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Specific Binding of Streptavidin onto the Nonbiofouling Titanium/Titanium Oxide Surface through Surface-Initiated, Atom Transfer Radical Polymerization and Bioconjugation of Biotin

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Abstract: Chemical modification of titanium/titanium oxide (Ti/TiO_2) substrates has recently gained a great deal of attention because of the applications of Ti/TiO_2-based materials to biomedical areas. The reported modification methods generally involve passive coating of Ti/TiO_2 substrates with protein-resistant materials, and poly(ethylene glycol) (PEG) has proven advantageous for bestowing a nonbiofouling property on the surface of Ti/TiO_2. However, the wider applications of Ti/TiO_2-based materials to biomedical areas will require the introduction of biologically active moieties onto Ti/TiO_2, in addition to nonbiofouling property. In this work, we therefore utilized surface-initiated polymerization to coat the Ti/TiO_2 substrates with polymers presenting the nonbiofouling PEG moiety and subsequently conjugated biologically active compounds to the PEG-presenting, polymeric films. Specifically, a Ti/TiO_2 surface was chemically modified to present an initiator for atom transfer radical polymerization, and poly(ethylene glycol) methacrylate (PEGMA) was polymerized from the surface. After activation of hydroxyl groups of poly(PEGMA) (pPEGMA) with *N,N'*-disuccinimidyl carbonate, biotin, a model compound, was conjugated to the pPEGMA films. The reactions were confirmed by infrared spectroscopy, X-ray photoelectron spectroscopy, contact angle goniometry, and ellipsometry. The biospecific binding of target proteins was also utilized to generate micropatterns of proteins on the Ti/TiO_2 surface.

Keywords: titanium/titanium oxide, atom transfer radical polymerization, biological applications of polymers.

Introduction

Due to the high mechanical strength, corrosion resistance and biocompatibility, titanium/titanium oxide (Ti/TiO_2) and its alloys have been investigated for orthopedic and dental implants, and other biomedical applications.[1] In these applications, the first intimate interactions between Ti/TiO_2 and bioactive molecules occur at the surface of Ti/TiO_2 in the body; therefore, it is crucial to control chemical and biological properties of the surface of Ti/TiO_2 for its facile applications to biomedical areas. Several methods for modifying/controlling the surface properties of Ti/TiO_2 have been developed.[2] Among them, chemical approaches have recently gained a great deal of attention, especially focusing on giving nonbiofouling property to Ti/TiO_2 substrates, because non-specific adsorption of biomolecules onto Ti/TiO_2 is seriously high. Non-specific adsorption of biomolecules to the oxide surface of Ti/TiO_2 is a generally occurring phenomenon, when Ti/TiO_2-based materials are exposed to biological environments.[3] The non-specifically adsorbed proteins can interfere with desired functions of biomedical devices, such as blood-contacting devices and surgical tools.[4] On the other hand, a desired, strong adhesion/recognition between Ti/TiO_2 and biologically active materials is found to be difficult to achieve because of its low bioactivity. Although Ti/TiO_2 is a widely-accepted material for supporting damaged bones, strong and close adhesion of the bones to Ti/TiO_2 has not been achieved so far.[5] Based on the fact mentioned above, much effort has been made for improving the surface

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properties of Ti/TiO_2 with chemical compounds, exemplified by grafting with functional polymers,[6] formation of self-assembled monolayers (SAMs),[7] adsorption of monolayer/multilayer polyelectrolytes,[8] formation of supported phospholipid bilayers.[9] Among the chemical attachment methods, grafting with functional polymers has been attempted very recently. For example, Messersmith *et al.* demonstrated surface-initiated polymerization of a monomer bearing oligo (ethylene glycol) (OEG) methyl ether from Ti/TiO_2 surfaces in order to give them nonbiofouling property.[10] They used the catecholic initiator, and showed reduced cell adhesion onto the polymer-grafted substrates. They also showed the effect of lengths of EG side chains on the cell-fouling resistance.[11] As another approach to the functionalization of Ti/TiO_2 surfaces, Tosatti *et al.* used poly(alkyl-phosphonates):[12] they prepared copolymers carrying both PEG and phosphonate groups, and anchored copolymers onto the Ti/TiO_2 surfaces by grafting-onto approach. The copolymer-anchored Ti/TiO_2 surfaces also showed good resistance to protein adsorption. Albeit much effort mentioned above, these cases focused on the generation of only nonbiofouling Ti/TiO_2 surfaces.

