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Letters

Construction of Nonbiofouling Surfaces by Polymeric Self-Assembled Monolayers

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The synthesis of a poly(ethylene glycol) (PEG)-grafted surface-reactive random copolymer and its self-assembled structure on Si/SiO_2 substrates for construction of nonbiofouling surfaces are reported. The copolymer, poly(TMSMA-r-PEGMA), which is comprised of an "anchor part" (trimethoxysilane) and a "function part" (PEG), was synthesized by a radical polymerization reaction. The copolymer spontaneously formed monolayers on Si/SiO_2 wafers with average thicknesses of 11 Å. Tapping mode atomic force microscopy (AFM) revealed that the surface of the polymer monolayers was smooth with an average roughness of 1.3 Å (root-mean-square). The protein resistance of the polymer monolayers on Si/SiO_2 wafers was evaluated using insulin, lysozyme, and fibrinogen. For all tested proteins, the polymer monolayers showed significant reduction (up to 98%) in nonspecific protein adsorption compared to the unmodified Si/SiO_2 wafers. In addition, cell adhesion as probed using 3T3 fibroblasts was significantly reduced on the polymer-coated glass substrates in comparison to unmodified glass substrates.

Introduction

The construction of protein- or cell-resistant surfaces is a crucial requirement for medical or analytical devices that contact biological fluids.[1] Such devices include biosensors,[2] chip-based diagnostic assays,[3] affinity chromatography columns,[4] and biomaterials used for implants and tissue engineering.[5] One strategy to reduce biofouling adsorption is surface modification by poly(ethylene glycol) (PEG) which has protein- and cell-repellent properties.[6] Previous attempts at grafting PEG onto oxide surfaces

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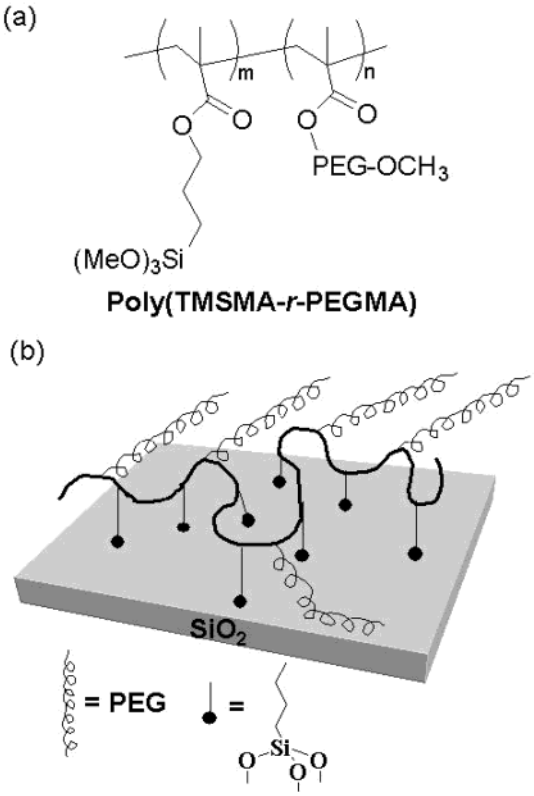
have involved covalent bond formation processes such as by chemical coupling reactions[7] and UV-induced graft polymerizations.[8] However, both approaches require the pregeneration of functional groups on the substrate surfaces by multistep heterogeneous reactions. An alternative is direct PEG modifications onto bare oxide surfaces using a PEG-grafted copolymer either by physical adsorption[9] or by electrostatic interaction.[7,10] Nonbiofouling surfaces can also be prepared either using self-assembled monolayers (SAMs) of oligo(ethylene glycol)[11] or by the self-assembly of surface-reactive polymers[12] on gold. On oxide surfaces, however, few studies have been reported using such SAMs.[13] Here we report an efficient approach to constructing nonbiofouling surfaces on bare oxide substrates by forming ultrathin polymeric self-assembled monolayers (PSAMs) via multiple covalent bonds. Polymeric self-assembly of surface-reactive block copolymers has been used for the construction of nano- and micropatterned polymer brushes on silicon dioxide.[14] We used a random copolymer composed of an "anchor part" (trialkoxysilane) and a "function part" (PEG). The chemical structure of the copolymer and its proposed self-assembled structure on a Si/SiO_2 surface are shown in Chart 1. Incorporation of the surface-reactive trimethoxysilyl group in the monomer can allow the copolymer to form multiple covalent bonds onto the oxide surface to provide multiple PEG immobilizations. Consequently, nonspecific protein adsorption and cell adhesion can be significantly reduced.

