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Direct Covalent Biomolecule Immobilization on Plasma-Nanotextured Chemically Stable Substrates

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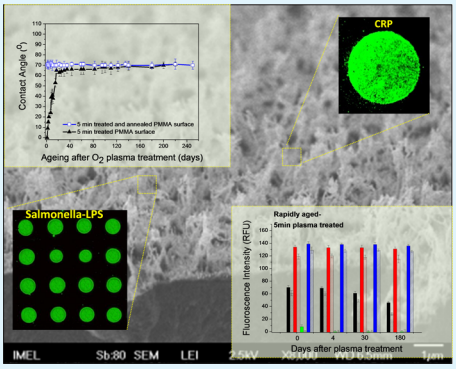
Supporting Information

ABSTRACT: A new method for direct covalent immobilization of protein molecules (including antibodies) on organic polymers with plasma-induced random micronanoscale topography and stable-in-time chemical functionality is presented. This is achieved using a short (1−5 min) plasma etching and simultaneous micronanotexturing process, followed by a fast thermal annealing step, which induces accelerated hydrophobic recovery while preserving important chemical functionality created by the plasma. Surface-bound biomolecules resist harsh washing with sodium dodecyl sulfate and other detergents even at elevated temperatures, losing less than 40% of the biomolecules bound even at the harshest washing conditions. X-ray photoelectron spectroscopy, secondary-ion mass spectrometry, and electron paramagnetic resonance are used to unveil the chemical modification of the plasma-treated and stabilized surfaces. The nanotextured and chemically stabilized surfaces are used as substrates for the development of immunochemical assays for the sensitive detection of C-reactive protein and salmonella lipopolysaccharides through immobilization of the respective analyte-specific antibodies onto them. Such substrates are stable for a period of 1 year with ambient storage.



KEYWORDS: *plasma nanotexturing, covalent immobilization, stable-in-time desirable chemical functionality, antibodies, C-reactive protein, salmonella lipopolysaccharides*

1. INTRODUCTION

Immobilization of biomolecules on surfaces is the first step for the preparation of a large variety of functional substrates, including microarrays, cell arrays, microfluidics for diagnostic purposes, biocompatible surfaces for medical applications such as implants, and many more. A biomolecule is said to be "immobilized" to a solid support if it is either physically adsorbed to the support via van der Waals forces and hydrogen-bonding or electrostatic interactions or covalently attached to active surface groups and cannot be removed from the support by washing with buffers and/or detergents.[1] Covalent immobilization is considered to result in higher biomolecule activity, reduced nonspecific adsorption, and greater stability of immobilized biomolecules compared to that by physical adsorption.[1,2] Providing chemically stable-in-time surfaces/substrates, which allow increased biomolecule immobilization, is a hot research topic with important commercial applications.

Organic polymer surfaces are chemically inert, and thus they require chemical modification in order to permit covalent immobilization of biomolecules. Common organic polymer treatment techniques used for modification of their surface include standard wet chemistry (e.g., application of oxidizing agents) or dry treatment (e.g., UV irradiation/ozone, corona discharge, and plasma).[3−6] Other wet methods include layer-by-layer assembly of charged molecules or polyelectrolytes, which combined with photolithography can lead to patterns of active sites.[7,8] Commercial high-binding polystyrene (PS) surfaces[9] are produced using irradiation to introduce carboxyl groups, thus increasing the protein binding capacity compared to that of the untreated PS.

Plasma processing is widely used for organic polymer surface functionalization prior to biomolecule immobilization[10] because of its high throughput and target-oriented chemistry.[11−15] In most cases, plasma is used to create functional groups such as NH_x, SO_x, or C═O, COOH, and OH groups by employing appropriate gas mixtures, which etch the substrate or deposit

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material on it.[10,16−18] The introduced functional groups are subsequently used for biomolecule attachment through the appropriate chemistry. Surface groups created by the plasma are usually chemically unstable-in-time (a phenomenon often termed "aging", or hydrophobic recovery for plasma-hydrophilized surfaces), and thus surfaces have to be used immediately after treatment.[17] For this reason, plasma-etched surfaces are less frequently used for biomolecule immobilization,[19−25] compared to plasma polymerized surfaces.[17,26−37]

An exception to this rule is the work by Bilek et al.,[2,38] who proposed an energetic ion-assisted plasma process (plasma immersion ion implantation) in order to produce surfaces chemically stable for months containing free radicals, which can directly immobilize biomolecules covalently on the surface. Nevertheless, the proposed method applies very high voltages (several kilovolts) and needs a specialized apparatus rather than the typical plasma processing equipment.

Little work has been done concerning the role of micro- and nanotopography on biomolecule attachment on surfaces.[39,40] In our previous works, we have observed the formation of controlled surface roughness (texture) on various polymers as a result of plasma processing. We used the term "nanotexture" to describe the plasma-induced roughness comprising high-aspect-ratio micro/nanostructures[41,42] and have extensively studied the effect of oxygen-, fluorine-, and fluorocarbon-containing plasmas on the wetting and biomolecule attachment properties on these surfaces through physical adsorption.[43−51] These surfaces, however, suffer from progressive aging, as indicated by contact-angle measurements, resulting in considerable changes with respect to biomolecule immobilization: Thus, to achieve repeatable results, a certain period of time (e.g., 20−30 days) should pass after plasma treatment prior to using the treated surfaces for biomolecule immobilization. In addition, storage of the surface for several months results in a change of their contact angle (aging)[49,52] and reduces their capacity to immobilize biomolecules.

Here, we present a method to create surfaces with stable-in-time (i.e., nonaging) chemical functionality and high-surface-area micro/nanotopography on which biomolecules can be covalently bound with a high density per projected surface area. To achieve this goal, we use plasma micro/nanotexturing followed by a short annealing step, which induces accelerated hydrophobic recovery while preserving the important chemical functionality created by the plasma. We measured constant contact angles for the annealed surfaces over a period of 1 year, showing that progressive aging was completely prevented. We probed the chemical functionality of the surfaces through reaction with activated molecules, proving that after annealing a significant number of carboxyl groups are found on the surface and remain constant for almost 1 year. To gain insight on the surface chemical composition of both the stabilized and nonstabilized plasma-treated surfaces (and subsurface region), we examined them by employing X-ray photoelectron spectroscopy (XPS), time-of-flight secondary-ion mass spectrometry (TOF-SIMS), and electron paramagnetic resonance (EPR) measurements. We demonstrated covalent binding of protein biomolecules on the surface together with physical adsorption, as evidenced by remarkable resistance to washings with sodium dodecyl sulfate (SDS) and other detergent solutions, because more than 60% of the initially immobilized biomolecule amount remains on the surface. Finally, we demonstrated 2 orders of magnitude more sensitive immunoassays using such surfaces as supports for immobilization of analyte-specific antibodies compared to flat untreated surfaces. As an example, two immunoassays for determination of C-reactive protein (CRP) and salmonella lipopolysaccharides (LPSs) are demonstrated. These stabilized plasma-nanotextured substrates are expected to find widespread application in microarrays, in lab-on-a-chip devices for chemical or biochemical analysis, in diagnostic applications, as well as in medical devices such as stents, implants, and drug-delivery devices.