We have previously utilized the surface-initiated, atom transfer radical polymerization (SI-ATRP) of EG-containing monomers from flat surfaces of gold, glass and silicon wafers, to fabricate a highly efficient platform for anchoring biomolecules.[13] For example, we generated thin films of poly(PEG methacrylate) (pPEGMA) with different thicknesses by SI-ATRP, and subsequently attached biotin onto the polymeric surfaces after the activation of the hydroxyl groups with *N,N'*-disuccinimidyl carbonate (DSC).[13b] In this work, we extended our reported strategy of achieving both nonbiofouling property and functionalizability to the surface of Ti/TiO_2. Ti/TiO_2 was chemically modified to present a catechol-based ATRP initiator, and PEGMA was polymerized from the surface.[14] After activation of hydroxyl groups of pPEGMA with DSC, biotin was conjugated to pPEGMA films as a model compound. The pPEGMA-grafted surfaces were found to be highly resistant to non-specific adsorption of proteins both before and after the conjugation steps, and the highly specific binding of streptavidin onto the biotin-presenting, pPEGMA surface was achieved. The specific binding of target proteins was also utilized to generate micropatterns of proteins on the Ti/TiO_2 surface.

Experimental

Materials. Copper(I) bromide (CuBr, 99.999%, Aldrich), 2,2'-dipyridyl (bpy, 99+%, Aldrich), 2-(3,4-dihydroxyphenyl)ethylamine hydrochloride (dopamine hydrochloride, 98%, Aldrich), 2-bromo-2-methylpropionyl bromide (98%, Aldrich), triethylamine (99.5%, Aldrich), poly(ethylene glycol) methacrylate (PEGMA, M_n: 360, Aldrich), *N,N'*-disuccinimidyl carbonate (DSC, Aldrich), 4-(dimethylamino)pyridine (DMAP, 98%, Fluka), phosphate-buffered saline (PBS, Sigma), fibrinogen (fraction I from human plasma, plasminogen-free, Sigma), lysozyme (grade III from chicken egg white, Sigma), streptavidin (Pierce), tetramethylrhodamine isothiocyanate (TRITC)-conjugated streptavidin (Pierce), 5-(biotinamido)-pentylamine (Pierce), 2-aminoethanol (99+%, Aldrich), absolute methanol (99.9%, Merck), and methylene chloride (CH_2Cl_2, HPLC grade, Merck) were used as received. Ultrapure water (18.3 MΩ·cm) from the Human Ultra Pure System (Human Corp., Korea) was used.

Characterization. IR spectra were obtained in single-reflection mode using a dry N_2-purged Thermo Nicolet Nexus FT-IR spectrophotometer equipped with the smart SAGA (smart apertured grazing angle) accessory. The *p*-polarized light was incident at 80° relative to the surface normal of the substrate, and a narrow band mercury-cadmium-telluride (MCT) detector cooled with liquid nitrogen was used to detect the reflected light. We averaged 2000 scans to yield the spectra at a resolution of 4 cm^{-1}, and all the spectra were reported in the absorption mode relative to that of a clean Ti/TiO_2 surface. The X-ray photoelectron spectroscopy (XPS) study was performed with a VG-ScientificESCALAB250 spectrometer (U.K.) with a monochromatized Al Kα X-ray source. Emitted photoelectrons were detected by a multichannel detector at a takeoff angle of 90° relative to the surface. During the measurements, the base pressure was 10^{-9}-10^{-10} Torr. Survey spectra were obtained at a resolution of 1 eV from three scans, and high-resolution spectra were acquired at a resolution of 0.05 eV from 5 to 20 scans. The thickness of monolayers and polymeric films was measured with a Gaertner L116s ellipsometer (Gaertner Scientific Corporation, IL) equipped with a He-Ne laser (632.8 nm) at a 70° angle of incidence. A refractive index of 1.46 was used for all the films. Fluorescence images were acquired on an IX 71 fluorescence microscope (Olympus, Japan). Contact angle measurements were performed using a Phoenix 300 goniometer (Surface Electro Optics Co., Ltd., Korea).

