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Facile Method for Selective Immobilization of Biomolecules on Plastic Surfaces

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A key aspect of biochip and biosensor preparation is optimizing surface attachment of biomolecules. Here, we report a facile approach for selectively immobilizing biomolecules on amphiphilic polymer-coated plastic surfaces with antibiofouling properties. To modify plastic surfaces, we synthesized two types of random copolymers by radical polymerization, which consisted of three parts: an anchoring group; a PEG component, which acted as a repellent of nonspecific biomolecules; and a functional group, to which biomolecules were conjugated. Dodecyl- and benzyl-based copolymers were highly soluble in water, presumably due to the presence of multiple PEG groups, and could easily coat the model plastic surface (polystyrene) in an aqueous environment. The antibiofouling property of each polymer-coated plastic surface was examined by measuring the extent of nonspecific protein adsorption using bovine serum albumin (BSA). Both polymer-coated plastic surfaces showed a very low level of BSA adsorption relative to that of an uncoated plastic surface (control). Finally, we showed that streptavidin and antibodies, as representative biomolecules, could be selectively immobilized on the polymer-coated plastic surfaces imprinted with biotin and protein A, respectively, by microcontact printing, exhibiting an intense signal with low background.

Introduction

Immobilization or conjugation of bioactive molecules onto material surfaces is a key step in the construction of biosensors and biochips.[1−3] One of the most popular methods for accomplishing this is the use of self-assembled monolayers (SAMs) formed on gold, indium tin oxide, or silicon oxide surfaces.[4] Recently, a novel method based on polymeric self-assembled monolayers (pSAMs) was developed for selective immobilization of proteins or cells on silicon oxide and indium tin oxide substrates.[5] This facile method yielded reproducible immobilization and reduced nonspecific adsorption of biomolecules compared with conventional SAMs.[6] Although a number of methods are available for modifying metallic and oxide surfaces,[4−6] only a few are suitable for plastic surfaces.[7] One such method is to physically adsorb proteins onto plastic surfaces through van der Waals interactions between hydrophobic protein residues and plastic surfaces[8] or through electrostatic interactions between charged proteins and poly(L-lysine)-coated plastics.[9] However, such physical adsorption methods often lack reproducibility in terms of the number and spatial density of adsorbed biomolecules because the immobilization process is uncontrolled.[8] In addition, the intrinsic hydrophobic character of plastic surfaces results in a high level of nonspecific adsorption (biofouling) of target biomolecules during bioassays.[10] Because plastics (e.g., cyclic olefin copolymer [COC] and polystyrene) are becoming widely used in commercially available cell culture and enzyme-linked immunosorbent assay (ELISA) plates, and are the main materials used to construct Lab-on-a-Chip biosensors, there is an increasing need for a technology platform capable of efficient, reproducible immobilization of biomolecules on plastic surfaces. In general, these plastic surfaces lack modifiable chemical functional groups; thus, a novel approach that departs from conventional strategies is needed. Very recently, our group reported that the amphiphilic polymers, poly(DMA-*r*-mPEGMA-*r*-MA) and poly(BMA-*r*-mPEGMA-*r*-MA) (Scheme 1a) composed of hydrophobic residues, poly(ethylene glycol) (PEG), and carboxylic acid were readily dispersible and could robustly coat carbon nanotubes via van der Waals interactions in aqueous medium.[11] On the basis of these previous findings, we reasoned that these same amphiphilic

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polymers could coat hydrophobic plastic surfaces and be used to selectively immobilize biomolecules through chemical means. Here, we report a facile approach to constructing patterns of biomolecules on amphiphilic polymer-coated plastic surfaces that are trifunctional, exhibiting surface-anchoring, bioreactivity, and antibiofouling properties.

