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Protein-Resistant Cross-Linked Poly(vinyl alcohol) Micropatterns via Photolithography Using Removable Polyoxometalate Photocatalyst

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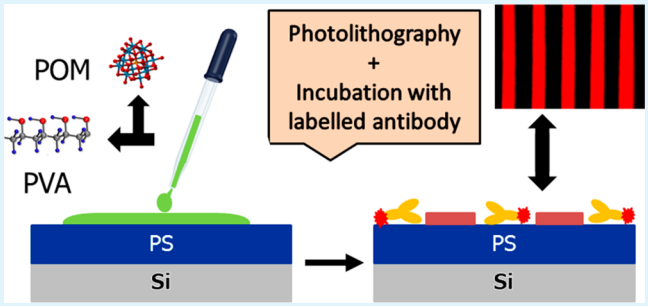
Supporting Information

ABSTRACT: In the last years, there has been an increasing interest in controlling the protein adsorption properties of surfaces because this control is crucial for the design of biomaterials. On the other hand, controlled immobilization of proteins is also important for their application as solid surfaces in immunodiagnostics and biosensors. Herein we report a new protein patterning method where regions of the substrate are covered by a hydrophilic film that minimizes protein adsorption. Particularly, poly(vinyl alcohol) (PVA) cross-linked structures created by an especially developed photolithographic process are proved to prevent protein physisorption and they are used as a guide for selective protein adsorption on the uncovered areas of a protein adsorbing substrate such as polystyrene. The PVA cross-linking is induced by photo-oxidation using, as a catalyst, polyoxometalate ($H_3PW_{12}O_{40}$ or α-$(NH_4)_6P_2W_{18}O_{62}$), which is removed using a methyl alcohol/water mixed solvent as the developer. We demonstrate that the polystyrene and the cross-linked PVA exhibit dramatically different performances in terms of protein physisorption. In particular, the polystyrene areas presented up to 130 times higher protein binding capacity than the PVA ones, whereas the patterning resolution could easily reach dimensions of a few micrometers. The proposed approach can be applied on any substrate where PVA films can be coated for controlling protein adsorption onto surface areas custom defined by the user.



KEYWORDS: *poly(vinyl alcohol) cross-linking, protein adsorption, photo-oxidation, polyoxometalates, 12-tungstophosphoric acid, ammonium 18-tungstodiphosphate*

1. INTRODUCTION

Surface functionalization methods play a key role in the nanobiotechnology field because they represent an indispensable processing step in fabrication of biomolecule microarrays and bioelectronic devices, as well as in tissue engineering and fundamental studies on biomolecule and cell interactions and their function in specific environments. In addition to application-specific biomolecule deposition and patterning processes, the design of surfaces that exhibit minimized protein adsorption is also a scientific issue of great interest for both in vitro and in vivo applications including fabrication of biosensors, microfluidics, implanted devices, and drug-delivery carriers.[1−8] These surfaces are usually referred in the literature as nonbiofouling, nonfouling, inert, or nonadsorbing, and although the specific requirements for such surfaces can differ with respect to the final application, the target in all cases is to avoid undesirable coupling to certain surface areas of proteins existing in the biological medium.

To this end, the chemical modification of surfaces with protein repulsive hydrophilic moieties is the most commonly employed approach. The hydrophilic materials so far used include molecules such as poly(ethylene glycol) (PEG) and oligo(ethylene glycol) (OEG),[9,10] tetraglyme,[11] dextran,[12,13] mannitol,[14] and glycerol dendrons.[15] Also, zwitterionic materials such as poly(sulfobetaine methacrylate),[16,17] poly(carboxybetaine methacrylate),[18] and poly(2-methacryloyloxyethyl phosphorylcholine)[19,20] are commonly used nonbiofouling materials. Coating or modification of surfaces with these molecules has been demonstrated to reduce nonspecific protein adsorption and cell and/or bacterial adhesion as well as biofilm formation. The nonfouling properties of such coatings/modifications are thought to result from both their chemical and physical properties.[21] In particular, the highly hydrated layer formed acts both as a physical and energy barrier that prevents protein adsorption on the surface. Furthermore, the protein adsorption resistance of these surfaces is enhanced by

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steric repulsion, especially when long-chain polymers are used for the surface modification.[22]

Although very successful in preventing protein adsorption, the mentioned surface modification approaches usually involve multiple steps including a first modification of the surface with silanes followed by reactions for covalent immobilization of hydrophilic moieties.[12,16] The process is further complicated when the spatially controlled modification of the surfaces is desirable. In this case, patterning of surfaces can be achieved by microcontact printing, microfluidic patterning and liquid-phase printing, photolithography, plasma polymerization combined with photolithography or laser ablation, photoimmobilization on photochemically generated pattern, stencil-assisted patterning, or inkjet printing.[3,23] Among these methods, those based on photolithography for the definition of nonbiofouling areas offer the advantages of high fidelity pattern replication at the micrometer and submicrometer range and of easier integration in a mass fabrication process. In this respect, the application of polymers with protein repellent properties that can be processed by photolithography is expected to facilitate the creation of surfaces with spatial control of their nonfouling/protein adsorption properties.[24]

A new approach to create patterns with controlled protein adsorption properties onto a surface by employing a patternable hydrophilic polymer film is reported here. Particularly, a poly(vinyl alcohol)-based photosensitive film is applied in order to create protein repulsive structures. Poly(vinyl alcohol) (PVA) has been previously used to modify flat surfaces intended to be used as substrates in biosensors in order to reduce the nonspecific protein adsorption and/or provide functional groups for covalent coupling of biomolecules.[25] In addition, the formation of a polymer hydrogel having the mechanical and optical properties required for the production of soft contact lenses and biomedical implants by UV cross-linking PVA with acrylic and methacrylic acid has been reported.[26] Moreover, PVA has been used to modify polystyrene-based materials in order to create stationery phases for liquid chromatography.[27−29] It should be noted though that PVA films, especially the ones of submicrometer thickness, are highly water-soluble and usually during exposure to protein solutions undesirable desorption of PVA molecules is taking place.[27] Thus, in order to be used as surface coating for biomedical or bioanalytical applications, cross-linking or modification of the PVA molecules is required to render the film insoluble to aqueous media. The most important problem of hydrophilic polymers in that respect is that they may turn from nonadsorbing to adsorbing upon chemical modification.[30]