Synthesis of the Polymerization Initiator. To a round-bottom flask were added dopamine hydrochloride (1 g), triethylamine (1.46 mL), and DMF (10 mL), and the mixture was stirred for 30 min at 0 °C. 2-Bromo-2-methylpropionyl bromide was then slowly added to the mixture, and the resulting mixture was stirred for 2 h at room temperature. The volatile materials were evaporated under reduced pressure, and the resulting crude product was purified by silica gel column chromatography (CH_2Cl_2:methanol = 9:1, 78%). The product was characterized by NMR and mass spectroscopy. ^1H NMR (400 MHz, $CDCl_3$): δ 6.89 (3H, bs), 6.71 (1H, d, J = 8.0 Hz), 6.67 (1H, s), 6.46 (1H, d, J = 8.0 Hz), 3.36 (2H, m), 2.60 (2H, dd, J = 6.9, 7.0 Hz), 1.81 (6H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.12, 144.32, 142.91, 130.15, 120.22, 115.44, 115.10, 61.91, 41.59, 32.02, 31.45. MS: calculated m/z for $C_{12}H_{16}BrNO_3$ 301.0301, found 301.0207.

Synthetic Procedures. Ti/TiO_2 substrates were prepared by thermal evaporation of 100 nm of Ti onto silicon wafers.

Prior to use, Ti/TiO_2 substrates were cleaned in acetone and methanol with sonication. The substrates were further cleaned by using an oxygen plasma cleaner (Harrick PDC-002, medium setting, 1 min). The SAMs of the ATRP initiators were prepared by immersing a Ti/TiO_2 substrate in a CH_2Cl_2 solution of the ATRP initiator (1 mg/mL) overnight. After formation of the SAMs, the substrate was rinsed with CH_2Cl_2 several times and then dried under a stream of argon.

Before SI-ATRP, the monomer, PEGMA, was purified by column chromatography on basic aluminum oxide. The initiator-attached Ti/TiO_2 substrate was placed in a Schlenk flask, and then the Schlenk flask was degassed under vacuum and purged with argon. To another Schlenk flask were added CuBr (0.014 g) and 2,2′-dipyridyl (0.031 g), and the mixture was degassed under vacuum and purged with argon. To the Schlenk flask containing the catalysts, were added degassed water (3.26 mL), methanol (5.38 mL), and purified PEGMA (3.26 mL). The resulting solution was transferred to the Schlenk flask containing the initiator-attached Ti/TiO_2 substrate by using a syringe. The resulting mixture was stirred for 3 h at room temperature, and the pPEGMA-coated Ti/TiO_2 substrate was taken, rinsed with methanol, and dried under a stream of argon.

The pPEGMA-coated Ti/TiO_2 substrate was placed in a flask, and a 10-mL DMF solution of DSC (0.1 M) and DMAP (0.1 M) was added to the flask. The activation reaction was carried out for 3 h at room temperature. The Ti/TiO_2 substrate was taken, rinsed with DMF, and dried under a stream of argon.

A PBS (pH 7.4, 2 mL) buffer solution of 5-(biotinamido)-pentylamine (0.5 mM) was prepared in a conical tube. The substrate coated with DSC-activated pPEGMA films was immersed in the solution without stirring for 3 h at room temperature, taken from the solution, rinsed thoroughly with PBS buffer, and dried under a stream of argon.

Nonbiofouling and specific binding properties of the biotin-presenting surfaces were investigated with PBS buffer solutions (0.1 mg/mL) of fibrinogen, lysozyme and streptavidin as model proteins. After 2-h immersion of the substrate in each solution, the substrate was washed with distilled water and dried under a stream of argon.

Microcontact Printing (μCP) and Pattern Generation. A PDMS stamp was prepared according to the literature method using Sylgard 184 silicon elastomer (Dow Corning).[13b] Before its use, the PDMS stamp was oxidized by an oxygen plasma cleaner (Harrick PDC-002, medium setting) for 1 min. The oxidized PDMS stamps were inked by spin-casting with a 1 mM PBS buffer (pH 7.4, 10 mL) of 5-(biotinamido)pentylamine for 1 min at 1,000 rpm. The inked stamp was brought into contact with the DSC-activated pPEGMA films for 1 min. After μCP of 5-(biotinamido)pentylamine, the sample was immersed immediately in a PBS buffer of 2-aminoethanol (1 mM). After 3 h, the sample was taken and rinsed with distilled water. For the visualization of the pattern, the sample was incubated in a solution of TRITC-conjugated streptavidin (0.1 mg/mL) in PBS buffer (pH 7.4) containing 0.1% (w/v) bovine serum albumin and 0.02% (v/v) Tween 20 at room temperature. After 2 h, the sample was taken, and washed several times with PBS buffer and distilled water. Fluorescence microscopy was used to examine the streptavidin-bound surface.