Chart 1. (a) Chemical Structure of the (Trimethoxysilyl)propyl Methacrylate and PEG Methacrylate Random Copolymer, Poly(TMSMA-*r*-PEGMA) and (b) Schematic Diagram of Self-Assembled Monolayers of the Copolymer on SiO_2 Surface



Experimental Section

Materials. 3-(Trimethoxysilyl)propyl methacrylate, poly(ethylene glycol) methyl ether methacrylate (average M_n = ca. 475), and 2,2′-azobisisobutyronitrile (AIBN) were purchased from Aldrich Chemical Co. (Milwaukee, WI). NIH 3T3 fibroblasts, Dulbecco's modified Eagle's medium, fetal bovine serum, and other cell culture supplies were purchased from American Type Culture Collection (Manassas, VA). All organic solvents were used as received. All substrates used herein such as Si/SiO_2 wafer, poly(dimethylsiloxane) (PDMS), and glass slide were first cleaned using detergent, followed by washing with deionized water and methanol several times. Prior to formation of the polymeric films, substrates were treated with an O_2 plasma at 0.15 Torr for 1 min to generate −OH groups as well as to clean the surfaces unless specially noted as "no plasma treatment".

Measurements. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Bruker instrument (Avance DPX 400). Organic phase gel permeation chromatography (GPC) was performed using a Hewlett-Packard 1100 series isocratic pump, a Rheodyne model 7125 injector with a 100 μL injection loop, and two PL-Gel mixed-D columns in series (5 μm, 300 × 7.5 mm, Polymer Laboratories, Amherst, MA). $CHCl_3$ was used as the eluent at a flow rate of 1.0 mL/min. The thicknesses of the monolayer films were measured with a Gaertner L116A ellipsometer (Gaertner Scientific Corporation, IL) at a 70° angle of incidence. A refractive index of 1.46 was used for all films, and a three-phase model was used to calculate thicknesses. A Ramé-Hart goniometer (Mountain Lakes, NJ) equipped with video camera and monitor was used to measure contact angles in both the advancing and receding modes (~1 μL/s) on drops of ~3 μL in volume. Reported values represent averages of at least three independent measurements. X-ray photoelectron spectroscopy (XPS) spectra were obtained using a Kratos AXIS Ultra Imaging X-ray photoelectron spectrometer with a monochromatized Al K X-ray source and a 160 mm concentric hemispherical energy analyzer for acquisition of spectra and scanned images, lateral resolution down to 20 μm. The spot size was 300 by 700 μm. Scanning force micrographs (1.0 × 1.0 μm^2) were performed in tapping mode on a NanoScope III Dimension (Veeco Instruments Inc., Woodbury, NY) in air. The scan rate was 1 Hz, and 256 lines were scanned per sample. Some of the images shown were flattened but not further manipulated. Tapping mode tips, NSC15-300 kHz, were obtained from MikroMasch (Portland). Data were processed using Nanoscope III 4.31r6 software (Veeco Instruments Inc., Woodbury, NY).