Experimental Section

Materials. Dodecyl methacrylate (DMA), benzyl methacrylate (BMA), methacrylic acid (MA), poly(ethylene glycol) methyl ether methacrylate (mPEGMA, average $M_n \approx 475$), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), and 2,2′-azobisisobutyronitrile (AIBN) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). (+)-Biotinyl-3,6,9-trioxaundecanediamine (biotin-NH_2) and tetramethylrhodamine isothiocyanate (TRITC)-labeled streptavidin were purchased form Pierce (Rockford, IL, USA). A biotin-conjugated mouse antirabbit IgG monoclonal antibody/3,3′,5,5′-tetramethylbenzidine (TMB) substrate reagent set and skim milk were purchased from BD Biosciences (San Diego, CA, USA). Flat-bottom, 96-well assay plates were purchased from Falcon, Becton Dickinson (Franklin Lakes, NJ, USA). Streptavidin was purchased from New England Biolabs Inc. (Beverly, MA). Horseradish peroxidase (HRP)-conjugated antimouse IgG antibody was purchased from Abbiotec (San Diego, CA, USA). Streptavidin-coated 96-well flat-bottom plates were purchased from R&D systems (Minneapolis, MN, USA). Protein A was purchased from Biovision (San Francisco, CA). Fluorescein isothiocyanate (FITC)-labeled bovine serum albumin (BSA) was purchased from Biomeda (Foster City, CA, USA). Chicken anti-BSA was purchased from OEM Concepts (Saco, ME, USA). Petri dishes were purchased from SPL Life Science (Kyounggi-do, Korea). All organic solvents were used as received without further purification.

Measurements. ^{1}H NMR (300 MHz) spectra were recorded on a JEOL JNM-LA300WB FT-NMR (Tokyo, Japan). Organic-phase gel permeation chromatography (GPC) was performed using a Waters 1515 series isocratic pump and a Rheodyne model 7725 injector with a 100 μL injection loop at a flow rate of 0.4 mL/min. An Aphoenix300 contact angle and surface tension analyzer (Surface electro optics, Kyunggi-do, Korea) equipped with video camera and monitor was used to measure contact angle. X-ray photoelectron spectroscopy (XPS) spectra were recorded using a Kratos AXIS Ultra Imaging X-ray photoelectron spectrometer with a monochromatized Al−K X-ray source.

Synthesis of Amphiphilic Polymers. The detailed procedure for synthesizing amphiphilic polymers was described in our previous report.[11] Briefly, prior to polymerization, neat mPEGMA was passed over an inhibitor-removal column (Sigma-Aldrich, Milwaukee, WI, USA). DMA or BMA (3.5 mmol, 3.5 equiv), mPEGMA (3.5 mmol, 1.663 g, 3.5 equiv), MA (3 mmol, 0.258 g, 3 equiv), and AIBN (16.5 mg, 0.1 mmol, 0.1 equiv) were placed in a vial and dissolved in 10 mL of tetrahydrofuran (anhydrous, 99.9%, inhibitor-free). The resulting homogeneous solution was degassed for 15 min by bubbling with a stream of N_2 gas, after which the vial was sealed with a Teflon-lined screw cap. The polymerization reaction was carried out at 70 °C for 24 h. The final product solution was stored at 4 °C. ^{1}H NMR (300.40 MHz, $CDCl_3$): poly(DMA-*r*-mPEGMA-*r*-MA) $\delta = 4.05$ (br, 2H, CO_2-CH_2 of PEGMA), 3.82 (br, 2H, CO_2-CH_2 of DMA), 3.66 (s, 30H), 3.40 (s, 3H), 2.0−1.71 (br, 10H), 1.5−1.1 (br, 20H), 0.87 (br, 3H); poly(BMA-*r*-mPEGMA-*r*-MA) $\delta = 7.30$ (s, 5H), 4.93 (br, 2H, CO_2-CH_2 of BMA), 4.05 (br, 2H, CO_2-CH_2 of PEGMA), 3.66 (s, 30H), 3.40 (s, 3H), 2.0−1.71 (br, 10H), 0.87 (br, 3H), 0.72 (br, 4H). GPC: poly(DMA-*r*-mPEGMA-*r*-MA), $M_n = 19\,581$ with $M_w/M_n = 1.88$; poly(BMA-*r*-mPEGMA-*r*-MA), $M_n = 21\,831$ with $M_w/M_n = 1.97$.

