMA and P(2F-4F) introduced PEO chains on the surface. The PEO chains predominantly segregated on the top surface when exposed to an aqueous environment. Moreover, it was found that incorporation of P(2F-4F) to the dip-coating polymers promoted PEO segregation. Thus the terpolymer-modified surfaces exhibited resistance to nonspecific protein adsorption that originated from the excluded volume effect and the high mobility of water-soluble PEO chains.

XPS was also used to detect protein adsorption, as shown in the N_{1s} spectra in Figure 4.[18] A bare PET surface exhibited an N_{1s} peak at 402 eV because of the adsorbed BSA. The N_{1s} peak is indicative of the relative amount of proteins present.[19] By contrast,

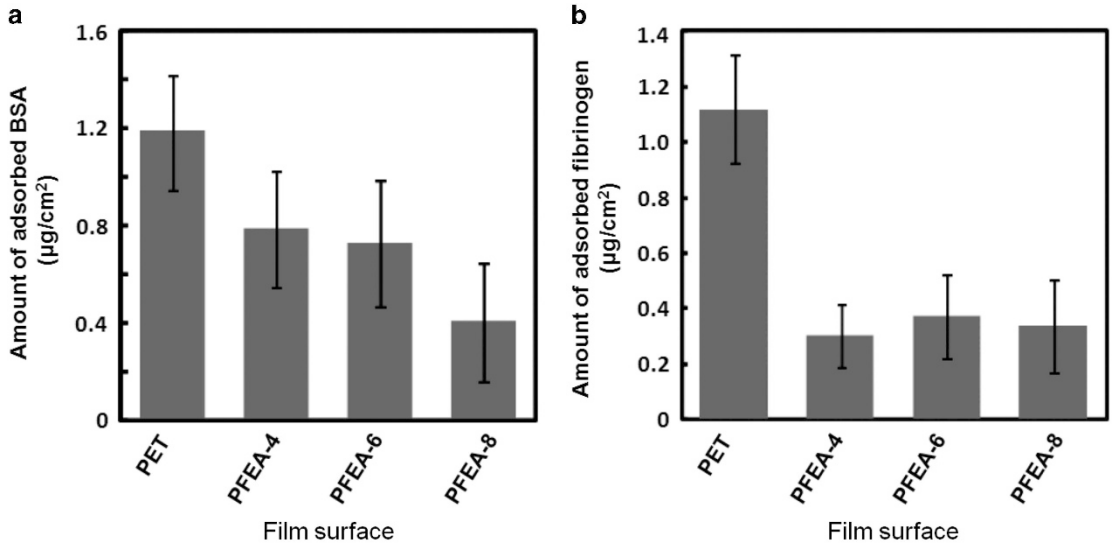


Figure 3 Amounts of (**a**) BSA and (**b**) fibrinogen adsorbed on bare and modified PET surfaces. The surface modification was performed by dip coating a mixture of P(MMA/PFEA-n/PEO-OH), P(2F-4F) and PMMA. Error bars represent s.ds.

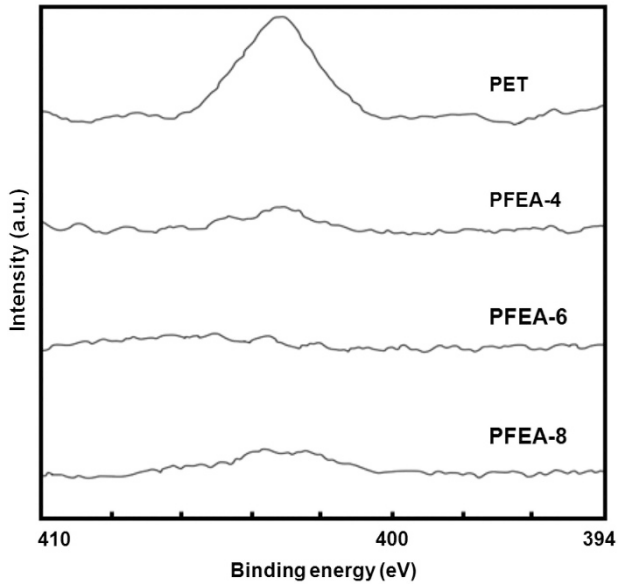


Figure 4 XPS N_{1s} narrow spectra of bare PET and terpolymer-modified surfaces after BSA adsorption. PFEA-4, PFEA-6 and PFEA-8 represent PET films coated with a mixture of P(MMA/PFEA-n/PEO-OH), P(2F-4F) and PMMA.

the terpolymer-modified surfaces exhibited significantly lower N_{1s} intensities, indicating reduced BSA adsorption.