Synthesis of the Copolymer Comprising 3-(Trimethoxysilyl)propyl Metharylate (TMSMA) and Poly(ethylene glycol) Methyl Ether Methacrylate (PEGMA): Poly(TMSMA-*r*-PEGMA). Prior to polymerization, neat PEGMA was flowed through the inhibitor removal column (Aldrich Chemical Co.). PEGMA (4.75 g, 10 mmol, 1 equiv), TMSMA (2.5 g, 10 mmol, 1 equiv), and AIBN (16.5 mg, 0.1 mmol, 0.01 equiv) were placed in a vial and dissolved in tetrahydrofuran (anhydrous, inhibitor free, 99.9%, 10 mL). The mixture was degassed for 20 min using an Ar gas stream, after which the vial was sealed with a Teflon-lined screw cap. The polymerization reaction was carried out at 70 °C for 24 h. After evaporation of solvent under vacuum, the polymer was obtained as a viscous liquid. ^{1}H NMR (400 MHz,

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$CDCl_3$): δ = 4.13 (br, 2H, CO_2-CH_2 at PEGMA), 3.92 (br, 2H, CO_2-CH_2 at TMSMA), 3.66 (s, 30H), 3.63−3.55 (s, 9H; m, 2H), 3.40 (s, 3H), 2.0−1.71 (br, 6H), 1.04 (br, 2H), 0.87 (br, 4H), 0.66 (br, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 177.5, 176.3, 71.8, 70.5, 68.3, 66.8, 63.8, 58.9, 50.6, 44.7, 21.5, 18.2, 5.3. FTIR (cm^{-1}, neat): 2943, 2874, 1729, 1453, 1350, 1247, 1107, 950, 853, 822.

Cell Culture on the Polymer-Coated Glass Slides. The copolymer-coated glass slides (ca. 1 cm × 1.5 cm dimensions) were prepared by immersing the slides in the copolymer solution (10 mg/mL in methanol) for 2 h followed by washing with methanol and then by curing in a 120 °C oven for 15 min. The coated glass slides were sterilized by UV radiation for 20 min. Subsequently each glass slide was placed in a 24-well plate, soaked in growth medium for 30 min, and replaced with fresh medium. Each well was seeded with 100 000 NIH 3T3 fibroblasts and grown at 37 °C: 5% CO_2 in Dulbecco's modified Eagle's medium, 90%; fetal bovine serum, 10%; penicillin, 100 units/mL; streptomycin, 100 μg/mL.

Results and Discussion

The random copolymer, poly(TMSMA-*r*-PEGMA) was synthesized quantitatively by the radical polymerization reaction of commercially available 3-(trimethoxysilyl)-propyl methacrylate (1.0 equiv) and PEG methyl ether methacrylate (1.0 equiv) in THF at 70 °C for 24 h (0.01 equiv of AIBN as an initiator). The molecular weight of poly(TMSMA-*r*-PEGMA) was M_n = 26 000 with M_w/M_n = 1.88 as measured by gel permeation chromatography (GPC) relative to monodisperse polystyrene standards. The feed ratio of the two monomers was initially 1 to 1. By comparing the integration value of the peak at δ = 4.13 (CO_2-CH_2 at PEGMA) with that of the peak at δ = 3.92 (CO_2-CH_2 at TMSMA) in the ^1H NMR spectrum, the molar ratio of the two monomer units in the copolymer was calculated to be the same as the corresponding feed ratio.

Polymeric self-assembled monolayers (PSAMs) of the copolymer onto substrates exposing a SiO_2 surface[15] were prepared by immersing the substrates in a methanol solution of the copolymer (5−10 mg/mL) at ambient temperature followed by washing with methanol. No ultrasonic washing procedure was required. The PSAMs were then cured at 120 °C for 15 min. When Si/SiO_2 wafers were immersed in the copolymer solution (5 mg/mL in methanol) for 1 h, ultrathin films of the polymer were generated with an average ellipsometric thickness of ca. 11 Å.[16] The thicknesses of the films remained unchanged after immersion times up to 48 h (see Figure S1a), whereas it is known that monomeric trialkoxysilyl groups can give different coating thickness with different immersion times.[17] In addition, the formation of the PSAMs was not dependent on concentrations of the polymer up to 50 mg/mL (see Figure S1b). This observation indicates that the adsorbed polymer layers can limit further attachment of additional polymer chains to the surface. Furthermore, we did not observe any thickness change of the PSAMs after baking at 120 °C for 2 h, autoclaving for 1 h, or incubation in PBS (pH 7.4) at 37 °C for 2 weeks.