In the current work, stable cross-linked PVA patterns are demonstrated that exhibit negligible protein physisorption as shown upon incubation with protein solutions. These cross-linked PVA structures were fabricated through photolithography on well-known protein adsorbing hydrophobic polymeric surfaces, leading to the successful selective adsorption of proteins. The PVA film is applied onto a substrate coated with a protein adsorbing hydrophobic film such as polystyrene and transformed to a stable, non-water-soluble layer by cross-linking initiated by photo-oxidation. Selected polyoxometalates such as $H_3PW_{12}O_{40}$ or α-$(NH_4)_6P_2W_{18}O_{62}$ are used as photo-oxidation catalysts to initiate reactions that result in PVA cross-linking. A similar formulation had been proposed in the past for a photoresist working in a double layer configuration for DUV lithography with the 12-tungstophosphoric acid also acting as the etch-resistant component.[31] Nevertheless, this photoresist did not find wide acceptance in the area of photolithography, probably due to the unconventional developer solutions that were required to prevent polyoxometalate leaching during the development step. On the contrary, in the present investigation, we make use of the fact that the photocatalyst, 12-tungstophosphoric acid or ammonium 18-tungtodiphosphate, can be effectively removed from the film after patterning using a water/methanol mixture as the developer. Thus, a PVA cross-linked film stable against aqueous solutions and, at the same time, acting as an effective barrier against protein adsorption is obtained. The negligible protein adsorption onto the patterned cross-linked PVA as well as the relatively small effect of PVA film patterning process to the protein adsorption properties of the polystyrene underlayer were demonstrated either through direct adsorption of fluorescently labeled biomolecules or by using a biotin/streptavidin assay. The resistance of cross-linked poly(vinyl alcohol) films to nonspecific protein adsorption could further pave the way to better understand nonfouling properties of hydrophilic polymers and facilitate the creation of nonadsorbing regions that could be integrated in bio-microelectromechanical systems (Bio-MEMs).

2. EXPERIMENTAL SECTION

2.1. Materials. Poly(vinyl alcohol) (PVA; Mw 13 000−23 000, 98% hydrolyzed), polystyrene (PS; Mw 280 000), and 12-tungstophosphoric acid (commonly also named as phosphotungstic acid) hydrate ($H_3PW_{12}O_{40}\cdot xH_2O$), were obtained from Aldrich. The ammonium 18-tungstodiphosphate, α-$(NH_4)_6P_2W_{18}O_{62}\cdot xH_2O$, was synthesized according to well-established methods.[32] The 12-tungstophosphoric acid and the ammonium 18-tungstodiphosphate belong to the class of the polyoxometalate compounds, also referred as POMs. An epoxidized cresol-formaldehyde novolac was purchased from Shell Chemical Company (named as Epikote 164). The commercial polymer was fractionated and the medium fraction was used with the abbreviation EPN (Mn = 1.277, Mw = 2.438, and I = Mw/Mn = 1.9). AZ5214, a novolac-diazonaphthoquinone-type photoresist, and the developer AZ726 MIF (0.26 N aqueous solution of tetramethylammonium hydroxide) were purchased from Clariant GmbH (Wiesbaden, Germany). Streptavidin labeled with Alexa Fluor 546 (AF546), goat antirabbit IgG antibody labeled with Alexa Fluor 546 (AF546), and goat IgG were purchased from Molecular Probes, Inc. (Eugene, OR, USA). Rabbit γ-globulins (RgG) and bovine serum albumin (BSA, Cohn fraction V, RIA grade) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Biotinylated BSA (b-BSA) was prepared according to a published method.[33] Deionized water with a resistivity of 15 MΩ cm^{-1} prepared from a Milli-RO plus apparatus (Millipore) was used where necessary.

2.2. Characterization Methods. The spectroscopic characterization of the films was carried out using an Ultraviolet−Visible PerkinElmer Lambda 40 spectrophotometer and a Fourier transform infrared (FTIR) Bruker Tensor 27 spectrometer. The surface topography was characterized by atomic force microscopy (AFM, Veeco CPII) in the tapping mode. The scanned area was 1 × 1 μm with 512 × 512 pixel resolution. The root-mean-square (rms) surface roughness was calculated from the software of the instrument. The contact angles were measured using a GBX Digidrop contact angle measurement system. The surfaces wetting properties were characterized with sessile droplets at ambient conditions using the Windrop program. Deionized water was used for contact angle measurements using 5 μL droplets. The thickness of polymeric films was measured using an Ambios technology XP-2 stylus profilometer.

2.3. Preparation of Substrates. Silicon wafers were coated with thin polymeric films to render them able for protein immobilization through adsorption. Three different polymeric materials were tested: polystyrene (PS), the commercial photoresist AZ5214, and epoxy-novolac EPN. The creation of stable and defect-free thin film underlayers was necessary in order to achieve high selective protein

adsorption on the patterned surfaces. Thus, prior to the spin-coating of the more hydrophobic PS and AZ5214, the silicon wafers were modified with 1,1,1,3,3,3-hexamethyldisilazane (HMDS) in order to make the surface hydrophobic through its interactions with the silanol groups.[34] Also, the HMDS primer seals out the moisture and enhances the adhesion between the sample and the polymeric films.

Particularly, for the preparation of the substrates, the following procedures were applied:

In the case of PS films, initially, HMDS was spin-coated at 4000 rpm for 1 min onto a silicon wafer. Then a 2% w/w PS solution was prepared in methyl isobutyl ketone and it was spin-coated at 2000 rpm for 1 min onto the hexamethyldisilazane-modified silicon wafer and then thermally treated at 120 °C for 5 min.

In the case of the AZ5214 films, HMDS was also spin-coated at 5000 rpm for 30 s onto a silicon wafer. The AZ5214 resist was diluted 10 times in methyl isobutyl ketone and then spin coated at 3000 rpm for 30 s onto the HMDS-modified silicon wafer and thermally treated at 200 °C for 1 h.

A 5% w/w EPN solution was prepared in propylene glycol methyl ether acetate and spin-coated on a silicon wafer at 4000 rpm for 1 min (priming with HMDS was not necessary in this case to get good quality films) and then it was thermally treated at 110 °C for 3 min.

2.4. Photolithographic Processes. The resist formulations were prepared using always fresh PVA solutions in order to avoid stability problems arising from the hydrolysis of PVA in aqueous environment. In particular, the solutions used in this study were up to 2 days old. The resist formulation adopted in the final protocol for the protein adsorption experiments consisted of 4.3% (w/w) PVA and 14.4% (w/w) 12-tungstophosphoric acid in deionized water. This resist was then spin-coated on the polymer modified silicon surfaces at 4000 rpm for 30 s. The surfaces were then thermally treated at 70 °C for 1 min (postapplied bake, PAB) and exposed in a contact-printing mode to deep UV light using a Hg−Xe lamp (Oriel Instruments, Stratford, CT). After that, the film was thermally treated at 88 °C for 3 min (postexposure bake, PEB). Development was performed by immersion for 2 min in a 1:1 (v/v) methanol/deionized water solution followed by washing with deionized water and drying under N_2 flow. Additional developers were also tested as described in detail in the Results and Discussion section. Formulations with lower 12-tungstophosphoric acid content were also tested, and especially the formulation with 8.2% (w/w) PVA and 14.4% (w/w) 12-tungstophosphoric acid (see results in the Supporting Information).