Preparation of Amphiphilic Polymer-Coated Polystyrene Surfaces. Commercially available Petri dishes were cut into specific sizes and immersed in a solution of amphiphilic polymers in distilled water (20 mg/mL) at ambient temperature, followed by washing with distilled water.

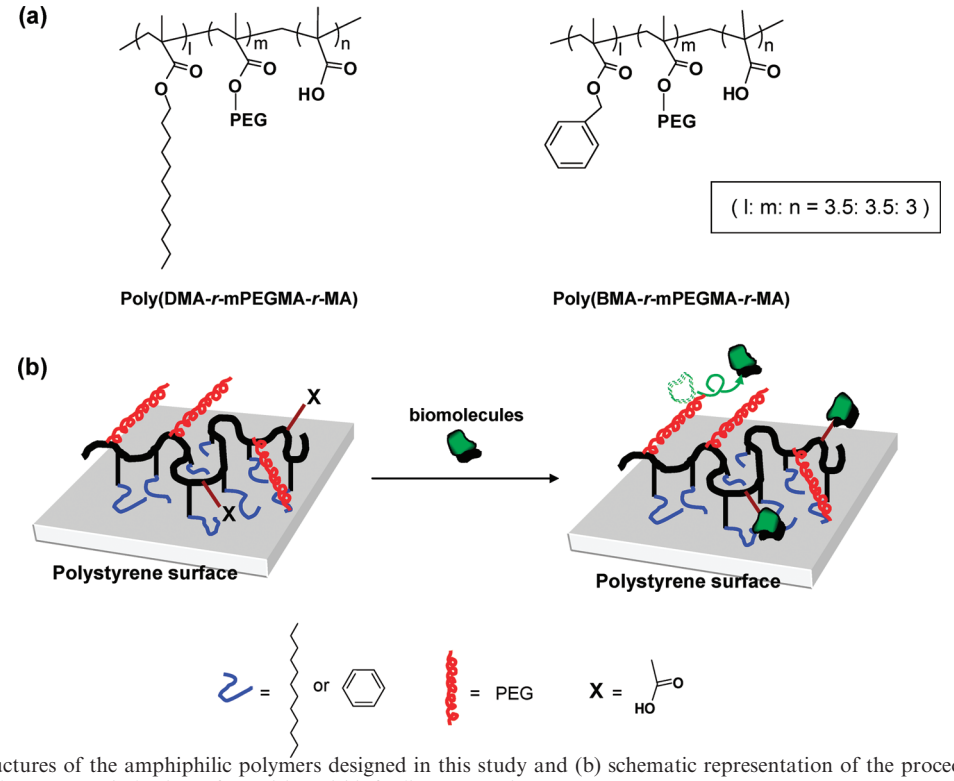
BSA Adsorption onto Amphiphilic Polymer-Coated Polystyrene Surfaces. The amphiphilic polymer-coated polystyrene surfaces were immersed in a solution of BSA in phosphate buffered saline (PBS) (0.25 mg/mL, pH 7.4) for 2 h, followed by washing with distilled water and air-drying. The degree of BSA adsorption onto the polystyrene surfaces was measured using high-resolution N (1s) XPS analysis.