The surface topography of the PSAMs of the polymer on Si/SiO_2 wafers was examined by tapping mode atomic force microscopy (AFM). Three different positions per substrate were taken. Figure 1a clearly shows the surface of the PSAMs on O_2 plasma treated Si/SiO_2 wafers was very smooth with average roughness of 1.3 Å (root-mean-square) and had a uniform topographical pattern with a vertical distance of 3−5 Å from the base polymeric layer upon cross-sectional analysis.[18] On the basis of ellipsometric data that revealed an average thickness of ca. 11 Å for the PSAMs, the thickness of the base polymer layer could be derived to be less than 6 Å. In addition, copolymer aggregates were not detected for the O_2 plasma treated substrate (Figure 1a), whereas randomly distributed polymer aggregates (about 30 nm wide and 5 nm high) could be detected for the untreated substrate (no plasma treatment) (Figure 1b). Such aggregation may be attributed to the cross-linking between unreacted trimethoxysilyl groups in the copolymer chains. It is anticipated that the O_2 plasma treatment generates more Si−OH groups on the surface of the substrate, resulting in a lower fraction of trimethoxysilyl groups that remained unreacted with the surface after the formation of the PSAMs in comparison to the untreated substrate (no plasma treatment). Therefore, this result suggests that plasma treatment enhances the uniformity of the PSAMs on Si/SiO_2 substrates.

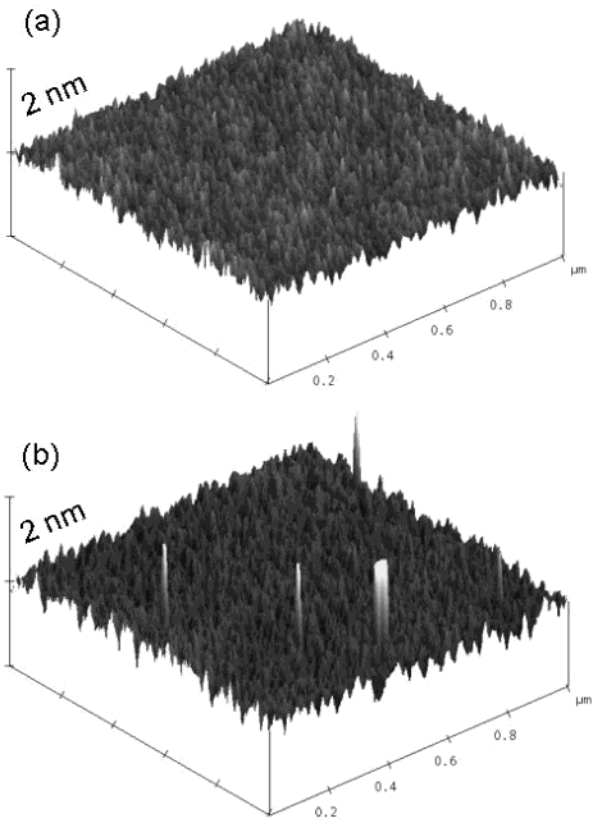

Figure 1. Tapping mode AFM height images of poly(TMSMA-*r*-PEGMA) SAMs on: (a) O_2 plasma treated-Si/SiO_2 wafer and (b) untreated-Si/SiO_2 wafer (no plasma treatment).