Results and Discussion

The chemical functionalization of Ti/TiO_2 surfaces with cell-adhesive peptides, such as Arg-Gly-Asp (RGD) and bone morphogenetic proteins (BMPs), has recently drawn attention. For example, Schwartz *et al.* developed a simple but versatile method for forming uniform monolayers of phosphonic acids on Ti/TiO_2, and chemically attached the RGD peptide onto the surface.[15] Menzel *et al.* prepared BMP-2-conjugated phosphonic acid monolayers:[7e] the hydroxyl- and carboxylic acid-containing phosphonic acids were assembled on Ti/TiO_2, and BMP-2 was attached to the Ti/TiO_2 surface using *N,N′*-carbonyldiimidazole (CDI) and *N*-hydroxysuccinimidyl (NHS) groups, respectively. These examples demonstrated methods for attaching biologically active moieties onto Ti/TiO_2, but the systems lacked the nonbiofouling property; it is desirable to have both properties, nonbiofouling and post-functionalizable properties, for facile applications of materials to many technologically important areas, including implants, prosthetic devices, and biosensors. In this work, we applied our reported protocols to Ti/TiO_2 surfaces for achieving the two properties. The experimental procedure consists of three simple steps: (1) immobilization of ATRP initiators onto Ti/TiO_2 surfaces, (2) SI-ATRP of PEGMA from the Ti/TiO_2 surfaces, and (3) activation of pPEGMA films and subsequent bioconjugation (Figure 1). pPEGMA has been investigated as a coating material, because of the biologically important functions of PEG: PEG side chain of the polymer, is resistant to non-specific adsorption of proteins and cells[16] as well as nontoxic and nonimmunogenic.[17] In addition, the chemical functionalizability of the hydroxyl group, terminal group of PEG, has proven advantageous over other polymers for achieving highly biospecific interactions at surfaces by us and others.[13b-d,14] ATRP was adapted for polymerizing PEGMA from the surface of Ti/TiO_2, because ATRP has several advantages over other polymerization techniques, including the precise control over the molecular weight of polymers (the thickness of functional polymer layers in this study) and mild polymerization conditions.[18]

Surface-Initiated, Atom Transfer Radical Polymerization of PEGMA and Activation of pPEGMA Films. The use of catechols has been well-developed for surface modifications of Ti/TiO_2. It is believed that strong bidentate charge-transfer complexes are formed between OH groups of catechols and Ti/TiO_2 surfaces.[19] The SAMs of the ATRP initia-

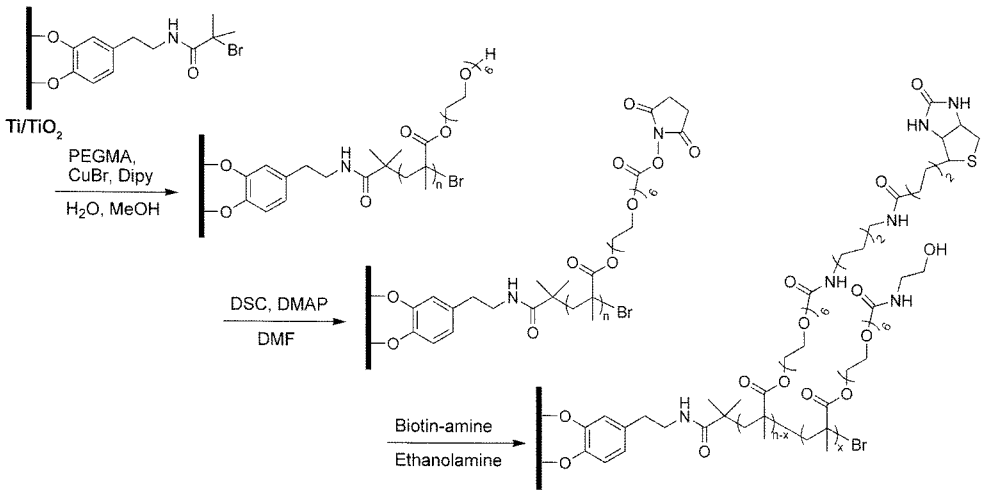

Figure 1. The procedure for the formation of functional Ti/TiO_2 surfaces.