Microcontact Printing (μCP). In the first step, the amphiphilic polymer-coated polystyrene surfaces were treated with a freshly prepared 1:1 mixture of EDC (400 mM) and NHS (100 mM) in distilled water for 20 min to convert the carboxylic group to a reactive NHS ester. A poly(dimethylsiloxane) (PDMS) stamp was first cleaned using detergent, then washed with deionized water and ethanol several times, and treated with O_2 plasma (Expanded Plasma Cleaner; Harrick Plasma Corp., Ithaca, NY) for 30 s to generate hydroxyl groups and further clean the surface. After inking a PDMS stamp with a 50×50 μm^2 circle pattern using biotin-NH_2 (10 mM in ethanol), the stamp pattern was transferred to the polymer-coated polystyrene surfaces by contact printing for 1 h. After removal of the stamp, the polystyrene substrate was immersed immediately in a sodium borate buffer (pH 9.0) and incubated for 2 h to hydrolyze unreacted NHS esters. The substrate was subsequently transferred to a solution of TRITC-labeled streptavidin (0.1 mg/mL) in PBS (pH 7.4), incubated at ambient temperature for 1 h, and then washed several times with PBS and distilled water. The patterns of streptavidin were visualized ($\lambda_{ex} = 547$ nm, $\lambda_{em} = 572$ nm) using a Leica DMRBE microscope (Leica Microsystems AG, Wetzlar, Germany) equipped with 200× and 400× objectives and TRITC-optimized filter sets (Omega Optical Inc., Brattleboro, VT, USA).

Preparation of a Protein A Pattern on Polystyrene Surfaces. Amphiphilic polymer-coated plastic surfaces were activated by EDC/NHS reagents and contact printed with protein A (100 μg/mL in PBS, pH 7.4) for 1 h using the same procedures and PDMS stamp described above. Thereafter, the substrate was immersed immediately in a sodium borate buffer (pH 9.0) and incubated for 2 h. Anti-BSA (10 μg/mL in PBS) was added to the protein A pattern and incubated for 1 h at ambient temperature. After washing with PBS, FITC-labeled BSA (50 μg/mL in PBS) was added to the patterned surfaces and incubated for 1 h. The patterns of BSA were visualized using a Leica DMRBE microscope (Leica Microsystems AG, Wetzlar, Germany) equipped with 200× and 400× objectives and FITC-optimized filter sets (Omega Optical Inc., Brattleboro, VT, USA) ($\lambda_{ex} = 494$ nm, $\lambda_{em} = 521$ nm).

Enzyme-Linked Immunosorbent Assay (ELISA). Commercially available 96-well flat-bottom assay plates were coated by adding 100 μL of a solution of amphiphilic polymers (20 mg/mL in distilled water) to each well and incubating for 1 h at ambient temperature. Each well was washed four times with 150 μL of deionized water. The carboxylic groups on amphiphilic polymer-coated surfaces were converted to reactive NHS esters by treatment with 100 μL of EDC (400 mM) and NHS (100 mM), respectively, for 20 min at ambient temperature, and then washed four times with cold distilled water. Thereafter, 100 μL of biotin-NH_2 (1 mg/mL in ethanol) was added to each well and allowed to react for 1 h, and then washed three times with 150 μL of ethanol. Sodium borate buffer (150 μL, pH 9.0) was then added immediately to each well and incubated for 2 h to hydrolyze unreacted NHS esters, after which the solution was decanted, and 100 μL of streptavidin in PBS (10 μg/mL) was added to each well. After incubating for 1 h, each well was washed three times with 150 μL of PBS, and 100 μL of biotin-conjugated mouse antirabbit IgG monoclonal antibody at different concentrations in PBS was added to each well. After incubating for 1 h, each well was washed with 150 μL of PBS containing 0.1% Tween-20 using Immuno Washers (Nunc A/S, Roskilde, Denmark), and 100 μL

Scheme 1.[a]



[a] (a) Chemical structures of the amphiphilic polymers designed in this study and (b) schematic representation of the procedure for immobilizing biomolecules on a polymer-coated plastic surface with anti-biofouling properties.

of HRP-conjugated antimouse IgG antibody in PBS (1 μg/mL) was then added to each well. After incubating for 1 h and subsequently washing with PBS, 100 μL of TMB substrate solution was added to each well. The enzymatic reaction stopped after 30 min by adding 100 μL of 1 M HCl. The intensity was calculated from absorbance values obtained at 450 nm using an automated ELISA reader.