Since poly(TMSMA-*r*-PEGMA) is a random copolymer, it is difficult to predict the structure of its self-assembled monolayers on oxide surfaces. According to the thickness data, however, it seemed that most of PEG side chains would stretch over the surface rather than align vertically on the surface, as the expected film thickness for the latter structure would be at least 20 Å. The polymer-coated films were also characterized by glancing angle X-ray photoelectron spectroscopy. Table 1 summarizes elemental compositions of the unmodified and the polymer-coated

(15) See Materials section for the substrate preparation.

(16) Advancing and receding water contact angles of the PSAMs on a silicon wafer were 62° ± 1° and 46° ± 3°, respectively.

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(18) See Supporting Information (Figure S2) for AFM 2D sectional images. All PSAMs on the Si/SiO_2 wafers were prepared under the same conditions unless specially noted: immersion in methanol solution of the copolymer (5 mg/mL) for 1 h.

Table 1. Elemental Composition of the PSAMs on Si/SiO_2 Wafer Measured by XPS[a]

	elemental composition (%)		
substrate	O	C	Si
unmodified Si/SiO_2	38 ± <1	7 ± <1	55 ± <1
PSAMs on Si/SiO_2	34 ± <1	34 ± 3	32 ± 2

[a] Take-off angle was 55° from the surface normal.

Table 2. High-Resolution XPS C(1s) Composition at 0° and 55° Takeoff Angles from Surface Normal

	composition (%)		
takeoff angle, deg	CH_2	C—O	C(═O)O
0	30 ± 3	61 ± 3	9 ± <1
55	35 ± 2	56 ± 2	9 ± <1

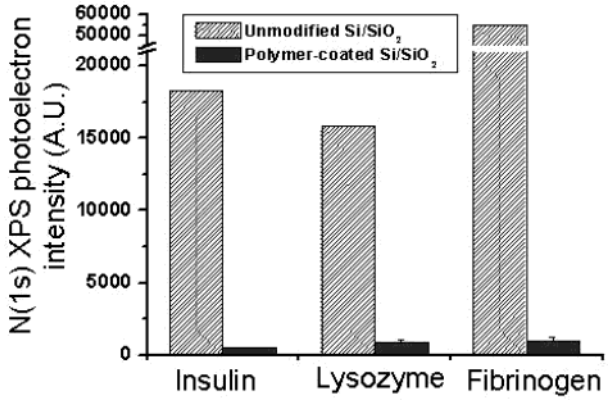

Figure 2. Protein adsorption on control (unmodified) and poly(TMSMA-*r*-PEGMA) SAMs on Si/SiO_2 wafer. The relative amount of each protein on the surface is denoted by its N(1s) XPS photoelectron intensity.

Si/SiO_2 surfaces. After the Si/SiO_2 was modified with the polymer, the carbon content was increased by 25% and the silicon content was decreased by 23%. High-resolution C(1s) XPS compositional data shown in Table 2 further confirms the formation of the PSAMs on Si/SiO_2 wafers.[19] Three carbon species (hydrocarbon, ether carbon in the PEG chain, and carbonyl carbon) were observed. If the PEG chains align vertically from the surface, it is expected that the ratio of C—O in the PEG moieties to O—C═O in the polymer backbone would increase with increasing takeoff angle. However, as takeoff angle was changed from 0° to 55° (from surface normal), the ratio decreased slightly, suggesting that the PEG chains lie horizontally over the surface as also suggested from the thickness data.

To evaluate the protein resistance characteristics of the polymeric SAMs, we immersed the polymer-coated Si/SiO_2 wafer in several protein solutions (0.25 mg/mL in PBS, pH 7.4) for 2 h. The degree of nonspecific protein adsorption onto the PSAMs was obtained from the high-resolution N(1s) XPS spectrum (Figure 2). For all three proteins studied, the PSAMs showed significantly lower (up to 98%) protein adsorption in comparison to the unmodified Si/SiO_2 wafer. For both a positive-charged protein (lysozyme, pI = 11.1) and a partially negative-charged protein (insulin, pI = 5.4), the polymer-coated substrates were highly resistant to protein adsorption by 95% and 97%, respectively. In addition, we observed about 98% resistance toward fibrinogen (pI = 5.5) adsorption, which is abundant in blood plasma and serum and is a well-known protein for its stickiness. Thickness changes

(19) See Supporting Information (Figure S3) for high-resolution C(1s) X-ray photoelectron spectra and peak analysis.