2. EXPERIMENTAL SECTION

2.1. Materials and Methods. Poly(methyl methacrylate) (PMMA) plates of 2 and 0.5 mm thickness were purchased from IRPEN (Barcelona, Spain) and Goodfellow GmbH (Stadt Bad Nauheim, Germany), respectively. Samples were ultrasonically cleaned in isopropyl alcohol and then in deionized (DI) water prior to plasma processing in order to remove any possible contamination.

Plasma processes were performed in (a) a helicon-type high-density plasma reactor (MET) by Alcatel (at 13.56 MHz; referred to in the text as reactor 1), providing radio-frequency (RF) power of up to 2000 W and (b) a low-density Nextral reactive-ion-etching (RIE) parallel-plate plasma reactor with a RF of 13.56 MHz (referred to in the text as reactor 2). We used two reactors in order to show that the results do not qualitatively depend on the mechanism of plasma excitation (capacitive or inductive). PMMA samples were treated in oxygen (O_2) plasma under highly anisotropic etching conditions (reactor 1, O_2 pressure of 0.75 Pa, flow of 100 sccm, plasma power of 1900 W, and bias voltage of −100 V; reactor 2, plasma power of 400 W and pressure of 10 mTorr). An annealing step was employed on a hot plate in air at 110 °C for 30 min for 2-mm-thick PMMA substrates (T_g 119 °C) or 100 °C for 2 h for 0.5-mm-thick PMMA substrates (T_g 109 °C). Several authors have attempted to stabilize the contact angle by applying different storage conditions.[53−56]

A FEI Company scanning electron microscope (samples viewed at 80° tilt) was used for surface topographical characterization of the plasma-treated surfaces. Water contact angles were measured by a GBX-DIGIDROP apparatus at ambient atmospheric conditions. DI water droplets of 5 μL were used for all contact-angle measurements.

XPS spectra were recorded on a PHI 5000 Versa Probe II (ULVAC-PHI, Chigasaki, Japan) system using a microfocused (100 μm, 25 W) Al Kα X-ray beam for photoelectron takeoff angle Θ = 45°, which is defined as an angle between normal to the surface and the axis of the XPS analyzer lens. A dual-beam charge neutralizer was used to compensate for the charge-up effect. High-resolution spectra were collected with an analyzer pass energy of 46.95 eV. The operating pressure in the analytical chamber was less than 5×10^{-7} Pa. All XPS peaks were referenced to the neutral (C−C) C 1s peak at a binding energy of 284.8 eV. Spectral backgrounds were subtracted using the Shirley method.

The PMMA surfaces prior to and after plasma treatment and further stabilization were analyzed using the TOF-SIMS 5 (ION-TOF GmbH) instrument, equipped with a 30 keV bismuth-liquid-metal-ion gun. Bi_3 clusters were used as the primary ions. The dose density deposited on the surface was identical for all samples and lower than 10^{12} ions/cm^2 to ensure static mode conditions. Negative-ion TOF-SIMS spectra were acquired from three different nonoverlapping spots (500 μm × 500 μm area). A pulsed, low-energy electron flood gun was used for charge compensation. The mass resolution $m/\Delta m$ > 6400 at the C6 (m/z 60) peak was maintained for all spectra. Principal component analysis (PCA) was performed on chosen TOF-SIMS signals characteristic for PMMA to enhance the detection of subtle differences in surface chemistry between different samples. Prior to PCA, the intensities of the selected peaks from each spectrum were normalized to the sum of their intensities and then mean-centered. PCA was performed using the PLS Toolbox (eigenvector Research, Manson, WA) for *MAT-LAB* (MathWorks, Inc., Natick, MA).

EPR measurements were obtained with an extensively upgraded former Bruker ER-200D spectrometer interfaced to a personal computer and equipped with an Oxford ESR 900 cryostat, an Anritsu MF76A frequency counter, a Bruker 035 M NMR gaussmeter, and an

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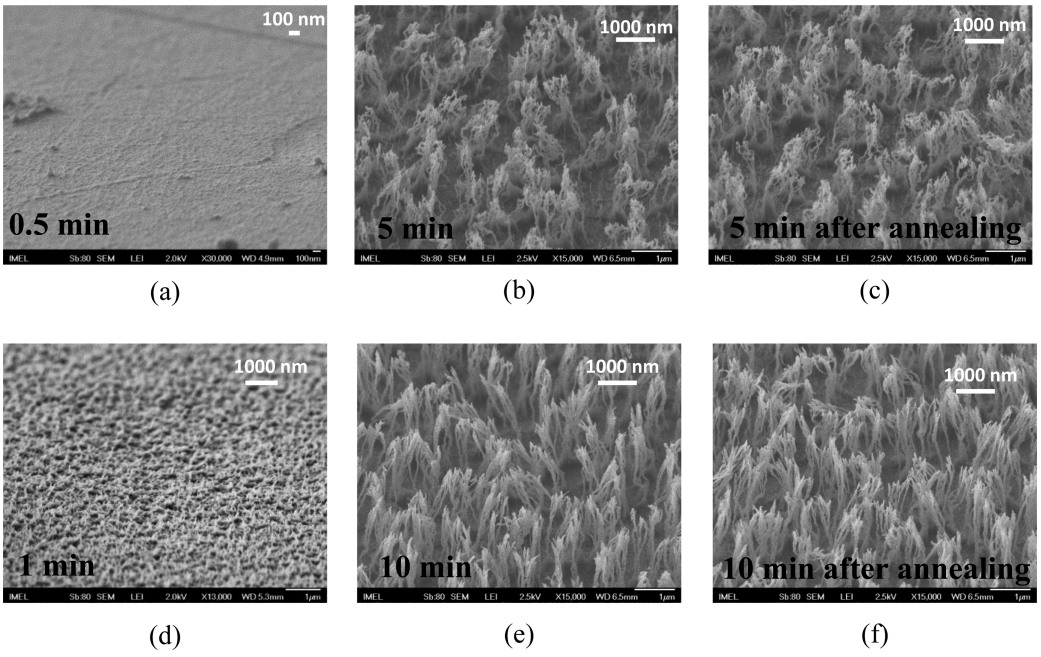

Figure 1. SEM images of PMMA surfaces O_2-plasma-etched in a high-density helicon-type (ICP) plasma reactor with an alumina dome and an electrode for (a) 30 s (a practically flat surface), (b) 5 min, and (c) 5 min followed by annealing and in a reactive ion etcher with a quartz electrode for (d) 1 min, (e) 10 min, and (f) 10 min followed by annealing. Note that no deformation of the micronanostructures created by O_2 plasma etching is observed after the annealing process. Conditions: baking on a hot plate in air at 110 °C for 30 min for 2-mm-thick PMMA substrates (T_g 119 °C) or 100 °C for 2 h for 0.5-mm-thick PMMA substrates (T_g 109 °C).