In addition, a formulation consisting of 4.3% (w/w) PVA and 13% (w/w) ammonium 18-tungtodiphosphate in deionized water was also investigated. In this case, the solution was spin-coated on the polymer modified silicon surfaces at 4000 rpm for 30 s. The surfaces were then thermally treated at 90 °C for 1 min (postapplied bake, PAB) and exposed in a contact-printing mode to deep UV light using an Hg−Xe lamp (Oriel Instruments, Stratford, CT). After that, the film was thermally treated at 90 °C for 3 min (postexposure bake, PEB). Development was performed by immersion for 2 min in 1:1 (v/v) methanol/deionized water solution followed by washing with deionized water and drying under N_2 flow.

Overall, the proposed photolithographic process is presented in Figure 1.

2.5. Fluorimetric Evaluation of Selective Protein Adsorption. The selective protein adsorption onto PS/PVA surfaces was evaluated either by direct immobilization of a fluorescently labeled protein (goat antirabbit IgG labeled with AF546) (Figure 2A) or through a model binding assay involving immobilization of b-BSA and detection with AF546 labeled streptavidin (Figure 2B).

For the direct immobilization of the fluorescently labeled antibody, a 25 μg/mL AF546 goat antirabbit IgG antibody solution was prepared in carbonate buffer, 50 mM, pH 9.2, and used for coating of silicon wafer dies covered either with PS or PVA/PS for coating times ranging from 30 min to 22 h. Then the pieces were washed extensively with 50 mM carbonate buffer, pH 9.2, and distilled water and finally dried under N_2 flow.

For the b-BSA/AF546-labeled streptavidin assay, a 25 μg/mL b-BSA solution in 50 mM phosphate buffer, pH 7.4, was applied on

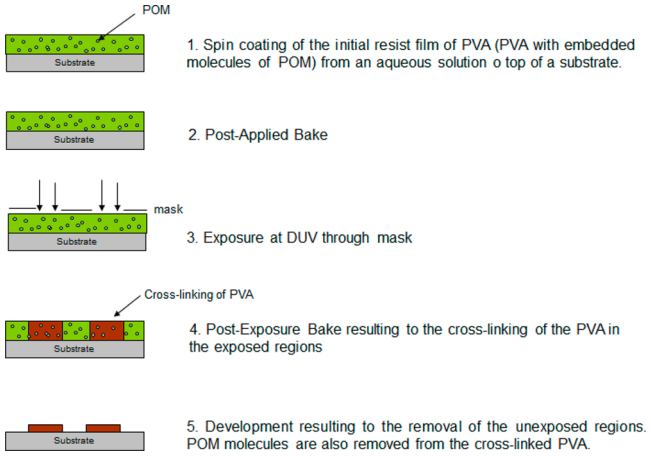

Figure 1. Proposed photolithographic scheme for patterning PVA via cross-linking. POM is either 12-tungstophosphoric acid or ammonium 18-tungstodiphosphate.

silicon dies covered either with PS or PVA/PS and incubated from 1 min to 22 h. Then, the dies were washed with a blocking solution (10 g/L BSA in 50 mM phosphate buffer, pH 7.4) and immersed in it for 1 h at room temperature. After that, the dies were washed with 50 mM phosphate buffer, pH 7.4, and distilled water, and dried on a N_2 stream. Then, they were immersed in a 5 μg/mL AF546 streptavidin solution in blocking buffer and incubated for 30 min at room temperature. Finally, the dies were washed extensively with 50 mM phosphate buffer, pH 7.4, distilled water, and dried on a N_2 stream.

Fluorescence images were obtained using the Axioskop 2 plus epifluorescence microscope (Carl Zeiss, Germany) equipped with a Sony Cyber-shot digital camera and processed using the Image ProPlus software (Media Cybenertics Co.). In each case, the net fluorescence values were determined by subtracting the values obtained by dies coated with the blocking protein only.

2.6. Label-Free Real-Time Monitoring of Selective Protein Adsorption. A custom-made experimental setup for label-free real-time monitoring of biomolecular interactions was used to further evaluate the selective protein adsorption to PS substrate vs the cross-linked PVA film. The setup is based on white light interference spectroscopy[35] and it is developed in collaboration with ThetaMetrisis S.A. (Athens, Greece). Silicon surfaces, on which a 1 μm thick thermal SiO_2 layer was grown, were modified with a PS or PS/cross-linked PVA layer as described above. The surfaces were mounted on a custom-made cartridge, allowing continuous solution flow at a constant rate and at the same time the reflectance spectrum was monitored. For each surface, two adsorption experiments were performed. The first consisted of running through the cartridge sequentially: phosphate buffer 50 mM, pH, 7.4, goat IgG solution 25 μg/mL in the same buffer, and finally, phosphate buffer. In the second one, pooled human serum diluted 100- and 10 times, respectively, with phosphate buffer 50 mM, pH 7.4, was run over each surface for 30 min. Phosphate buffer was run prior to and after the introduction of each serum dilution for approximately 10 min.

3. RESULTS AND DISCUSSION

3.1. Development of Photolithographic Process. The use of PVA as an agent to prevent protein adsorption onto the underlayer is based on the notion that the highly hydrophilic nature of this molecule will result on a protein repulsive layer. In this work, the creation of a stable, non-water-soluble PVA layer onto the polymer coated silicon wafer was pursuit through cross-linking of PVA molecules after their deposition as a thin film onto the surface. The proposed approach for PVA cross-linking is based on the modification of a photolithographic process reported in the past for either deep ultraviolet (DUV)

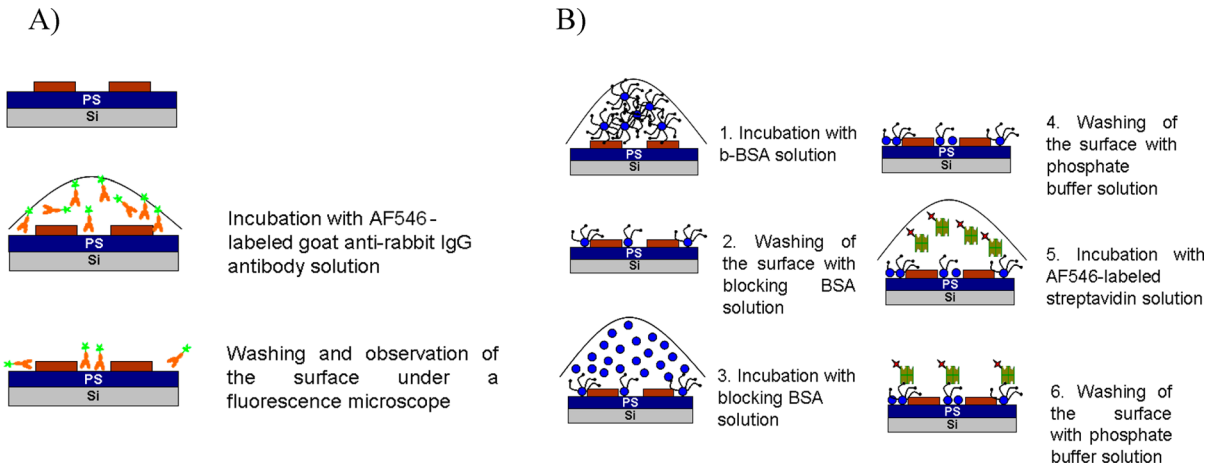

Figure 2. (A) Direct immobilization process of an AF546 labeled goat antirabbit IgG antibody on a PS film guided by cross-linked PVA structures. (B) Selective immobilization of b-BSA on a PS underlayer guided by photopatterned PVA structures and detection through a fluorescently labeled streptavidin.