Results and Discussion

There are several drawbacks associated with conventional methods for immobilizing biomolecules on plastic surfaces,[7–9] including the lack of reproducibility and a high level of nonspecific adsorption (biofouling) of biomolecules during bioassays that results in a poor signal-to-noise ratio.[12] To resolve such shortcomings, we attempted to modify plastic surfaces using amphiphilic polymers with antibiofouling properties. The chemical structures of the two amphiphilic polymers are shown in Scheme 1a. The two polymers are composed of three parts: a hydrophobic moiety, which serves to anchor the polymer on the plastic surface; a poly(ethylene glycol) methacrylate (PEGMA) component that acts as a protein repellent; and carboxylic acid, which acts as a functional group to conjugate biomolecules through formation of a covalent bond. We have previously shown that random amphiphilic copolymers are capable of coating carbon nanotubes via van der Waals interactions; the resulting carbon nanotubes are highly dispersible, functionalizable, and protein-repellent in aqueous media.[11] Our expectation was thus that these amphiphilic polymers would be similarly capable of coating hydrophobic plastic surfaces in aqueous medium, forming stable polymer films that could immobilize biomolecules and exhibit minimal biofouling (Scheme 1b). Each polymer was synthesized from the corresponding monomers by radical polymerization using a molar feed ratio of 3.5:3.5:3, as described in our previous report.[11]

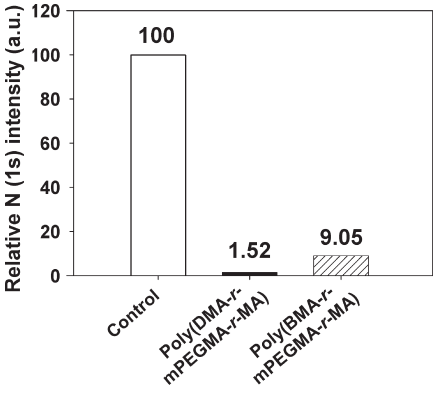


Figure 1. Antibiofouling effect of polymer-coated plastic surface assessed by XPS. High-resolution N (1s) XPS intensity of the control (unmodified plastic surface) and polymer-coated plastic surfaces measured after BSA adsorption for 2 h. The relatives amount of protein adsorption onto each surface are calculated as the percentage of protein adsorption onto an unmodified, bare plastic surface.

To examine the ability of amphiphilic polymers to coat plastic surfaces, we used a commercially available polystyrene substrate as a model plastic surface. Polymer coating was achieved by immersing a polystyrene plate in an aqueous solution of the copolymer for 1 h at ambient temperature, followed by washing with distilled water as described in the Experimental section. Each polymer-coated polystyrene surface was characterized by measuring the static water contact angle before and after polymer coating. The water contact angles decreased drastically from the

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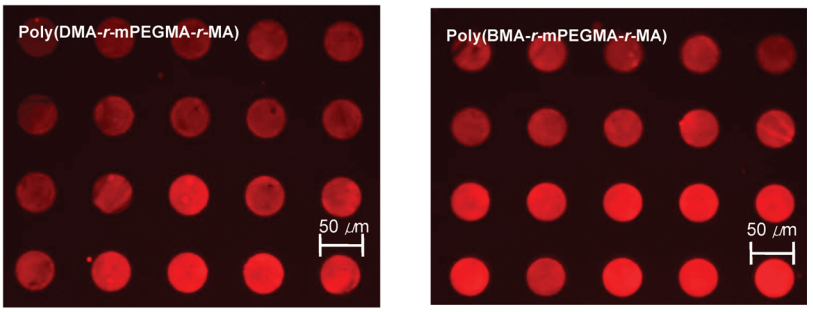
(a)



(b)

polymer	Poly(DMA-*r*-mPEGMA-*r*-MA)	Poly(BMA-*r*-mPEGMA-*r*-MA)
Fluorescent intensity (a.u.)	150 ± 24	180 ± 34