SR830 digital lock-in amplifier by Stanford Research. The perpendicular 4102ST cavity was used, and the microwave frequency was 9.41 GHz.

2.1.1. Probing of the Surface Functionality after Plasma Processing. To identify the presence of carbonyl groups on the surfaces, the surfaces were reacted with a 25 mM solution of a water-soluble biotin compound containing a poly(ethylene glycol) (PEG) spacer arm and a terminal primary amine (amine-PEG_3-biotin, Pierce Inc., Rockford, IL). In all cases, 80 μL droplets of each solution were deposited onto the surfaces and incubated for 2 h at room temperature (RT) in a humidity chamber. Then, the surfaces were washed with a 0.1 M $NaHCO_3$ solution, pH 8.5, and immersed in a 10 g/L bovine serum albumin (BSA) solution in 50 mM phosphate buffer, pH 7.4 (blocking solution), for 1 h at RT in order to cover the remaining free binding sites of the surface. After that, the surfaces were washed three times with 50 mM phosphate buffer, pH 7.4, and reacted with a 2.5 μg/mL AF546-labeled streptavidin solution in a blocking solution for 30 min at RT. Subsequently, the surfaces were washed extensively five times with 50 mM phosphate buffer, pH 7.4, and distilled water and dried under a stream of nitrogen (N_2). Fluorescence images were acquired with an Axioskop 2 Plus epifluorescence microscope (Carl Zeiss) equipped with a Sony Cyber-Shot 8-bit digital camera and processed with *ImagePro Plus* software (Media Cybernetics, Inc.).

For detection of the carboxyl groups, two approaches were followed: (a) direct incubation with a solution containing 25 mM amine-PEG_3-biotin and 5 mM *N*-[3-(dimethylamino)propyl]-*N*′-ethylcarbodiimide hydrochloride (EDC; Sigma-Aldrich) in 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 5; (b) a two-step approach involving (1) activation of the carboxyl groups by incubation with a solution containing 10 mM EDC and 5 mM *N*-hydroxysulfosuccinimide (sulfo-NHS; Pierce Inc.) in 0.1 M MES buffer, pH 5, for 1 h at RT and (2) coupling of amine-PEG_3-biotin from a 25 mM solution in a 0.1 M $NaHCO_3$ solution, pH 8.5, for 2 h at RT. All samples were then washed, blocked, and reacted with AF546-labeled spreptavidin for detection of the immobilized biotin moieties as described above.

Surfaces were also incubated with a 25 mM D-biotin (Sigma-Aldrich) solution in 0.1 M $NaHCO_3$, pH 8.5, for 2 h at RT and then subjected to washing, blocking, and fluorescently labeled streptavidin as described above. This was done in order to exclude binding of biotin onto the surfaces from sites other than the amine group in the biotin derivative used to identify carbonyl and/or carboxyl groups on the plasma-treated PMMA surfaces. For the stability studies, surfaces were stored for up to 180 days and assayed as discussed above at certain periods of time.

2.1.2. Immobilization of Biomolecules. The immobilization of proteins on PMMA surfaces (untreated, O_2-plasma-treated, and O_2-plasma-treated and annealed) was performed by the manual deposition of 1 μL droplets of a 100 μg/mL RgG solution in 10 mM phosphate buffer, pH 7.4, and incubation up to 24 h at RT in a humidity chamber. After washing with 10 mM phosphate buffer, pH 7.4 (washing buffer), some samples were immersed in a (5%) SDS solution for 10 min at 90 °C. All samples were washed three times with washing buffer and then immersed in a 10 g/L BSA solution in 50 mM phosphate buffer, pH 7.4 (blocking solution), for 1 h at RT, in order to cover the remaining free protein-binding sites of the surface. Then, the surfaces were washed three times with washing buffer, and the immobilized protein molecules were detected through reaction with a 5 μg/mL AF488-labeled goat anti-rabbit IgG solution prepared in a blocking solution for 1 h at RT. Here, the relevant increase of biomolecule binding on the plasma-treated versus nontreated surfaces is determined through fluorescence measurements in order to show the advantage of our method. Quantitative measurements of radioactive labeling are also planned in the near future. Finally, the surfaces were washed extensively five times with washing buffer and distilled water and dried under a stream of N_2. Fluorescence images were acquired and processed as mentioned above. For the stability studies, surfaces were stored for up to 280 days and assayed as discussed above at certain periods of time.

2.1.3. Immunoassays Directly on a Plasma-Micronanotextured Surface. Affinity-purified rabbit polyclonal antibodies against CRP (Sripps Laboratories Inc.) or salmonella LPS (AbD Serotec) were immobilized on the surfaces through incubation with a 100 μg/mL solution in 50 mM phosphate buffer, pH 7.4, overnight at RT. The substrates were then washed with 10 mM phosphate buffer, pH 7.4 (washing buffer), and immersed in a 10 g/L BSA solution in 50 mM