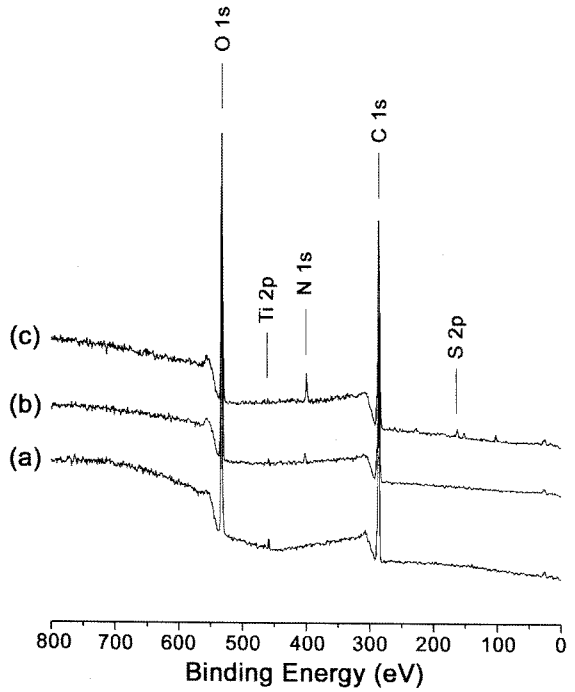

Figure 2. XPS spectra of (a) pPEGMA-coated, (b) DSC-activated, and (c) biotin-conjugated Ti/TiO_2 substrates.

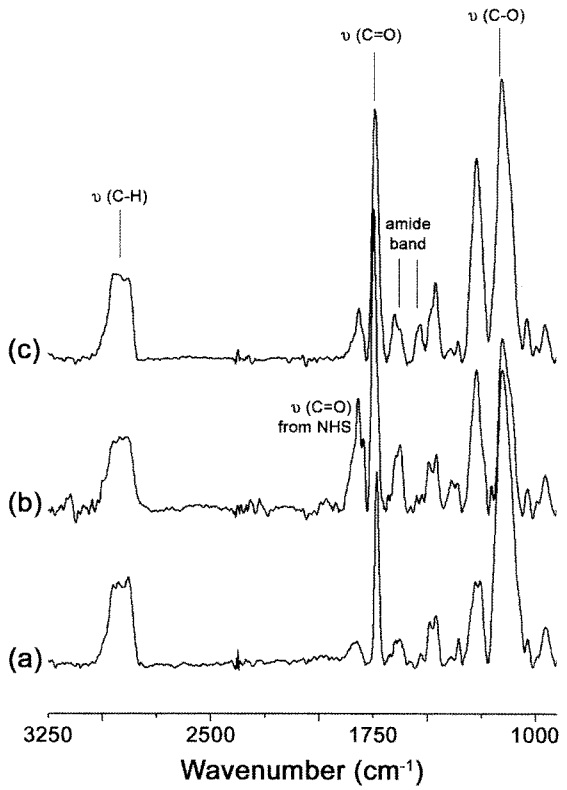

Figure 3. IR spectra of (a) pPEGMA-coated, (b) DSC-activated, and (c) biotin-conjugated Ti/TiO_2 substrates.

tor were formed by immersing a Ti/TiO_2 substrate in CH_2Cl_2 solution of the initiator for overnight at room temperature. The thickness of the resulting bromide-terminated SAMs was measured to be 14.6±0.7 Å by ellipsometry, and the water static contact angle was 63.1°. The XPS spectrum showed a new peak at 69.76 eV (Br 3d), indicative of the presence of the SAMs on Ti/TiO_2 surfaces (data not shown). After 3 h of SI-ATRP, a 17-nm-thick pPEGMA film was obtained. We used this pPEGMA film for further study, because our previous results indicated that the binding capacity of pPEGMA films was maximized and saturated at about 20 nm.[13b] The water static contact angle was changed to 45.8° after the polymerization, indicative of relatively hydrophilic nature of the pPEGMA surface. Although the XPS spectrum did not show any characteristic peaks (Figure 2(a)), the IR spectrum showed peaks at 2870-2960 (C-H stretching), 1730 (C=O stretching), and 1149 cm^{-1} (C-O-C stretching) (Figure 3(a)).