Figure 2. (a) Fluorescence microscope images of TRITC-labeled streptavidin patterns obtained after incubating with a polymer-coated plastic surface containing 50 × 50 μm^2 circle-patterns of biotin−amine imprinted by μCP. (b) Mean fluorescence intensities of immobilized TRITC-labeled streptavidin patterns obtained as in (a).

original polystyrene substrate value of 130 ± 7° to 74 ± 1° and 79 ± 2° for the poly(DMA-*r*-mPEGMA-*r*-MA)- and poly(BMA-*r*-mPEGMA-*r*-MA)-coated surfaces, respectively, indicating that polymer layers had formed and suggesting that the hydrophilic PEG component was exposed, as illustrated in Scheme 1b. There was little change in the contact angles of two polymer layers up to 1 week when treated with distilled water. On the other hand, after 1 h incubation in an 1% aqueous solution of sodium dodecylsulfate, the contact angle of the poly(DMA-*r*-mPEGMA-*r*-MA)-coated surface increased from 79° to 88°, whereas little change was observed for the poly(BMA-*r*-mPEGMA-*r*-MA)-coated surface; the latter polymer layers seem to be stable in the presence of surfactants but the former layers are not so.

The ability of the polymer coating layer to block nonspecific protein adsorption was evaluated using BSA as a model plasma protein. Each substrate was immersed in a solution of BSA for 2 h and then was characterized by XPS. The degree of nonspecific BSA adsorption, calculated by measuring N(1s) nitrogen intensity in XPS data and expressed relative to BSA adsorption to the uncoated polystyrene surfaces, was approximately 1.5% and 9.1% for poly(DMA-*r*-mPEGMA-*r*-MA)- and poly(BMA-*r*-mPEGMA-*r*-MA)-coated surfaces, respectively (Figure 1). These results suggest that both amphiphilic polymers are capable of blocking unwanted, nonspecific protein adsorption onto plastic surfaces due to the exposure of multiple PEG groups.

To examine the suitability of the amphiphilic polymer-coated plastic surfaces for immobilization of biomolecules, we prepared a micropattern of biomolecules using μCP, a soft lithographic technique.[13] In the first step, the carboxylic group in the polymer-coated polystyrene surfaces was converted into the corresponding NHS ester by treatment with EDC/NHS reagents. Then, the amine-terminal biotin ink (biotin-NH_2, 10 mM in ethanol) was contact-printed onto the reactive polymer layers using a positive PDMS stamp with 50 × 50 μm^2 circular patterns. Because the amine groups of biomolecules can also react with the activated NHS ester group of the polymeric surfaces in the subsequent step, the resulting biotin-patterned plastic surfaces were immersed in borate buffer (pH 9.0) to hydrolyze unreacted NHS esters on the surfaces and then incubated with a solution of TRITC-labeled streptavidin as described in the Experimental section. Figure 2a shows the fluorescence microscopic images of the TRITC-labeled streptavidin pattern, in which streptavidin was selectively immobilized on the biotin-patterned areas with a high signal-to-noise ratio. A comparison of the relative signal intensity of the circle areas for the two polymers (Figure 2b) showed that the fluorescence intensity for the poly(BMA-*r*-mPEGMA-*r*-MA)-coated surface (180 ± 34 au) was slightly higher than that for the poly(DMA-*r*-mPEGMA-*r*-MA)-coated surface showed (150 ± 24 au). Notably, the background signal on the nonpatterned areas was very low, resulting in high apparent signal-to-noise ratio. Using conventional approaches, it is often the case that either backprinting with PEG[14] or a precoating step with BSA[15] is necessary to minimize nonspecific adsorption of proteins onto the surfaces of biochips. However, because of the presence of PEG groups in the copolymers, there is no need for such extra steps in preparing our polymer-coated surfaces.[11]