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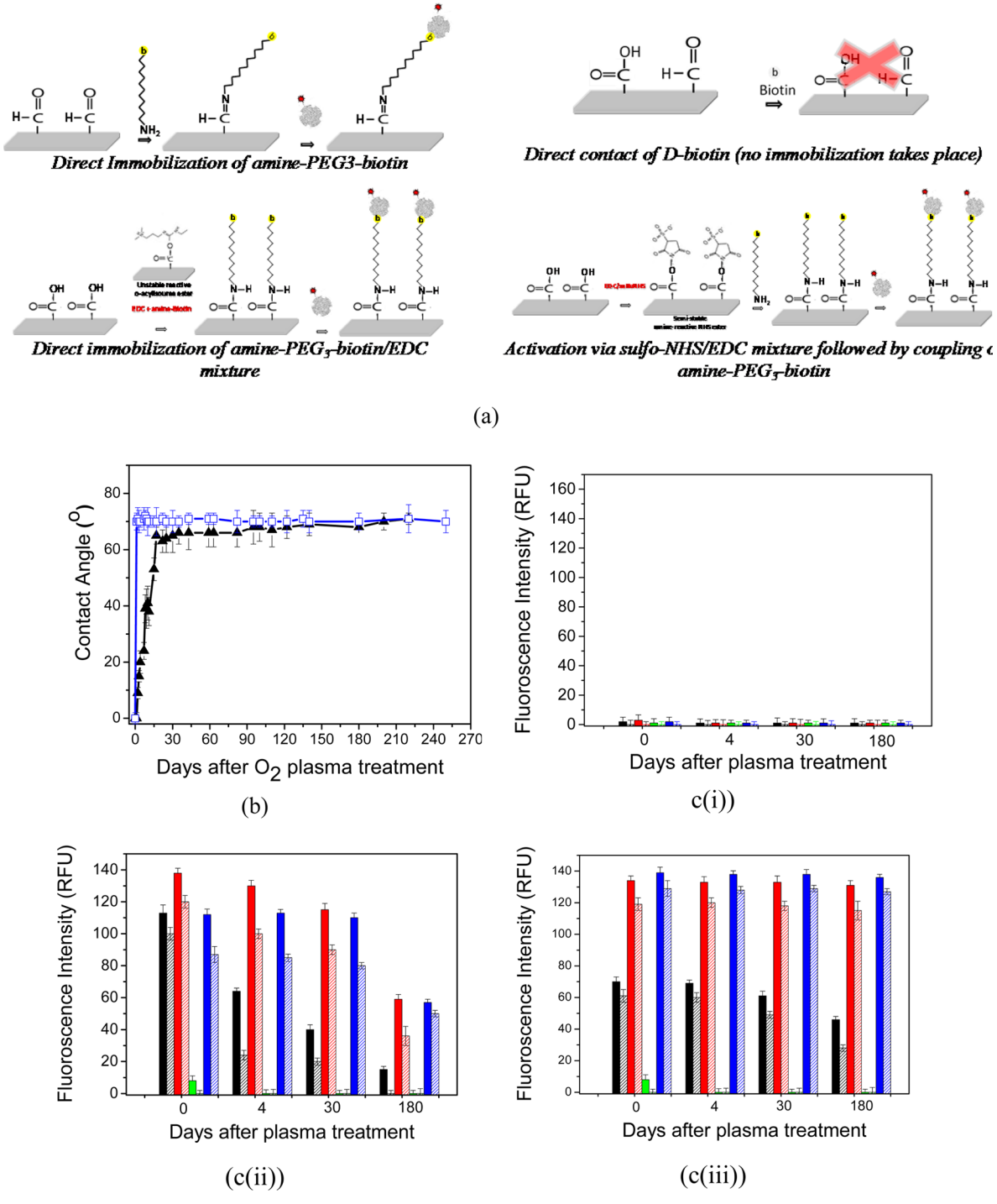

Figure 2. (a) Processes of covalent attachment and detection of biotin derivatives on plasma-etched PMMA surfaces. (b) Hydrophobic recovery (aging) of PMMA surfaces with and without thermal annealing after O_2 plasma micronanotexturing in reactor 1 for 5 min: nonannealed surfaces (▲); surfaces annealed at 110 °C for 30 min (□). (c) Fluorescence intensities of PMMA surfaces (i) untreated, (ii) 5-min-plasma-treated in reactor 1, and (iii) 5-min-plasma-treated in reactor 1 and thermally annealed after reaction with different biotin derivatives and detection with AlexaFluor 546-labeled streptavidin. Columns correspond to reaction with amine-PEG_3-biotin (black), amine-PEG_3-biotin/EDC (red), NHS/EDC and amine-PEG_3-biotin (blue), and biotin (green). Hatched columns correspond to surfaces washed with SDS after reaction. The figure of fluorescence values also reveals the stability of the covalently attached biotin derivatives following washing with SDS (hatched columns). The points in part b and the columns in part c (i−iii) represent mean values obtained from three surfaces, and the bars represent SDS.

phosphate buffer, pH 7.4 (blocking solution), for 2 h at RT. After that, the surfaces were washed three times with washing buffer and reacted for 1 h with CRP (0−100 ng/mL) or LPS (0−500 ng/mL) solutions in 10 mM phosphate buffer, pH 7.4, containing 10 mg/mL BSA, and 0.05% (v/v) Tween 20 (assay buffer). After washing as previously described, the substrates were incubated for 1 h with a 10 μg/mL biotinylated anti-CRP or anti-LPS antibody solution in the assay buffer. Visualization of the immunocomplexes bound on the surface was carried out using an ELF 97 Immunohistochemistry kit (Invitrogen Inc.) according to manufacturer instructions. Fluorescence images were acquired and processed as mentioned above.

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3. RESULTS AND DISCUSSION

3.1. Morphology of Plasma-Micronanotextured Surfaces after O_2 Plasma Etching and Thermal Annealing. O_2 plasma etching was used to micro/nanotexture PMMA surfaces and thus to create substrates with increased surface area for biomolecule immobilization. Plasma treatment of PMMA surfaces results in the formation of columnar micronanostructures that grow with etching time. Etching has been performed in two different reactors: (a) a helicon-type high-density plasma reactor (reactor 1) and (b) a RIE reactor (reactor 2). Similar trends were obtained using these two types of etching equipment (see the Materials and Methods section), suggesting independence from the plasma reactor type.

Micro/nanotexture formation is the result of sputtering from the reactor dome or powered electrode inducing micromasking on the organic polymer surface. XPS analysis of these surfaces has shown that they are contaminated with aluminum species in the form of aluminum oxide or oxyfluoride (reactor 1) or with silicon in the form of silicon oxides, usually on the order of a few percent (reactor 2), coming from the anodized aluminum or quartz electrodes, respectively.[49]

Figure 1 illustrates tilted-view scanning electron microscopy (SEM) images of PMMA surfaces treated in both reactors. For reactor 1, the images reveal the formation of densely packed 1-μm-high micro- and nanocolumns after 5 min of O_2 plasma treatment (part b). The 30-s-treated surface (part a) shows minimal topography compared with the 5-min-treated surface but is included in the surfaces under investigation to examine only chemical modification caused by plasma upon protein covalent binding. For reactor 2, the images reveal the formation of nano- and microcolumns with heights of 0.3 μm (part d) and 1.3 μm (part e) after 1 and 10 min of treatment, respectively. Moreover, in Figure 1, SEM images of PMMA surfaces treated for 5 min in reactor 1 (part c) or 10 min in reactor 2 (part f) and then annealed are also included. From these images, it is obvious that no deformation of the surface topography is observed after the annealing process.