Antithrombogenicity

Above, we discussed the adsorption of purified proteins on the modified surfaces. In practical terms, however, foulants are usually a mixture of proteins and other compounds. Because blood contains a wide variety of foulants that cooperatively adsorb on polymeric surfaces, we used whole human blood to examine the antithrombogenicity of the terpolymer-modified surfaces. Figure 5 shows no blood clots on the terpolymer-modified surfaces, whereas there were numerous clots on the bare PET surface. Clotting is typically caused by the aggregation and adhesion of platelets triggered by the adsorption of fibrinogen, a blood protein. The antithrombogenicity thus results from the protein (fibrinogen)-repellent terpolymer-modified surfaces.[20,21]

Resistance to cell adhesion

Cell adhesion to a polymeric surface is also known to be initiated by protein adsorption.[22] For example, proteins have a key role in the adhesion of L929 cells and in cell propagation.[23] Therefore we investigated L929 cell adhesion on bare PET and terpolymer-modified surfaces. Figure 6A shows cell adhesion on PET film surfaces, where the lower half of each sample was coated with terpolymers. After 2 days of culturing, a considerable number of cells adhered on the upper (bare PET) halves of the film surfaces (Figures 6Aa–c). By contrast, the terpolymer-modified surfaces (lower halves) exhibited negligible cell adhesion.

For quantitative evaluation of the cell adhesion, L929 cells that adhered on the film surfaces were counted over 3 days. Figure 6B shows the effect of culture time on the cell density on the bare PET and the terpolymer-modified surfaces. Cell densities on all of the modified surfaces were only one-eighth that of the bare PET film. Thus surface modification with terpolymers can prevent both cell adhesion and propagation. The PFEA-6-modified surface showed relatively high resistance to cell adhesion, which is attributed to the amount of PEO chains segregated on the surface. Indeed, Figure 2b shows a relatively large amount of PEO chains on the PFEA-6-modified surface. The reduced cell adhesion was most likely because of the resistance to protein adhesion by the PEO segregation.

Covalent immobilization of biotin on a low-fouling surface

For analytical chemistry and biosensing, covalent immobilization of specific antibodies, enzymes, oligonucleotides or ligands on low-fouling surfaces is critical. However, a low-fouling surface is usually less reactive or does not have reactive functional groups. Because the terpolymers contained hydroxy groups at the termini of the PEO chains, we oxidized the hydroxy groups (P(MMA/PFEA-6/PEO-OH)) into carboxy groups, which were used to immobilize biotin hydrazide. Figure 7 shows fluorescence images of bare PET and surface-modified films after immersion in an aqueous solution containing Alexa Fluor 647-labeled BSA (red) and Alexa Fluor 488-labeled streptavidin