After the formation of the pPEGMA films, the OH-termi-

nated side chains were activated with *N,N'*-disuccinimidyl carbonate (DSC) for selective conjugation of biomolecules to the pPEGMA films. The XPS and IR spectra indicated the successful transformation of terminal OH groups to NHS ester groups. The XPS spectrum showed a new peak at 400.34 eV (N 1s) from the NHS groups (Figure 2(b)), and the IR spectrum did intense C=O stretching peaks at 1816 and 1793 cm^{-1} (Figure 3(b)). After activation, the thickness increased to 20 nm.

Amide Coupling with Biotin. We investigated subsequent conjugation of the activated pPEGMA films with biomolecules. As a model of biomolecules, we chose biotin because biotin has a strong, biospecific interaction with streptavidin (K_D=10^{-15} M),[20] and the association between biotin and streptavidin is very rapid and unaffected by extremes of pH, organic solvents, and other denaturing agents. In addition, the presence of biotin can be confirmed and visualized with ease by complexation with fluorescent dye-conjugated streptavidin. A biotin compound bearing the amine group, 5-(biotinamido)pentylamine, was covalently attached to the pPEGMA films by the amide bond formation between the reactive NHS group and the amine group of the biotin compound. After the coupling reaction, 2-nm-increase in the thickness was observed; the thickness was 22 nm. The XPS spectrum showed a new peak at 161.51 eV (S 2p) from biotin, in addition to the relative increase of the peak intensity at 397.87 eV (N 1s) (Figure 2(c)). The IR spectrum showed two new peaks at 1650 and 1550 cm^{-1} (amide bands), which also indicated the successful coupling of biotin to the pPEGMA films (Figure 3(c)).

Biospecific Interactions and Pattern Generation. The nonbiofouling property of the Ti/TiO_2 substrates was tested with fibrinogen, lysozyme and streptavidin by using XPS and ellipsometry. Fibrinogen and lysozyme were used as model proteins, because fibrinogen has been known as a "sticky protein", and lysozyme is a positively charged globular protein under the PBS buffer of pH 7.4.[21] Streptavidin was a target protein for biotin-presenting Ti/TiO_2 surfaces. In the XPS experiments, the N (1s) peak was used as a guidance for the non-specific adsorption.[22] The bare Ti/TiO_2 substrate was used as a comparison. In the case of the pPEGMA film (before activation), the intensity of the N (1s) peak was below or close to the detection limit with all three proteins tested (Figure 4(a)). In contrast, the XPS intensity from the bare Ti/TiO_2 was significantly high after incubation with protein solutions. The XPS results indicated that the pPEGMA film was highly resistant to the adsorption of proteins.

Because the XPS analysis was not practically applicable to the biotin-conjugated pPEGMA films that had a large amount of N, the ellipsometric measurement was used as a complementary method for studying the nonbiofouling property of the biotin-conjugated pPEGMA film. Figure 4(b) shows the thickness changes after immersion of three Ti/TiO_2 substrates—bare Ti/TiO_2, pPEGMA film, and biotin-conjugated pPEGMA film—in the protein solutions. The ellipsometric measurements were in agreement with the XPS analysis. While the thickness increased by 10-40 Å for the bare Ti/TiO_2 substrate, the thickness increase was nearly negligible for the pPEGMA film: we observed rather a small thickness decrease after the immersion.