lithography[31] or the formation of metallic features by exploiting the presence of a polyoxometalate used as a photo-oxidation catalyst.[36] The cross-linking of PVA is initiated by the photoreduction of ammonium 18-tungtodiphosphate (Supporting Information scheme 1) or the photoreduction of 12-tungstophosphoric acid, commonly known as phosphotungstic acid, (Supporting Information scheme 2a),[37] which results in partial oxidation of the hydroxyl groups in the PVA molecules to carbonyls. Then, the PVA chains undergo dehydration during the postexposure bake and the products are condensed by Diels–Alder reactions during the same step to create a cross-linked PVA/12-tungstophosphoric acid film (Supporting Information scheme 2b)[31] or a cross-linked PVA/ammonium 18-tungtodiphosphate film, correspondingly. The formation of dienes and dienophiles during the postexposure bake can be monitored by UV spectroscopy.[31] (See also Supporting Information Table 1).

To use the above approach to create patterned surfaces for bioapplications, the efficient removal of the 12-tungstophosphoric acid or the ammonium 18-tungstodiphosphate from the final cross-linked PVA film is necessary because their presence could affect the protein binding properties of the cross-linked PVA films.[38] For achieving this goal, the modification of the processes followed in the past[31,36] by modification and optimization of the development step was crucial in order to effectively remove both the unexposed regions and the POM molecules from the exposed cross-linked PVA film and retain acceptable resolution performance. In addition the modification of the material formulation (i.e., POM/PVA ratio) and the processing parameters (exposure, thermal treatments) had to be considered since the amount of hydroxyl groups remaining in the cross-linked film is critical in this application for the prevention of protein adsorption.

For this investigation, 12-tungstophosphoric acid (known also as phosphotungstic acid) was selected as the photosensitizer because this compound is commercially available and the proposed method can be easily and widely implemented in Bio-MEMs fabrication. Nevertheless, in cases where the presence of an acid in the starting PVA film would be undesirable, 18-ammonium tungstodiphosphate can be used, which generates acid upon exposure,[39] although, in this case, a higher exposure dose is demanded for effective PVA cross-linking (~165 vs 75 mJ/cm^2).

The optimization of the photosensitive material formulation was first investigated using pure water as the developer. It was found that through the photolithography of the material formulation that contained 4.3% (w/w) PVA and 14.4% (w/w) 12-tungstophosphoric acid for which the molar ratio of POM to the PVA monomer was ~1:20 calculated by a rough estimation, i.e., without taking into account possible unhydrolyzed acetate groups and without determining hydration of POM, the best resolution was achieved, namely 2.5 μm cross-linked PVA structures. It should be noted that in compositions with lower POM content, we obtain higher contrast but the achieved resolution is lower, attributed to increased swelling of the PVA film in the developer due to lower cross-linking density at comparable doses (see Supporting Information Figure 1). It should be also noted that the quantity of POM plays a critical role to the consumption of alcoholic groups and therefore controls the interactions of the final cross-linked PVA structures with proteins. As discussed below (section 3.2), it is proved that the proposed formulation and process allow the retention of enough remaining alcoholic groups that prevent the physisorption of proteins.

To ensure that the POM is effectively removed from the cross-linking regions, different developers were investigated. In addition to deionized water, the commercially available developer AZ726 MIF (0.26N tetramethylammonium hydroxide), termed from this point forward as TMAH, and 1:1 (v/v) methanol/deionized water solution. Water was employed as the basis of all developers because both the non-cross-linked PVA and 12-tungstophosphoric acid are water-soluble. The 1:1 (v/v) methanol/deionized water solution was also selected for comparison because it was expected that the latter could result in a more facile removal of the 12-tungstophosphoric acid from the film due to the interactions of methanol with the POM molecules. Finally, the tetramethylammonium hydroxide (TMAH) was also expected to effectively remove the 12-tungstophsphoric acid molecules from the film because, due to its basic character, it can cause disintegration of the POM according to the following reaction:[38]