In the above biotin−streptavidin system, the relatively small biotin molecule was directly conjugated to the polymer-coated surface in the first steip, not the larger streptavidin. To determine whether larger protein molecules can also be conjugated directly to the activated surfaces, we used protein A as a model protein. Because it can capture the Fc region of an antibody, thus providing an active orientation of bound antibody, protein A has been extensively used as a platform for immobilizing several types of antibodies in numerous immunoassays.[16] Protein A was contact-printed onto each polymer-coated plastic surface using a positive PDMS stamp and, once immobilized, was incubated sequentially with anti-BSA antibody and FITC-labeled BSA as described in the Experimental section. Figure 3a shows a schematic representation of the entire surface modification and binding process for coating a polystyrene surface with poly(DMA-*r*-mPEGMA-*r*-MA). As expected, FITC-labeled BSA bound exclusively to the anti-BSA antibody-immobilized areas, indicating that protein A was successfully conjugated to these areas in the first step. Little nonspecific adsorption of BSA was

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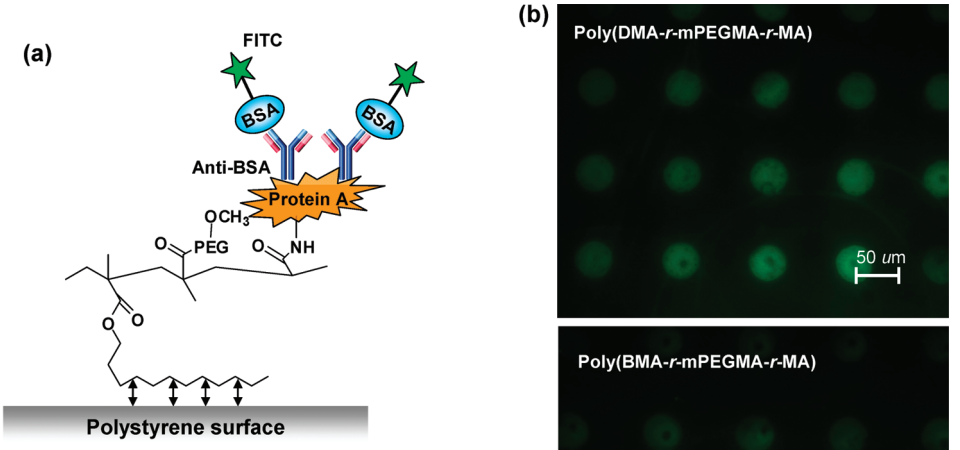

(c)

polymer	Poly(DMA-*r*-mPEGMA-*r*-MA)	Poly(BMA-*r*-mPEGMA-*r*-MA)
Fluorescent intensity (a.u.)	99 ± 15	53 ± 7

Figure 3. (a) Schematic representation of entire surface modification and detection of anti-BSA using protein A on a polymer-coated plastic surface. (b) Fluorescence microscope images of FITC-labeled BSA patterns obtained after incubating anti-BSA with a polymer-coated plastic surface containing $50 \times 50\ \mu m^2$ circle-patterns of protein A imprinted by μCP. (c) Mean fluorescent intensities of immobilized FITC-labeled BSA patterns obtained as in (b).