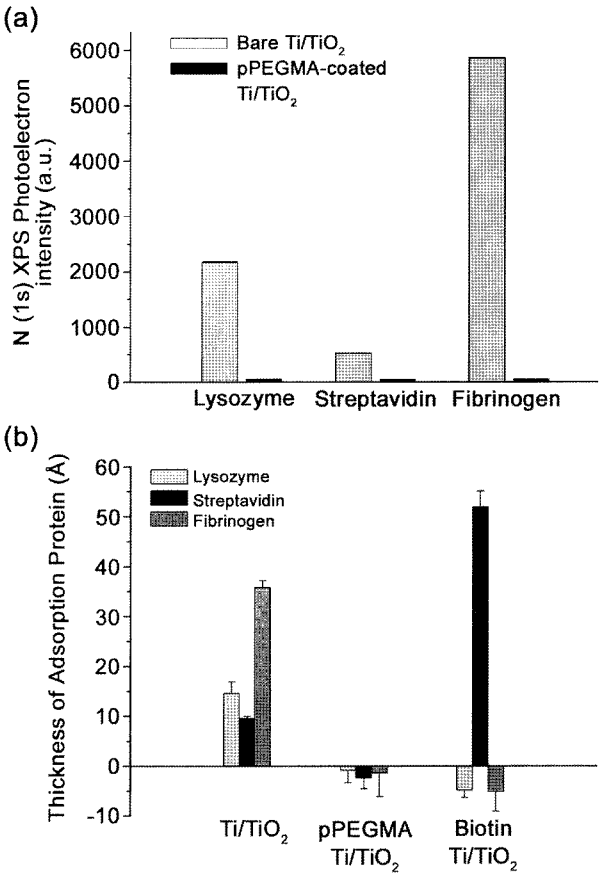

Figure 4. (a) Protein adsorption onto bare and pPEGMA-coated Ti/TiO_2 substrates. The amount of adsorbed proteins on the surface is denoted by N (1s) XPS photoelectron intensity. (b) Thickness changes for bare, pPEGMA-coated, and biotin-conjugated Ti/TiO_2 substrates, after protein adsorption experiments.

In the case of the biotin-conjugated pPEGMA film, we observed a highly biospecific binding of streptavidin to the surface (51-Å-increase); we did not observe any thickness increase with the other two proteins, lysozyme and fibrinogen. These results clearly indicated that the Ti/TiO_2 substrate possessed the biospecific binding property for streptavidin with nonbiofouling characteristics for the other proteins, which would be a crucial prerequisite for bioassays and functional implants and prosthetic devices. Because the binding of streptavidin to the biotin-conjugated pPEGMA film was highly specific, we applied a technique of microcontact printing for pattern generation. After contact-printing 5-(biotinamido)pentylamine on the DSC-activated pPEGMA film with a PDMS stamp (50-μm-wide lines separated by

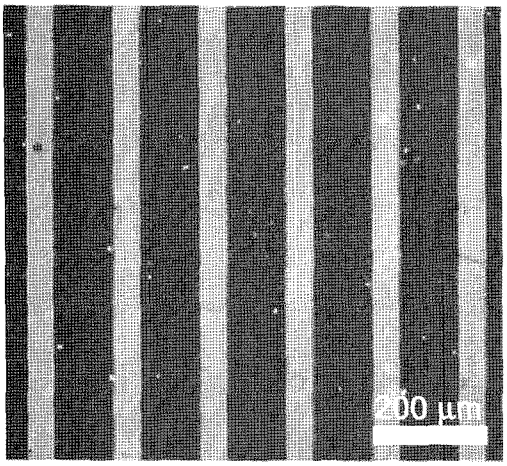

Figure 5. Fluorescence image of TRITC-conjugated streptavidin bound to micropatterens of biotin.

100 μm), the other areas were passivated with 2-aminoethanol. TRITC-conjugated streptavidin was used for visualization. We observed highly contrasted red color with black background (Figure 5), which also confirmed that our method was smoothly applied to the introduction of dual functions, nonbiofouling property and biospecficity, onto Ti/TiO_2 surfaces.

Conclusions

In summary, we demonstrated a method for introducing two important properties, nonbiofouling and functionalizable properties, onto Ti/TiO_2 surfaces by surface-initiated, atom transfer radical polymerization of PEGMA and subsequent activation of the hydroxyl groups of pPEGMA. With biospecific biotin-streptavidin recognition as a model, we clearly showed that our approach was suitable for the generation of functionalizable, nonbiofouling Ti/TiO_2 surfaces. The prepared surfaces had the streptavidin-specific binding property while no interactions with other proteins, such as fibrinogen and lysozyme. Moreover, the spatioselective binding of streptavidin was achieved by microcontact printing of biotin onto the pPEGMA-coated Ti/TiO_2, followed by backfilling with ethanolamine. We believe the method described in this paper would find its applications in biomedical areas, where Ti/TiO_2 has widely been being used.

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