$$[PW_{12}O_{40}]^{3-} + 23OH^- \rightarrow HPO_4^{2-} + 12WO_4^{2-} + 11H_2O$$

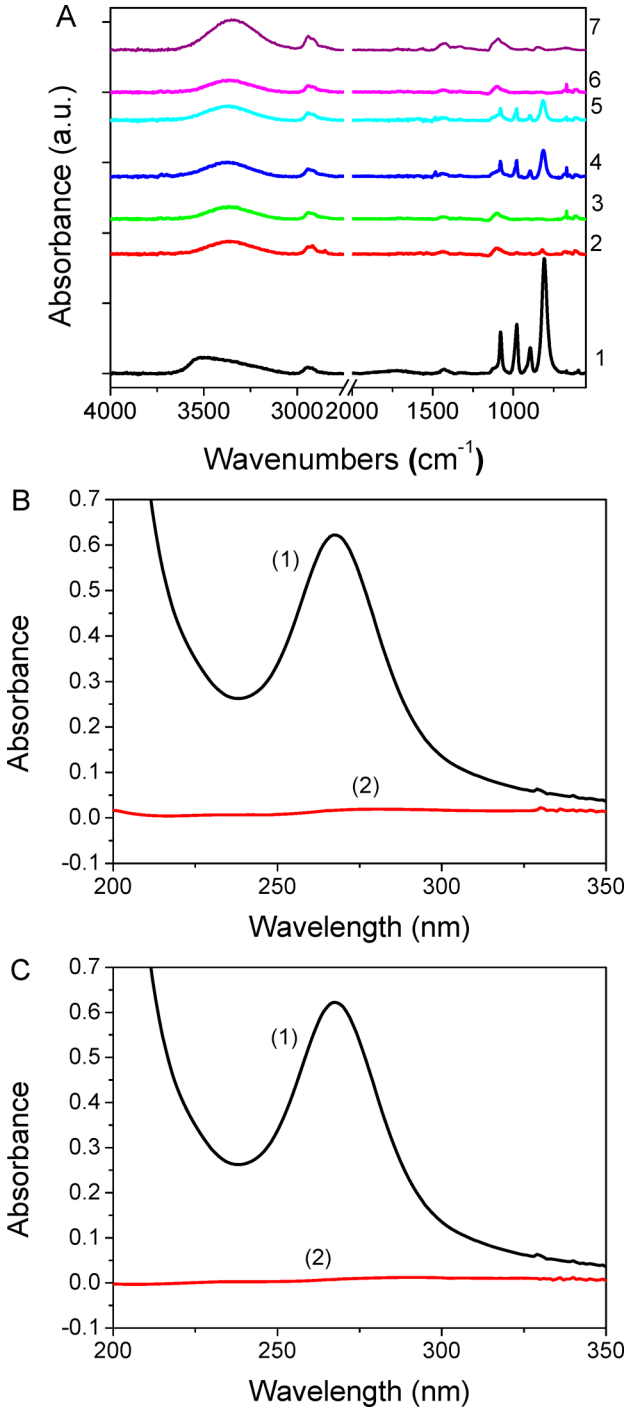

Figure 3. (A) FTIR spectra of the initial film (line 1), the film after development with deionized water for 2 min (line 2); 0.26 N TMAH solution for 2 min (line 3); 0.001 N TMAH solution for 2 min (line 4); TMAH 0.001 N solution for 12 min (line 5); or 1:1 (v/v) methanol/deionized water solution for 2 min (line 6) and PVA film without POM (line 7). (B) UV spectra of the initial film (black line 1) and the film after development in deionized water for 2 min (red line 2). (C) UV spectra of the initial film (black line 1) and the film after development in 1:1 (v/v) methanol/deionized water solution for 2 min (red line 2). The same spectrum (red line 2) was obtained for cross-linked PVA after development in 0.26 N TMAH solution.

TMAH was used in the development step at a concentration of 0.26 or 0.001 N prepared in deionized water. The 0.001 N TMAH solution was tested for two different development times

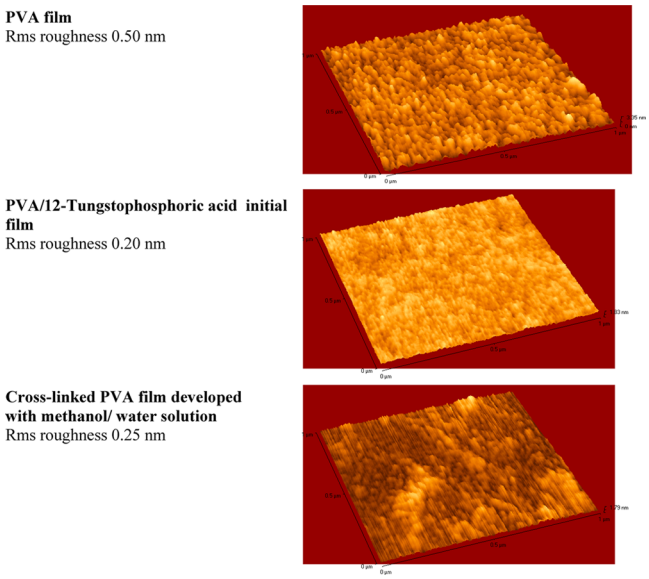

Figure 4. AFM micrographs for 1 μm size scans and feature dimensions of a PVA film (top image); PVA/12-tungstophosphoric acid initial film (middle image), methanol/deionized water developed cross-linked PVA film (bottom image).

in order to define the optimum conditions. The removal of 12-tungstophosphoric acid from the cross-linked film after development with the different solutions was evaluated by recording FTIR (4000−440 cm^{-1}) and UV (200−400 nm) spectra and comparing them with those of the initial film. The FTIR spectra of films developed with the different developers are presented in Figure 3A along with that of the initial film and the PVA film. The removal of the 12-tungstophosphoric acid was evaluated by comparing the intensity of its characteristic peaks at 793, 895, 977, and 1079 cm^{-1}.[40] It should be noted that the last peak at 1079 cm^{-1} is mainly attributed to the presence of 12-tungstophosphoric acid (Figure 3A line1) but it is also present in the PVA film without POM (Figure 3A line 7) due to the C−O stretching vibrations and it does not provide a safe indication for the POM removal. Furthermore, the peak assigned to the hydroxyl groups of PVA is observed at 3500 cm^{-1} in the initial resist film whereas it is shifted at 3350 cm^{-1} in the final cross-linked film. The presence of the 12-tungstophosphoric acid in the initial film results to formation of hydrogen bonds between the hydroxyl groups of PVA and the POM molecules. These findings are in accordance with previous reports claiming the creation of hydrogen bonds between the hydroxyl groups of PVA and POMs in hydrophilic hybrid membranes.[41,42] Thus, as it is shown in Figure 3A, the most efficient developers for the 12-tungstophosphoric acid removal were the 1:1 (v/v) methanol/deionized water solution and the 0.26 N TMAH solution. Deionized water was not able to fully remove from the cross-linked film. Finally, the 0.001 N TMAH solution failed to remove 12-tungstophosphoric acid, though it was used for a much longer time (12 min instead of 2 min employed in the case of all other developers), a result attributed to insufficient base strength for the $[PW_{12}O_{40}]^{3-}$ disintegration and to the possible formation of an insoluble salt of this anion with the tetramethylammonium cation.

The removal of the 12-tungstophosphoric acid from the cross-linked film after development was also confirmed by UV spectroscopy. As it is shown in Figure 3C, the peak at 270 nm attributed to the presence of 12-tungstophosphoric acid[40] is

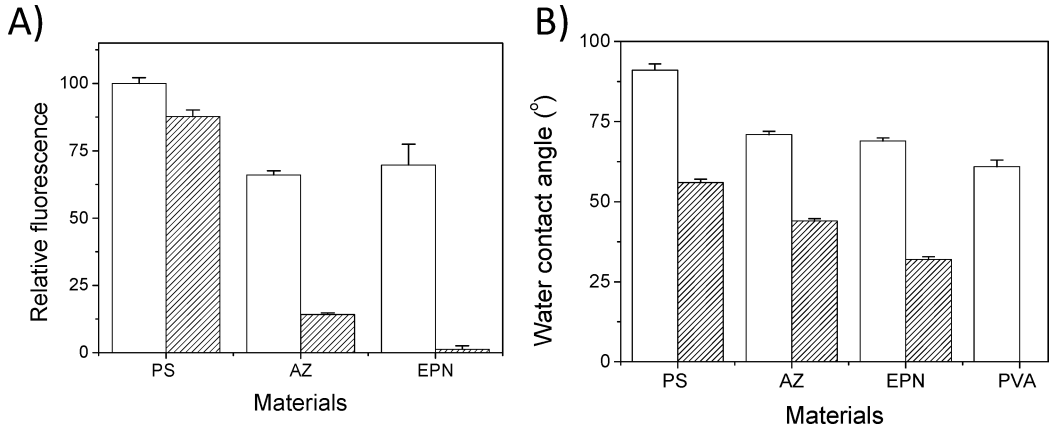

Figure 5. (A) Fluorescence intensity values obtained from PS, AZ5214, and EPN surfaces to which an AF546 goat antimouse IgG antibody has been adsorbed prior to (white columns) and after application/removal of PVA/12-tungstophosphoric acid film (hatched columns) expressed as % ratio of fluorescence provided by the untreated PS surface. Each point is the mean value of three measurements ± SD. (B) Water contact angle values obtained from PS, AZ5214, and EPN surfaces prior to (white columns) and after application/removal of PVA/12-tungstophosphoric acid film (hatched columns). Each point is the mean value of five measurements ± SD.