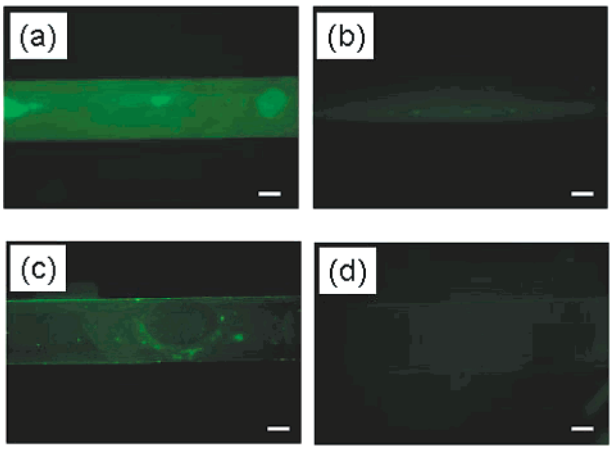

Figure 3. Fluorescence images on each part of capillary channel after flowing an aqueous solution of FITC-BSA (0.1 mg/mL, pH 7.4 PBS buffer) through the channel: (a) unmodified glass and (c) PDMS; (b) polymer-coated glass and (d) PDMS. The scale bar indicates 100 μm.

of the substrates before and after incubation in each protein solution were also obtained by ellipsometry (see Supporting Information). Similar protein-resistance trends were also observed with polymer-coated glass and poly(dimethylsiloxane) (PDMS) substrates.[20] The polymer films on PDMS and on glass had lower protein resistances (i.e., 81% and 94% for insulin, respectively) than on Si/SiO_2 wafers (95%) (see Supporting Information). This result is attributed to large differences in the extents of nonspecific protein adsorption on each unmodified substrate. According to the absolute peak intensity, however, the amounts of each protein adsorption on the PSAM surface are similar across all three substrates (see Figure S6).

Figure 3 shows one potential application of the present strategy for preparing nonbiofouling capillary channels as needed in electrophoresis, microfluidics. and bio-microelectromechanical system devices.[21] The microfluidic channels (80 μm height, 500 μm width) were fabricated using standard soft lithography approaches from PDMS and were placed onto a glass slide with adhesion between PDMS and glass being made using an O_2 plasma.[22] First, the microfluidic channel walls were coated by flowing the copolymer solution (10 mg/mL in methanol) through the channels for 2 h at a rate of 2 μL/min. During this procedure, swelling of PDMS was minimized by using methanol as solvent. Then fluorescein isothiocyanate labeled bovine serum albumin (FITC-BSA) (0.1 mg/mL in PBS, pH 7.4) was allowed to flow through the channel at a rate of 5 μL/min for 30 min. The channel was subsequently washed with PBS solution for 15 min. The PDMS and glass parts were separated, and fluorescence images were taken of each channel area. As shown in the fluorescence images, much less protein adsorption was observed on the polymer-coated channel walls on both glass (Figure 3b) and PDMS (Figure 3d) compared to those

(20) The polymer films on glass and PDMS substrates were prepared by immersing the substrates in a methanol solution of the copolymer (10 mg/mL) for 2 h and curing at 120 °C for 15 min. The polymer films were characterized by XPS at a 55° take-off angle (Figure S5) and the degree of nonspecific protein adsorption was obtained from the high-resolution N(1s) XPS spectrum at 55° angle (Figure S6). See Supporting Information for details.