3.2. Nanotextured PMMA Surfaces with Stable Chemical Functionality Capable of Covalent Binding of Biomolecules. Untreated PMMA exhibits a static contact angle of 65°. Further hydrophilization is induced by O_2 plasma processing. However, plasma hydrophilized surfaces are not chemically stable and suffer from a phenomenon known as hydrophobic recovery, i.e. a gradual increase of contact angle with time (also referred to as aging). In our previous work, we observed that aging of super hydrophilic plasma nanotextured PMMA surfaces is retarded for approximately one month, as compared to plasma treated but not nanotextured surfaces possibly due to the effect of the topography.[49] In this work, we propose a method that can create nanotextured PMMA surfaces with stable-in-time wetting behavior (for over one year) by employing an annealing step at 110 °C for 30 min for 2 mm thick (T_g 119 °C) or 100 °C for 2 h for 0.5 mm thick PMMA substrates (T_g 109 °C).

As shown in Figure 2b, PMMA surfaces treated for 5 min in reactor 1 exhibited superhydrophilicity for as long as 4 days and slowly aged to the initial contact angle over a period of 17 days. On the contrary, the contact angle of the 5-min-O_2-plasma-treated PMMA surface, which were subjected to thermal annealing, returned to the initial value immediately after the annealing step and remained stable for a long period of time. Similar results for reactor 2 are provided in the Supporting Information (SI; Figure S1).

In order to demonstrate the covalent immobilization capability of the plasma-etched PMMA surfaces compared to the untreated ones, we used different activated biotin derivatives designed to form covalent bonds with carboxyl (−COOH) or carbonyl (C═O) groups. The reaction schemes are shown in Figure 2a. More specifically, we expect that, if carbonyl groups are present on the surface, the $-NH_2$ group of the amine-PEG_3-biotin will covalently link directly to them without the need for any additional treatment. Carbonyl groups react with amines without activation to form Schiff bases that are not stable for a long time. However, because testing for the aldehyde groups on the surface was performed immediately after the formation of imine bonds, no stabilization was necessary. Also, surface treatment with the sulfo-NHS/EDC mixture followed by reaction with amine-PEG_3-biotin or the addition of an EDC/amine-PEG_3-biotin mixture is expected to lead to biotin immobilization on the surfaces through the carboxyl groups that are present on them. Finally, the surfaces were also reacted with a solution of D-biotin to exclude binding of the derivatives from sites other than their specific functional groups. The covalent immobilization of biotin and biotin derivatives was then detected through reaction with a fluorescently labeled streptavidin (AlexaFluor 546-labeled streptavidin). To further confirm covalent binding of the biotin moieties on the surfaces, the surfaces are subjected to washing with a SDS solution at 90 °C, a harsh and aggressive treatment able to remove noncovalently bound molecules.[2,57]

In Figure 2c (i−iii), the fluorescence intensities obtained from areas modified with different biotin derivatives prior to and after washing with SDS are provided for (i) untreated PMMA, (ii) 5-min-plasma-treated PMMA surfaces in reactor 1, and (iii) 5-min-plasma-treated PMMA surfaces in reactor 1 and annealed PMMA surfaces. All surfaces were tested over a period of 180 days. As shown in Figure 2, the untreated PMMA provided extremely low fluorescence values, confirming the absence of groups appropriate for covalent binding of biotin derivatives. On the other hand, the plasma-micronanotextured PMMA surfaces provided high fluorescence values, indicating the presence of both carbonyl and carboxyl groups available for reaction with appropriate biotin derivatives. There is, however, a distinct difference in the functionality of annealed and nonannealed plasma-treated PMMA surfaces. In the annealed surfaces, the carbonyl groups are less than those in the nonannealed surfaces, and their number slightly decreases with time, whereas the number of carboxyl groups remains stable over a period of 180 days. On the other hand, in the nonannealed surfaces, both the carbonyl and carboxyl groups are constantly decreasing with time, with their number reduced by approximately 70% after 180 days with respect to the value of fresh surfaces. This decline can be ascribed to the presence of unstable dangling bonds and oxidized forms of carbon such as carbonate groups after plasma treatment (see the XPS results), which decay with different rates in time. One can further observe that bound molecules on the stabilized annealed PMMA surfaces are not affected by harsh washing with detergent at 90 °C because the fluorescence intensity values from the SDS-treated surfaces were slightly lower (less than 10%) than the values obtained from nonwashed surfaces, suggesting covalent binding. It should also be noted that incubation with D-biotin provided very low signals (2−4% of the signal provided by areas reacted with amine-PEG_3-biotin),

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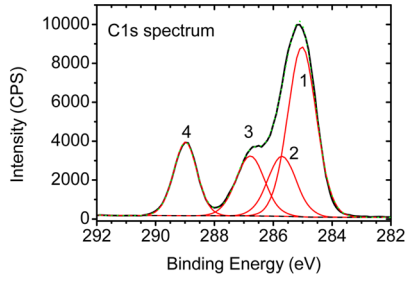
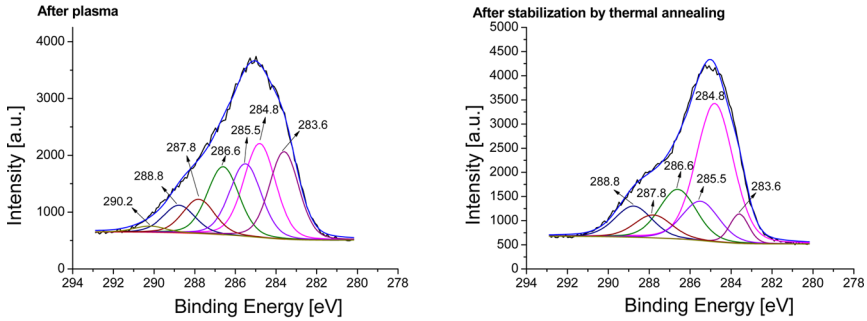
Table 1. Fitting Parameter for the C 1s Peak of O_2-Plasma-Treated PMMA