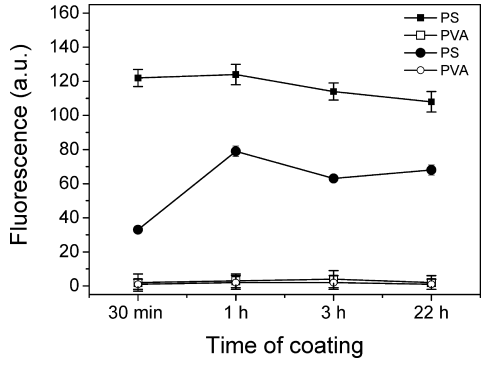

Figure 6. Fluorescence intensity values versus the coating time obtained from PS (closed symbols) and PVA (open symbols) areas after incubation with an AF546 labeled goat antirabbit IgG antibody (circles) or coating with b-BSA and reaction with AF546 labeled streptavidin (squares). Each point is the mean value of three measurements ± SD.

completely eliminated after development with 1:1 (v/v) methanol/deionized water solution and 0.26 N TMAH solution.

A significant reduction of PVA film thickness is also expected due to removal of the bulky 12-tungstophosphoric acid molecules from the network of cross-linked PVA film. Indeed, the average thickness of the initial nondeveloped PVA film (PAB) was 120 (±5) nm, while the film thickness was reduced to about 56 (±3) nm after development with 1:1 (v/v) methanol/deionized water solution or with 0.26 N TMAH solution. On the other hand, the developed film thickness was 60 (±3) nm after development with deionized water, and about 87 (±4) nm after development with 0.001 N TMAH solution, confirming the incomplete 12-tungstophosphoric acid removal in these cases.

The hydrophilicity of the cross-linked PVA film was evaluated by performing contact angle measurements. The measurements revealed an average water contact angle of about 60°. Because this value is relatively high for a hydrophilic film,

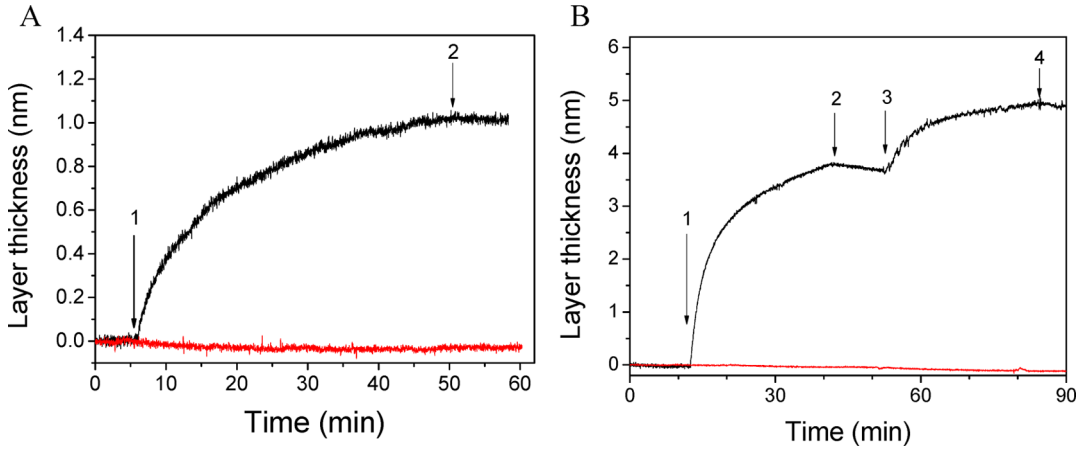

Figure 7. (A) Real-time monitoring of goat IgG adsorption from a 25 μg/mL solution on a PS (black line) and a PS/PVA surface (red line), respectively. The solutions run over the surface sequentially were the following: start to 1, phosphate buffer 50 mM, pH 7.4; 1 to 2, goat IgG solution 25 μg/mL in phosphate buffer 50 mM, pH 7.4; 2 to end, phosphate buffer 50 mM, pH 7.4. (B) Real-time monitoring of human serum adsorption on a PS (black line) and a PS/PVA surface (red line), respectively. The solutions run over the surface sequentially werethe following: start to 1, phosphate buffer 50 mM, pH 7.4; 1 to 2, human serum 100-times diluted in phosphate buffer 50 mM, pH 7.4; 2 to 3, phosphate buffer 50 mM, pH 7.4; 3 to 4, human serum 10-times diluted in phosphate buffer 50 mM, pH 7.4; 4 to end, phosphate buffer 50 mM, pH 7.4.