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(22) The picture of the microfluidic device is shown in Figure S7. For a reference how microfluidic channels are made, see: Beebe, D. J.; Mensing, G. A.; Walker, G. M. *Annu. Rev. Biomed. Eng.* **2002**, *4*, 262.

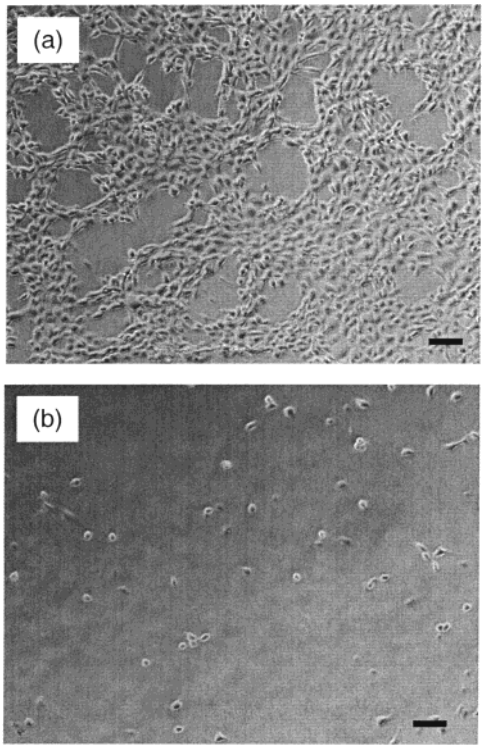

Figure 4. NIH 3T3 fibroblasts cultured on (a) unmodified glass and (b) the polymer-coated glass for 24 h with an initial seeding density of 5.3×10^4 cells/cm^2. The scale bar indicates 100 μm.

of unmodified glass and PDMS (parts a and c of Figure 3, respectively).[23]

To investigate the cell-resistant properties of the PSAMs, we carried out cell culture experiments. NIH 3T3 fibroblasts, which tend to be very adhesive and can make confluent monolayers, were seeded on unmodified glass and the polymer-coated glass. After 24 h, each glass substrate was gently washed with the same culture medium and optical microscopic images were taken. Fibroblasts grew on the unmodified glass slides to form confluent monolayers with density of ca. 1.4×10^5 cells/cm^2 (Figure 4a); however, the polymer-coated glass slide was highly resistant to cell adhesion as well as to cell spreading (Figure 4b) with density of ca. 4.3×10^3 cells/cm^2. This result may be attributed to high resistance of the copolymer layers to serum proteins in the cell culture medium.

In conclusion, we have demonstrated that nonbiofouling surfaces can be easily prepared on bare Si/SiO_2 substrates by forming polymeric self-assembled layers of a surface-reactive PEG-grafted copolymer. The PSAMs on Si/SiO_2 wafers were reproducibly generated under an ambient environment without special care for anhydrous conditions. The resulting ultrathin films showed a great reduction in nonspecific protein adsorption (up to 98%) and cell adhesion. The present approach may be potentially applied to other substrates such as metal oxides (e.g., Al_2O_3, TiO_2) and polymer surfaces. Since functional units other than PEG (e.g., biotin, fluorocarbons) can be also incorporated into the copolymer as monomer units, the combined copolymer system of the "anchor part" (trialkoxysilane) and the "function part" may have applications in the preparation of functional surfaces for medical and analytical devices.

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Supporting Information Available: Effects of immersion time and copolymer concentration on the thicknesses of the PSAMs, 2D AFM images of the PSAMs, high-resolution C(1s) X-ray photoelectron spectra of the PSAMs on Si/SiO_2 wafers, ellipsometric thickness changes in the PSAMs on Si/SiO_2 wafers after immersion into each protein, high-resolution C(1s) XPS spectra of polymer-coated PDMS and glass, protein resistance data for polymer-coated PDMS and glass, picture of the microfluidic device. This material is available free of charge via the Internet at http://www.acs.org.

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(23) Details of this work will be published separately.