		C*−Si−O	C−C, C−H	C*(CO)O, C*C(OH)O	C*OC═O	C*O	OC*═O	O_2C*═O	O total	Si*O_x	Si*CO	Si*O_y
PMMA	mean		33.1	13.9	13.7		12.9		26.5			
	SD		1.1	0.4	0.2		0.1		0.5			
plasma-treated	mean	8.0	8.1	6.4	5.9	2.9	2.3	0.5	50.7	5.6	4..6	4.8
	SD	0.8	0.4	0.2	0.1	0.1	0.1	0.0	0.2	0.2	0.4	0.3
stabilized	mean	1.9	16.2	4.4	5.9	2.5	3.6	0.0	50.6	8.0	2.5	4.4
	SD	0.3	0.3	0.4	0.3	0.2	0.3	0.0	0.1	0.5	0.1	0.4

		untreated PMMA		plasma-treated		stabilized	
	position	area [%]	SD	area [%]	SD	area [%]	SD
C−Si−O	283.6			23.3	2.3	5.6	0.8
C−C, C−H	284.8	45.1	1.2	23.8	1.0	47.0	0.7
C*(CO)O, C*C(OH)O	285.5	18.9	0.6	18.8	0.5	12.8	1.3
C*OC═O	286.6	18.6	0.3	17.3	0.3	17.0	0.9
CO	287.8			8.6	0.3	7.2	0.7
OC*═O	288.8	17.5	0.4	6.8	0.4	10.6	0.9
O_2C═O	290.2			1.5	0.1	0.0	0.0



(a)



(b) (c)

Figure 3. High-resolution XPS C 1s core-level surface spectra with fitted peaks of PMMA surfaces untreated (a) and treated in O_2 plasma (reactor 2) for 10 min prior to stabilization (b) and after stabilization by thermal annealing (c).

which were further reduced after washing with SDS, showing almost negligible nonspecific binding. These results suggest that plasma-micro/nanotextured and annealed PMMA surfaces possess a stable number of reactive and available carboxyl and carbonyl groups that can be used for covalent immobilization of biomolecules.

To gain insight on functional group formation on PMMA surfaces after nanotexturing with O_2 plasma, we examined nanotextured surfaces by EPR to detect free radicals that can form covalent bonds. EPR measurements are provided in the SI (Figure S3). The EPR signal from the nanotextured surfaces does not show the presence of detectable free radicals. This result is in agreement with the results from the Bilek group, which has shown that the EPR signal was negligible when the energy of the ions was below 500 eV (in our case, the ion energy is on the order of 100 eV). This negative result essentially confirms that in our case free radicals in the bulk cannot be responsible for covalent binding, although some surface dangling bonds are possible. Binding is probably due to the formation/presence of specific chemical groups, as discussed above. Thus, EPR was carried out for further confirmation of our conclusions.

In order to unveil the differences among the pristine, plasma-treated, and stabilized plasma-treated surfaces, a detailed XPS analysis was conducted. Table 1 and Figure 3 show the results of XPS curve fitting for the C 1s peaks for untreated (part a), plasma-treated (part b), and annealed plasma-treated (part c) surfaces. Peak positions and corresponding chemical states are taken from our previous work[49] and from the literature.[58,59] The C 1s spectrum of the untreated PMMA is the result of the contribution of four components: the hydrocarbon (C−C/C−H; number 1) at a binding energy of ~284.9 eV, the β-shifted

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carbon (due to their juxtaposition to O−C═O groups; number 2) at ∼285.6 eV, the methoxy group carbon (number 3) at ∼286.7 eV, and the carbon in the ester group (number 4) at ∼289.0 eV. A comparison of the peaks (see Table 1 and Figure 3) reveals additional contributions from oxidized carbon and silicon groups, as a result of plasma treatment. In particular, a strong oxidation and diminution in the quantity of C−C (284.8 eV) bonds is observed after plasma processing. The determined C/O ratio strongly decreases from 2.78 for untreated to 0.67 and 0.68 for plasma-treated and stabilized surfaces, respectively. Plasma processing causes breaking of the C−C bonds and creation of new bonds with oxygen including the carbonyl group (C═O at 287.8 eV), carbonate group (O_2C═O at 290.2 eV), and incoming sputtered electrode material (C−Si−O at 283.6 eV). It can also be noted that oxidation of the ester group (O−C═O at 288.8 eV) takes place because of its diminished contribution on plasma-treated and annealed surfaces compared to the untreated one. As can be clearly seen in the RIE reactor 2 results, in addition to oxygen bonding, C−Si−O bonds are formed. Additionally, the differences in the shape of the C 1s spectra reveal strong modifications of the PMMA surface after stabilization by thermal annealing.

During stabilization, reactions take place between the dangling bonds. Therefore, more C−C bonds (284.8 eV) are partially created, thus restoring the diminished C−C bindings, and a lower fraction of C−Si−O (283.6 eV) bonds are visible, possibly because of the formation of Si−O bonds. It can be noted (see Table 1) that the surface becomes rich in more stable forms of carbon- and oxygen-containing groups; e.g., a fraction of the carbonate group (O_2C═O at 290.2 eV) after stabilization is absent. The existence of more "saturated" bonds stabilizes the surface of the polymer. We note also that the surface contents in the C═O and COOH groups (287.8 and 286.6 eV) remain intact; i.e., the chemical functionality is preserved (see Table 1).

The results with molecules presented in Figure 2 and Figure S2 in the SI from the experiments on covalent bonding employing biotin-amine without and with surface activation via EDC/NHS for two different processing times and two different reactors can be used to determine how the ratio of CO to COOH groups is affected from the specific plasma conditions. The results show that, for annealed (nonannealed shown in parentheses) plasma-treated surfaces, the ratio CO/COOH is 0.37 and 0.52 (0.65 and 1.00) for processing times of 30 s and 5 min, respectively, in the ICP reactor and 0.61 and 0.82 (0.89 and 1) for processing times of 1 and 10 min in the RIE reactor. Thus, it can be concluded that with increasing processing time the CO/COOH ratio increases for both reactors. We note that the ratio values 0.82 (1) for the 10-min-processed samples in the RIE reactor determined by the molecules is close to the values 0.70 (1.26) obtained from XPS and shown in Table 1.