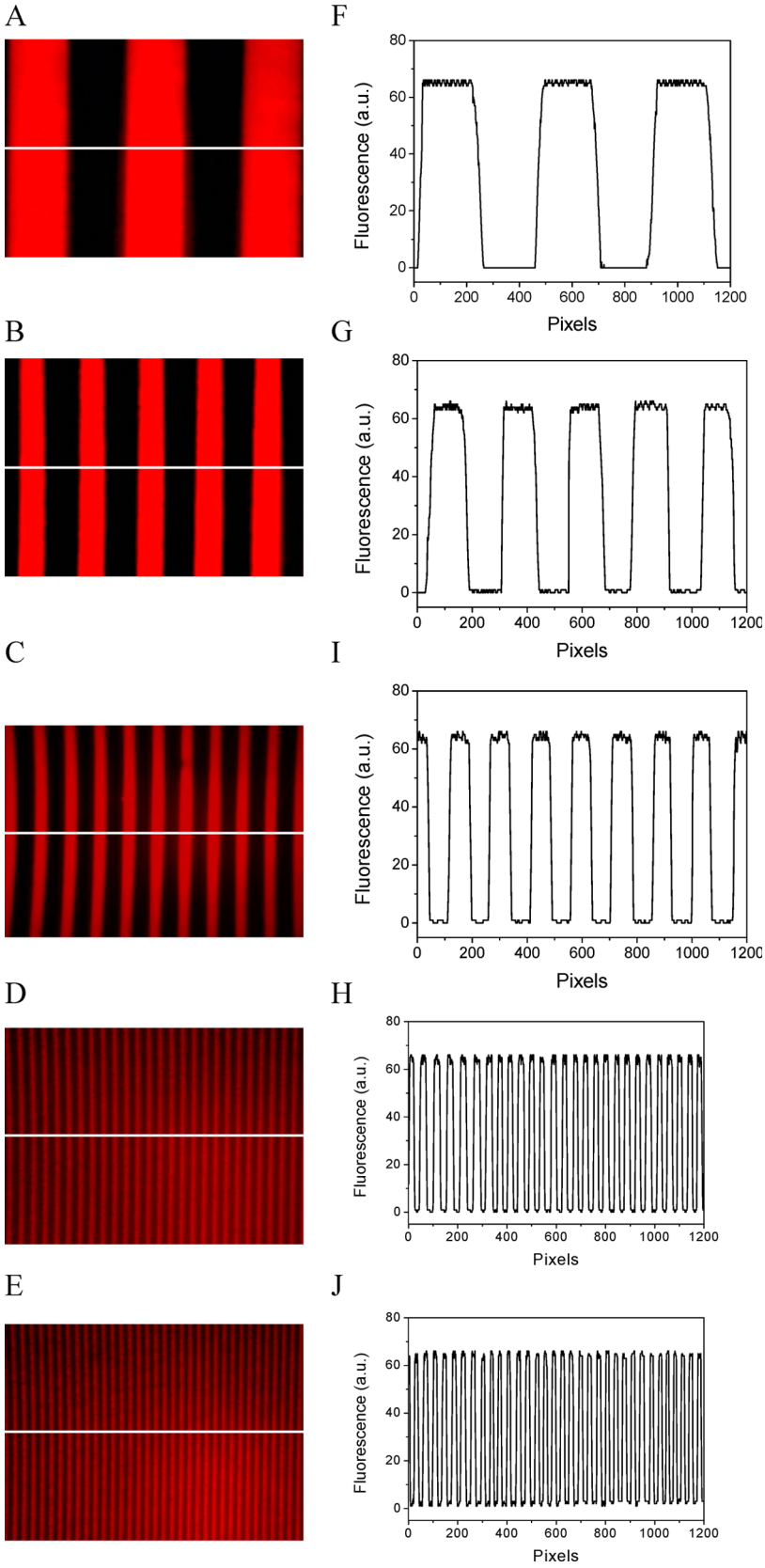

Figure 8. Fluorescence images (A–E) and fluorescence intensity plots (F–J) obtained from PS/PVA patterned substrates with line widths of 25 (A, B), 12.5 (C, D), 6.25 (E, F), 3.75 (H, G), and 2.5 μm (I, J). The right column plots (F–J) correspond to fluorescence intensity across the white lines in each image (A–E).

we investigated the film hydroxyl groups content. As it is shown in the FTIR spectrum (Figure 3A line 6), the characteristic peak at 3350 cm^{-1} assigned to the hydroxyl groups is present in the final PVA film, proving the preservation of a high

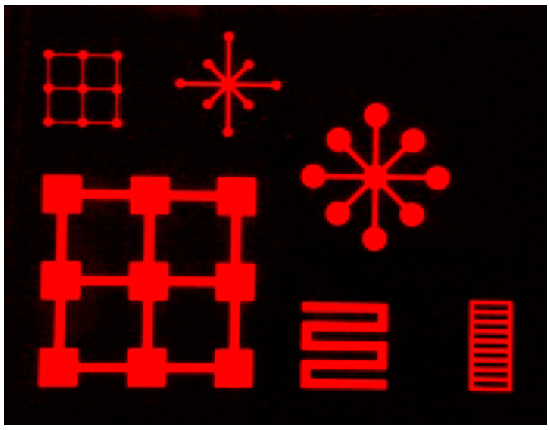

Figure 9. Biotinylated BSA patterns created on a PS substrate through photolithography of a PVA/12-tungstophosphoric acid film.

percentage (~64%) of initial hydroxyl groups in the final film. The possible formation of hydrogen bonds between the closely spaced hydroxyl groups of the cross-linked PVA could be responsible for the measured contact angle value. Nevertheless, as it will be shown below, these surfaces are characterized by a strong protein-repellent behavior.

Finally, the topography of PVA films was examined by using tapping mode atomic force microscopy (AFM) in order to have an indication of morphology changes after cross-linking and 12-tungstophosphoric acid removal. As it is shown in Figure 4, the rms roughness value of a pure PVA film is 0.50 nm, whereas the rms roughness value of the PVA/tungstophsphoric acid initial film is 0.20 nm and the rms roughness value of the cross-linked PVA film after development with methanol/deionized water is 0.25 nm a value close to the one of the original film. In other words, the development and removal of the bulky 12-tungstophosphoric acid molecules from the PVA film did not affect drastically the film's morphology.

3.2. Selective Protein Immobilization. According to the lithographic scheme proposed in Figure 1, patterned cross-linked PVA structures can be formed on selected substrates. To evaluate the nonprotein adsorbing properties of such PVA structures, we first selected, as substrates, polymeric films known for their protein physisorption capabilities[43] and we determined the effect of the deposition and processing of PVA/12-tungstophosphoric acid films on them to their adsorption properties. Thus, thin films of three different polymeric materials, namely polystyrene (PS), epoxy novolac (EPN), and the cresol-formaldehyde diazonaphthoquinone commercial photoresist AZ5214, were used as underlayers and evaluated with respect to their protein adsorption properties prior to and after the application and removal of the non-cross-linked PVA/12-tungstophosphoric acid film. As it is shown in Figure 5A, PS provided the highest protein capacity both prior to and after application/removal of non-cross-linked PVA/12-tungstophosphoric acid layer. The protein binding capacity of the underlayer film after application/removal of PVA/12-tungstophosphoric acid film was reduced with respect to that of the initial film by approximately 13, 80, and 98% for PS, AZ5214, and EPN, respectively. The observed reduced protein binding capacity of all three films after the application/removal of non-cross-linked PVA/12-tungstophosphoric acid films can be ascribed to incomplete removal of PVA molecules from these surfaces. Especially, for the most reactive surfaces, like EPN, there is a strong possibility that some PVA chains are grafted onto the EPN underlayer during the PAB or PEB that are performed at 70 and 88 °C, respectively, and not removed during the development step. The relatively small reduction of ~13% in the protein physisorption capacity of the PS film indicated was considered acceptable in the current study as providing enough binding for the proteins used here in contrast to the cross-linked PVA film. Nevertheless, more drastic approaches such as a slight plasma treatment could be investigated in cases where such a reduction needs to be further minimized.

The observed changes of the film properties are also in line with the water contact angle values measured after the application/removal of PVA/12-tungstophosphoric acid film as compared to those prior to the treatment (Figure 5B). In particular, the water contact angles of AZ5214 and EPN surfaces were 71° (±1°) and 69° (±1°), 44° (±1°), and 32° (±2°) prior to and after the application/removal of PVA/12-tungstophosphoric acid film, respectively. On the other hand, for the PS the water contact angle values were 91° (±2°) and 70 (±2°) prior to and after the application/removal of PVA/12-tungstophosphoric acid film, respectively. Despite this noticeable effect on the PS underlayer contact angle, because its protein binding properties were not severely affected, PS was selected as the most suitable protein adsorption layer in order to demonstrate PVA guided protein patterning.