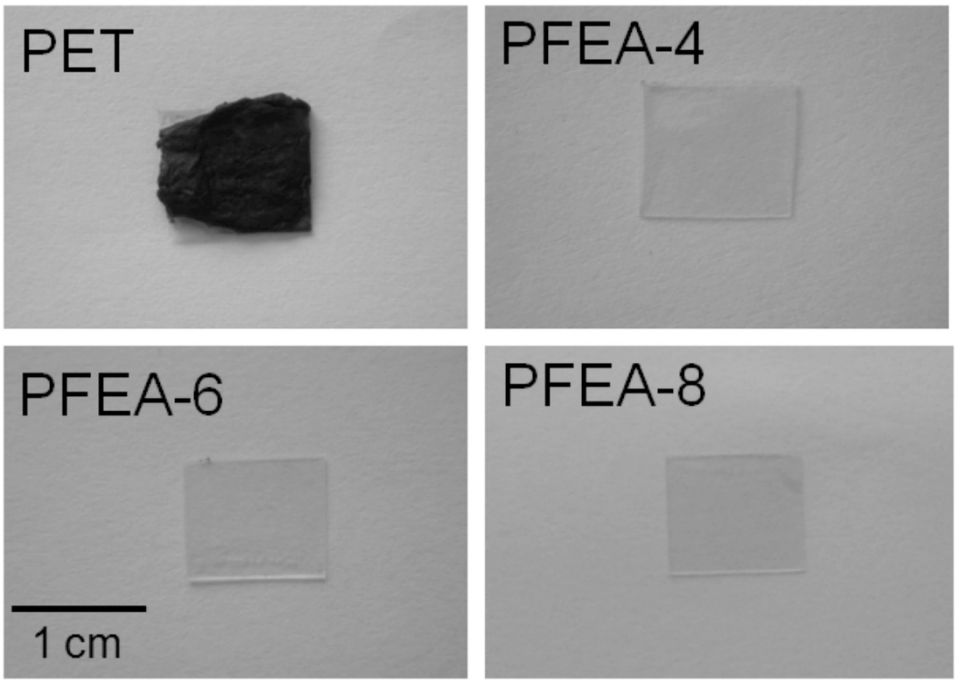


Figure 5 Optical photographs of bare and surface-modified PET films after immersion in whole human blood. PFEA-4, PFEA-6 and PFEA-8 represent PET films coated with a mixture of P(MMA/PFEA-n/PEO-OH), P(2F-4F) and PMMA. A full color version of this figure is available at the *Polymer Journal* online.

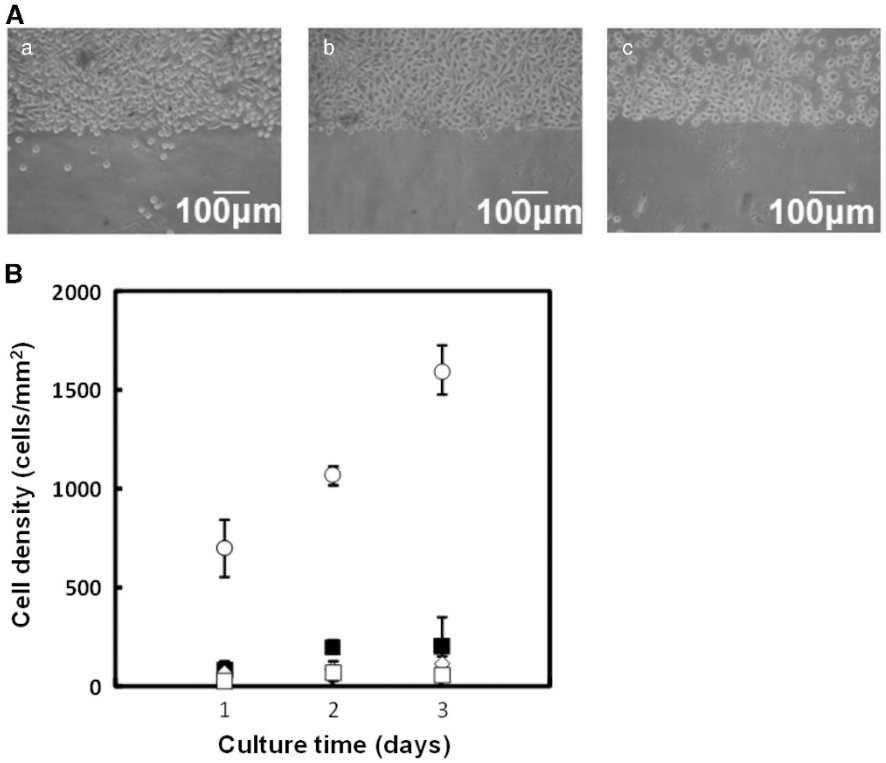


Figure 6 (**A**) Optical micrographs of L929 cells seeded and cultured for 2 days on PET films. The lower halves of the films were coated with a mixture of P(MMA/PFEA-n/PEO-OH), P(2F-4F) and PMMA. (a) P(MMA/PFEA-4/PEO-OH), (b) P(MMA/PFEA-6/PEO-OH) and (c) P(MMA/PFEA-8/PEO-OH). (**B**) Effect of culture time on the cell density of L929 cells on bare PET and surface-modified films. (○) Bare PET, (■) P(MMA/PFEA-4/PEO-OH), (□) P(MMA/PFEA-6/PEO-OH) and (◇) P(MMA/PFEA-8/PEO-OH).