To detect subtle variations in the surface chemistry, different TOF-SIMS signals are inspected simultaneously within PCA, as shown in the SI. The principal component 1 (PC1) scores clearly separate pristine PMMA, having positive PC1 values, from the plasma-treated samples, exhibiting negative PC1 scores (Figure S8a in the SI). PC1 is loaded negatively by O, OH, and O_2 secondary ions (Figure S8b in the SI), which is in accordance with an increase of the oxygen concentration from ∼26% for pristine PMMA to ∼50% for plasma-treated samples determined with XPS (Table 1). In addition, the principal component 2 (PC2) scores plot reveals a difference between the plasma-treated and plasma-treated/annealed samples. The PC2 loadings plot (FigureS7c in the SI) shows that the separation seen between samples is due to O, C_3H_3O, $C_4H_5O_2$, and $C_8H_{13}O_2$ (positive loadings on PC2), which are characteristic for plasma-treated PMMA, and C_2HO, CHO_2, C_4H, C_4HO, and C_3HO_2 (negative loadings on PC2), which are characteristic for plasma-treated/annealed samples. Information gained from analysis of the surfaces with XPS is in agreement with the increase of the contact angle for stabilized surfaces because the fraction of C−C aliphatic bonds, which are responsible for hydrophobic behavior, is 2 times higher after plasma treatment and annealing (see Table 1). Moreover, SIMS results together with PCA (PC2; Figure S8c in the SI) confirm the above observation because samples after plasma treatment show mainly O peaks, while samples after stabilization show an increase of secondary ion signals, which are characteristic of aliphatic fragments (e.g., C_4H).

The reproducibility of the fabrication of nanotextured and annealed surfaces was tested over a 1-year period by preparing eight batches and testing them through covalent coupling of amine-PEG_3-biotin after activation with EDC/NHS. Three different surfaces from each batch were used for testing. As is shown in Figure S4 in the SI, the results obtained were very repeatable because all of the values were within the range of ±2 standard deviations (SDs) of the mean value of all measurements, i.e., in the range of ±10% of the mean value obtained for the eight different batches.

3.3. Stable Covalent Immobilization of Rabbit γ-Globulins (RgGs) on O_2 Plasma-Treated PMMA Surfaces.

We demonstrate the capability of plasma-micronanotextured and annealed (110 °C for 30 min) PMMA surfaces to serve as substrates for stable and covalent immobilization of proteins through immobilization of RgGs. Immobilized molecules are detected through reaction with a fluorescently labeled (AlexaFluor 488) anti-rabbit IgG antibody. We determine the amount of protein covalently bound on the surface by determining the fluorescence signal from surfaces washed with SDS, an ionic surfactant capable of overcoming the forces responsible for physical adsorption, while not affecting the covalent bonds, as discussed therein.[2] In addition to the direct incubation of plasma-treated surfaces with the protein solution, protein was added to surfaces after activation of surface carboxyl groups through reaction with a mixture of EDC/NHS, in order to distinguish between the protein binding directly on the surface due to readily available reactive groups, i.e., the carbonyl groups, and the binding due to the activated by EDC/NHS carboxyl groups. To strengthen the generality of our results, immobilization results for another protein, namely, biotinylated BSA (b-BSA), are included in the SI (Figure S5).

The process of protein immobilization and detection is schematically depicted in Figure 4a (see the details in the Materials and Methods section). In Figure 4b, the fluorescence intensity of RgG-coated areas (100 μg/mL concentration) is shown as a function of the incubation time (1 and 24 h of incubation), on 5-min-plasma-treated and annealed PMMA surfaces (reactor 1), prior to and after washing with SDS. The plasma-treated PMMA surface shows significant protein uptake. After SDS washing, 5% and 40% of the initial protein is retained, when incubation with the protein solution has been performed for 1 and 24 h, respectively. On the other hand, the untreated surfaces were characterized from significantly lower protein binding capacity (approximately 30% compared to plasma-treated surfaces), which is attributed exclusively to physical adsorption because it is reduced to almost zero after

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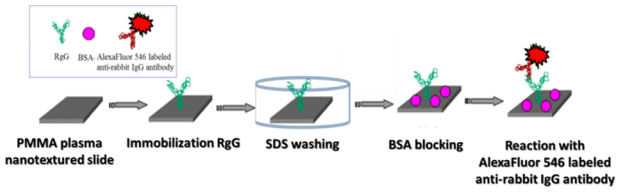
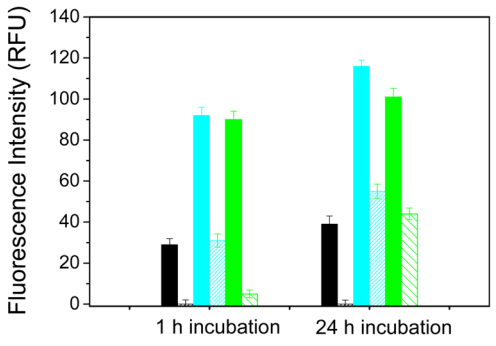


(a)



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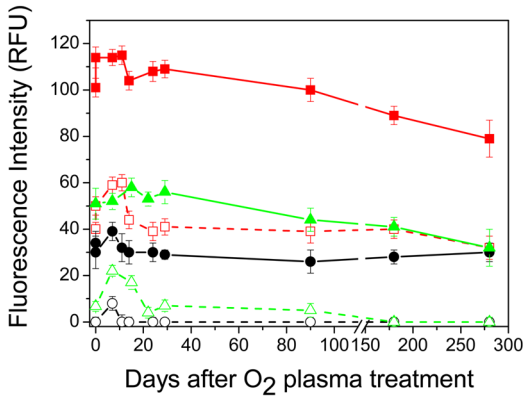

(c)

Figure 4. (a) Process of immobilization and detection of RgG on PMMA surfaces. (b) Fluorescence intensity values from areas on untreated PMMA (black columns), 5-min-O_2-plasma-treated PMMA surfaces (green columns), and 5-min-O_2-plasma-treated EDC/NHS-activated PMMA surfaces (blue columns) incubated with a RgG solution for 1 and 24 h, respectively, in reactor 1 (for reactor 2, see the SI). Immobilized RgG is detected in all cases through reaction with an AF546-labeled anti-rabbit IgG antibody. Filled columns correspond to surfaces nonwashed with SDS and hatched columns to surfaces washed with SDS. (c) Fluorescence intensity values obtained for PMMA surfaces incubated with a RgG solution for 24 h, after reaction with an AF546-labeled anti-rabbit IgG antibody as a function of the days after plasma treatment in reactor 1 for various plasma durations and after baking at 110 °C for 30 min. Filled symbols correspond to surfaces nonwashed with SDS and empty symbols to surfaces washed with SDS. Circles, triangles, and squares correspond respectively to untreated, 30-s-treated, and 5-min-plasma-treated surfaces, respectively.

washing with SDS. The EDC/NHS-activated plasma-treated PMMA surfaces also demonstrated significant retention of immobilized protein, typically 33% and 47% protein retention for incubation with the RgG solution for 1 and 24 h, respectively. From these results, it can be concluded that, in the freshly treated PMMA surfaces, direct protein immobilization without surface activation with EDC/NHS results from physical adsorption and covalent bonding to carbonyl groups. On the other hand, activation with EDC/NHS takes advantage of the carboxyl groups present on the surface to form stable amide bonds with the proteins, as evidenced from the higher amount of protein remaining after washing with SDS compared to nonactivated surfaces. Therefore, the plasma-treated and annealed surfaces are preferably activated with EDC/NHS prior to their use as substrates for antibody microarrays.