For the successful demonstration of the proposed process, the radiation dose required to create water insoluble and protein-repellent PVA structures was determined. For this purpose, PVA/12-tungstophosphoric acid films spun-off on a PS substrate were exposed for different time intervals to the Hg−Xe lamp and then the thickness of the film and protein binding capacity was evaluated. The evaluation of protein binding capacity was based on the fluorescence signal determined after incubation of the surfaces with an AF546 labeled goat antimouse IgG antibody for 1 h at RT. We observed that by increasing the exposure time, the cross-linked PVA film thickness increased accompanied by decrease of the protein adsorption on the created PVA structures (see Supporting Information Figure 3). These effects are attributed to increased cross-linking in the PVA film as the exposure time increases, leading to better coverage of the PS underlayer.

Almost negligible protein adsorption in combination with stable film thickness was achieved for exposure doses of about 40 mJ/cm^2. A higher exposure dose of 85 mJ/cm^2 was selected for the final protocol in order to ensure stability of the PVA structures and maximize the coverage without causing undesirable overexposure effects such as unacceptable reduction of hydroxyl groups, or even pattern degradation due to undesired exposure of the adjacent areas.

Afterward, the patterned surfaces were tested regarding the protein adsorption selectivity for different coating times ranged from 30 min to 22 h (overnight). As it is shown in Figure 6, there was no detectable fluorescence signal from PVA areas independently of the coating time, proving the nonprotein adsorbing property of the cross-linked PVA. On the other hand, the PS areas showed significant protein binding capacity that reached maximum plateau values after incubation for 1 h with either the labeled antibody or b-BSA solution. The immobilized b-BSA was detected through reaction with a fluorescently labeled streptavidin.

The highly selective protein adsorption onto the PS substrate vs the cross-linked PVA was also demonstrated in a label-free format using a custom-made experimental setup based on white

light interference spectroscopy. For this purpose, PS and PS/PVA films were created on silicon surfaces where a 1 μm thermal SiO_2 was grown. When white light strikes vertically the surface, an interference reflectance spectrum is created. Protein adsorption on the surface was monitored in real-time by recording the shift in the reflected interference spectrum due to the accumulation of protein molecules on the surface. As it is shown in Figure 7A, when a goat IgG solution was run over the two surfaces, a typical adsorption curve was obtained for the PS surface whereas no protein adsorption was observed for the cross-linked PVA surface. A similar behavior was observed when pooled human serum diluted with phosphate buffer was run over the two surfaces. In particular, when a 100-times diluted serum was run over the two surfaces, a layer increase of about 4 nm was observed for the PS surface after 30 min, whereas at the same time, a slightly negative response was obtained for the cross-linked PVA surface. After washing and introduction of a 10-times diluted serum, a second distinct layer thickness increase was obtained for the PS, which was much lower than the first one (approximately 1 nm), indicating that most of the surface binding sites have been already covered during the first probing with human serum (exponential part of adsorption curve between the arrows 1 and 2). On the other hand, no increase in layer thickness was observed over the whole experiment duration for the PVA surface. As in the case of goat IgG solution, a slight decrease in layer thickness was observed, which could be the result of molecules rearrangement in PVA film due to hydration. The behavior of the PVA film toward diluted human serum does not only support the negligible protein adsorption by this film but also its overall stability to complex biological media.

Finally, the selective immobilization process of b-BSA on PS underlayer guided by structures of cross-linked PVA is presented in Figure 8 where the ability to create structures of micrometer dimensions following the proposed photolithographic procedure is demonstrated. It was possible to achieve structures with dimensions down to 2.5 μm with high signal homogeneity within the protein adsorbing PS areas and clearly negligible signal from the cross-linked PVA areas, as it is demonstrated by the fluorescence intensity plots across the structures presented in Figure 8. In particular, after background correction to reduce the uneven lighting in the middle of the images, the variation of fluorescence intensity values within the PS structures was less than 5% of the mean value and the same was true for the variation of the mean fluorescence intensity values of between the similar width structures of PS.

An inherent advantage of lithographic processes is the freedom to create custom patterns by applying the appropriate mask. As it is shown in Figure 9, the proposed lithographic process provides the ability to create custom protein patterns onto surfaces with negligible protein adsorption in the surrounding area and due to relatively mild film bake and development conditions it could be applied to sensor surfaces.

4. CONCLUSIONS

A new approach for converting a surface resistant to protein adsorption by using a cross-linked PVA based film was demonstrated. The cross-linking of PVA is induced by using, as a photo-oxidation catalyst, a polyoxometalate compound. The cross-linked PVA films exhibit negligible protein physisorption, as it was observed upon incubation with protein solutions. In the selected process, the polyoxometalate is completely removed at the end of the photolithographic process, as it was confirmed by UV and FTIR spectroscopy whereas AFM has been employed to characterize the resulting film surface showing roughness similar to the one of the starting material if methanol/deionized water is used as the developer. The process capabilities concerning selective protein adsorption were demonstrated using, as a substrate, a polystyrene film deposited on a silicon wafer. Patterns of proteins have been created showing that cross-linked PVA structures resisted protein adsorption whereas polystyrene areas preserved their protein physisorption properties. According to the presented results, the proposed methodology is a non-substrate-specific process and can be applied to make any substrate protein-resistant while it can be used to create surfaces with distinct protein adsorption/protein resistance properties. The proposed photolithographic process could be integrated in a straightforward manner in Bio-MicroElectro-mechanical Systems (Bio-MEMs) and microarrays fabrication.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Cross-linking of PVA by the photoreduction of ammonium 18-tungstodiphosphate (Scheme 1) or the photoreduction of 12-tungstophosphoric acid (Scheme 2a) molecules. Cross-linking of the PVA after the dehydration taking place during PEB (Scheme 2b). Dienes that are formed during dehydration (Scheme 3). The oxidized PVA undergoes dehydration UV spectra of the PVA cross-linked films that were exposed and thermally treated (PEB) at different conditions (Table 1). Different material formulations (Figure 1). Fluorescent images showing the nonfouling properties of cross-linked PVA films (Figure 2). Optimization of the exposure dose (Figure 3). Selective adsorption of b-BSA on a PS underlayer guided by photopatterned PVA structures and detection through a fluorescently labeled streptavidin vs incubation time (Figure 4). This material is available free of charge via the Internet at http://pubs.acs.org.

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The paper was written through contributions of all authors. All authors have given approval to the final version of the paper.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AF546 = Alexa Fluor 546
AFM = atomic force microscopy
b-BSA = biotinylated bovine serum albumin
Bio-MEMs = bio-microelectromechanical systems
BSA = bovine serum albumin
EPN = epoxidized novolac of cresol-formaldehyde
OEG = oligo(ethylene glycol)
PAB = postapplied bake

PEB = postexposure bake
PEG = poly(ethylene glycol)
POM = 12-tungstophosphoric acid, polyoxometalate
PS = polystyrene
PVA = poly(vinyl alcohol)
RgG = rabbit γ-globulins
RT = room temperature
TMAH = tetramethylammonium hydroxide
UV = ultraviolet

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