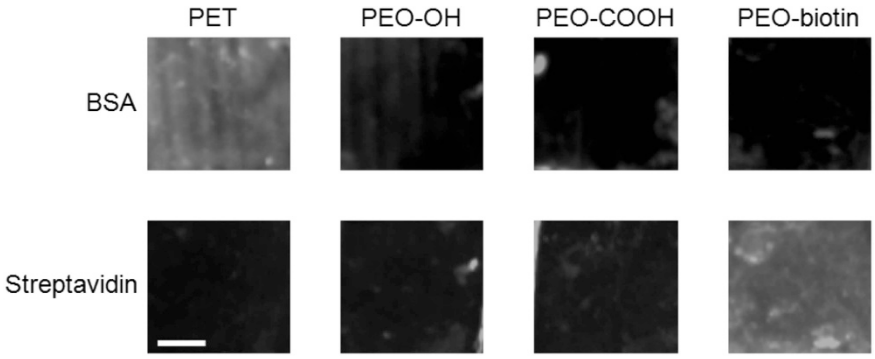


Figure 7 Fluorescence images of PET and terpolymer-modified surfaces that were treated with an aqueous solution containing Alexa Fluor 647-labeled BSA (red) and Alexa Fluor 488-labeled streptavidin (green). A mixture of P(MMA/PFEA-6/PEO-OH), P(2F-4F) and PMMA was used for the modification. PEO-COOH and PEO-biotin represent the terpolymer containing carboxy-terminated PEO and the terpolymer containing biotin-conjugated PEO, respectively. PET films were dip coated with a mixture of P(MMA/PFEA-6/PEO-OH), P(2F-4F) and PMMA (PEO-OH). The hydroxy groups at the termini of the PEO chains in the terpolymer were carboxylated, as illustrated in Scheme 1 (PEO-COOH). Then, biotin hydrazide was conjugated with the carboxy groups (PEO-biotin). The scale bar represents 2 mm. A full color version of this figure is available at the *Polymer Journal* online.

(green). The bare PET surface exhibited strong fluorescence from adsorbed Alexa Fluor 647-labeled BSA, whereas the terpolymer-modified surfaces (PEO-OH, PEO-COOH and PEO-biotin) exhibited weak fluorescence. Thus resistance to BSA adsorption derived from the PEO chains was maintained even after carboxylation of the PEO termini and conjugation of biotin hydrazide to the PEO termini.

Only the biotin-immobilized surface (PEO-biotin) displayed strong fluorescence from the Alexa Fluor 488-labeled streptavidin, whereas the bare PET surfaces and other terpolymer-modified surfaces did not. This result indicates carboxylation of the PEO termini and immobilization of biotin hydrazide on the PEO-biotin surface. The terpolymer-modified surface (PEO-OH) and the carboxylated surface (PEO-COOH) resisted streptavidin adsorption. Streptavidin adsorption was also inhibited on the bare PET film, possibly because of blocking by the large amount of adsorbed BSA.

CONCLUSION

We used methacrylate-based terpolymers, containing both R_f groups and PEO side chains, to dip coat PET films. The modified surfaces showed resistance to protein adsorption, antithrombogenicity and cell adhesion. The low-fouling effect resulted from the surface-segregated terpolymer PEO chains. Moreover, carboxylation of the PEO termini on the surface was demonstrated in addition to covalent immobilization of biotin on the PEO-segregated surface. The biotin-functionalized surface recognized streptavidin in the presence of other proteins. In conclusion, simple surface modification via dip coating of synthetic terpolymers can produce low-fouling and ligand immobilization.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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