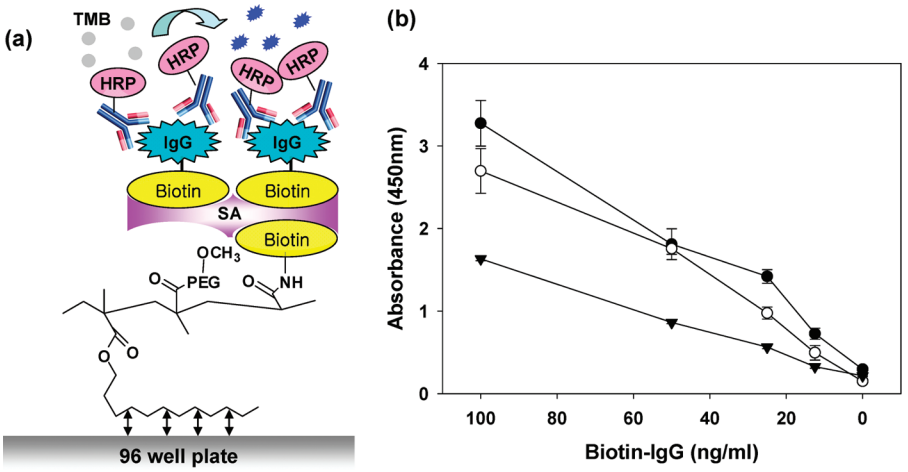

Figure 4. (a) Schematic representation depicting the entire process of surface modification and detection of biotin-conjugated mouse antirabbit IgG using our polymer-coated 96-well plate. (b) ELISA detection of adsorbed biotinylated mouse IgG using an automated ELISA reader. Immobilized biotinylated mouse IgG was detected by HRP-conjugated antimouse IgG. Poly(DMA-*r*-mPEGMA-*r*-MA) (●) and poly(BMA-*r*-mPEGMA-*r*-MA) (○) denote our polymer-coated streptavidin plate system. Control (▼) denotes a commercially available streptavidin plate.

detectable on areas lacking protein A conjugation (i.e., those containing only a hydrolyzed polymeric layer), resulting in high contrast between signal and noise. A comparison of the relative fluorescence intensity of the circle areas in images of each polymer-coated plastic surface, shown in Figure 3c, revealed that the signal intensity of the poly(DMA-*r*-mPEGMA-*r*-MA)-coated surface was higher than that of the poly(BMA-*r*-mPEGMA-*r*-MA)-coated surface (99 ± 15 versus 53 ± 7 au). Taken together, these results suggest that the present method of protein A immobilization is suitable for construction of protein chips on plastic surfaces.

To further demonstrate the usefulness of our polymer systems, we coated a 96-well polystyrene plate used routinely for biological studies and bioassays, such as ELISAs. Wells of a plain, uncoated 96-well plate were coated with poly(DMA-*r*-mPEGMA-*r*-MA) or poly(BMA-*r*-mPEGMA-*r*-MA), and then polymer-coated wells were sequentially modified by biotin-NH_2 and streptavidin. Biotin-conjugated mouse IgG was then immobilized and subsequently detected by the ELISA method using HRP-conjugated antimouse IgG. A schematic representation of the entire surface modification and ELISA method is shown in Figure 4a for poly(DMA-*r*-mPEGMA-*r*-MA)-coated 96-well plates. For comparison purposes, we also tested the same ELISA assay using a commercial streptavidin-coated 96-well plate as a positive control. Whereas the commercial streptavidin-coated 96-well plates required blocking with milk protein (2% w/v in distilled water) for 2 h to reduce nonspecific protein adsorption, our polymer-coated streptavidin plates needed no such additional step because the

existing PEG groups efficiently blocked nonspecific protein binding. Using this ELISA assay, we detected biotinylated mouse IgG (12.5−100 ng/mL) as a model analyte. Figure 4b shows absorbance curves for the ELISA assay as a function of biotinylated mouse IgG concentrations. Although all three plates showed linear responses proportional to analyte concentrations, our polymer-coated streptavidin plates exhibited larger slopes with an approximate detection limit of 12.5 ng/mL, whereas the detection limit of the commercial streptavidin plate was approximately 25 ng/mL. Although the differences in detection limits were not significant, the fact that our polymer-coated plates performed at least as well as commercial plates without requiring a blocking step demonstrates the potential of our polymer system for use in ELISA assays.

In conclusion, we have devised a facile method for selective immobilization of biomolecules on amphiphilic polymer-coated plastic surfaces that possess triple functionalities: surface-anchoring, bioreactive, and antibiofouling properties. Amphiphilic polymer films were formed efficiently on a model plastic surface via van der Waals interactions in aqueous environments. Biomolecules (i.e., proteins) were selectively immobilized on the amphiphilic polymer-coated plastic surfaces with little nonspecific adsorption, reflecting the repulsive influence of the PEG component. Together, these results indicate that the amphiphilic polymers described here may be suitable for a variety of applications involving biomolecule deposition and detection on plastic surfaces, including the construction of biosensors and biochips.

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