In Figure 4c, we examine the effect of the chemical stability of the surfaces after plasma treatment for up to 280 days, as well as the effect of the duration of the plasma treatment. Several samples are treated in O_2 plasma for 30 s and 5 min in reactor 1, then stabilized by annealing, and finally stored in ambient conditions. Pairs of samples are selected, activated by EDC/NHS, and coated with RgG on specific dates; one of the sample pairs is washed with SDS. The fluorescence intensity values obtained from such PMMA samples are shown in Figure 4c versus time after plasma treatment for either nonwashed or washed SDS surfaces. A surface treated for only 30 s is also included in the study; this surface has chemical modification because of O_2 plasma exposure but almost negligible topography. A trend generally observed is the increase in the fluorescence intensity with plasma treatment time up to about 3−4 times for 5 min treatment compared to untreated surfaces. Notice that the 30-s-treated sample that had almost negligible topography has almost 2 times higher protein binding capacity than the untreated sample. The plasma-treated PMMA surfaces show 40−85% protein retention compared to untreated PMMA surfaces after washing with SDS, which removes almost completely the proteins immobilized on untreated surfaces. This suggests that a covalent bond is formed and is accountable for the robust protein attachment. The percentage of protein that is not retained can be attributed to physical adsorption. Similar results with regard to protein retention after SDS washing are obtained for PMMA surfaces imposed to O_2 plasma treatment for 1 and 10 min in reactor 2 (data in the SI, Figure S6). In reactor 2, even after 1 min of plasma treatment, the nanotextured PMMA surface showed 85% protein retention compared to untreated PMMA surfaces.

Figure 4 demonstrates the excellent chemical stability of the plasma-treated surfaces for a period of 1 year after plasma treatment and annealing and the covalent binding on such surfaces due to resistance to hard washing. To further demonstrate that the protein immobilized on the micro-nanotextured surfaces is stable against harsh washings, we tested a series of other washing protocols in PMMA surfaces micronanotextured for 5 min in reactor 1 after incubation with the RgG solution for 24 h. The washing protocols that we used are summarized in Table 2. In detail, we applied (1) 5% (w/v) SDS at 90 °C for 10 min (discussed thus far), (2) 5% (w/v) SDS at RT for 1 h, (3) Hellmanex, a strong alkaline solution after dilution in phosphate buffer (PB; 2% v/v; it is stronger than SDS and similar detergents because it causes mild surface etching); (4) 0.05% v/v Tween 20, a polysorbate nonionic surfactant widely used as a detergent in washing solutions employed in binding assays, (5) acetonitrile after dilution in water (5% v/v), an organic solvent that can disrupt a combination of electrostatic or hydrophobic interactions of the adsorbed protein, as well as short-range molecular interactions such as van der Waals forces and hydrogen bonds. As can be concluded from the data presented in Table 2, the different washing protocols remove only a part of the immobilized RgG from the micronanotextured surfaces, with

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Table 2. Washing Protocols and the Corresponding Protein Retention Percentages Compared to Those of Nonwashed Surfaces

washing protocol	rinsing protocol	protein retention (%)
5% SDS, 10 min at 90 °C	5 times with 50 mM PB	40
5% SDS, 1 h at RT	5 times with 50 mM PB	70
0.5% Tween 20, 10 min at RT	5 times with 50 mM PB	92
2% Hellmanex, 10 min at RT	5 times with 50 mM PB	80
5% acetonitrile, 10 min at RT	3 times with DI water and 5 times with 50 mM PB	89

SDS being the most effective. Nevertheless, even in the case of harsh washing with SDS, a substantial amount of immobilized protein is retained on the plasma-treated surface. It should also be noted that washing with Tween 20, which is usually applied in binding reactions and immunoassays, results in the removal of less than 10% of the immobilized protein.

3.4. Immobilization of Polyclonal Antibodies against CRP and LPS and Application to CRP and LPS Immunoassays. The advantages of the O_2-plasma-treated micronanotextured and stabilized PMMA surfaces when used as substrates for immunoassays are demonstrated through the development of immunoassays for the detection of CRP and LPSs from *Salmonella enterica* serotype typhimurium (salmonella LPS). The immunoassay process for the detection of CRP or salmonella LPS is schematically depicted in Figure 5a and encompasses the following steps: (1) coating with the respective analyte-specific antibody through incubation with a 100 μg/mL solution for 24 h at RT, (2) washing and immersion for 2 h in a 10 g/L BSA blocking solution, (3) 1 h reaction with salmonella LPS or CRP calibrator solutions (0–500 ng/mL), (4) 1 h reaction with biotinylated analyte-specific antibodies, and (5) reaction with the ELF 97 Immunohistochemistry reagent according to the manufacturer's instructions (Invitrogen Inc.). We note that the antibodies were directly immobilized on the plasma-treated surfaces, without any additional surface functionalization.[60]

In Figure 5b, typical CRP calibration curves are presented, obtained using as the immunoassay support untreated PMMA surfaces and surfaces treated for 5 min in O_2 plasma in reactor 1, both annealed and nonannealed, and activated through EDC/NHS. We show that the micronanotextured plasma-treated surfaces provided considerably higher (15 times) fluorescence signals for the same CRP concentrations compared to the untreated ones, resulting in at least 50 times higher detection sensitivity for CRP. This improvement in the analytical performance of the assay indicates that the antibodies bound on the nanotextured surfaces after activation retained their immunoreactivity to a great extent compared to the untreated surfaces. Representative fluorescence images are provided of anti-CRP antibody spots created on (5ci) untreated, (5cii) plasma-treated but not annealed PMMA surfaces, and (5ciii) plasma-treated and stabilized via thermal annealing of the surfaces using a microspotter after reaction with a 50 ng/mL CRP solution. Notice that the stabilized (annealed) plasma-treated samples exhibit considerably higher fluorescence intensity signals compared to both micronanotextured and nonannealed, as well as the untreated PMMA surfaces.

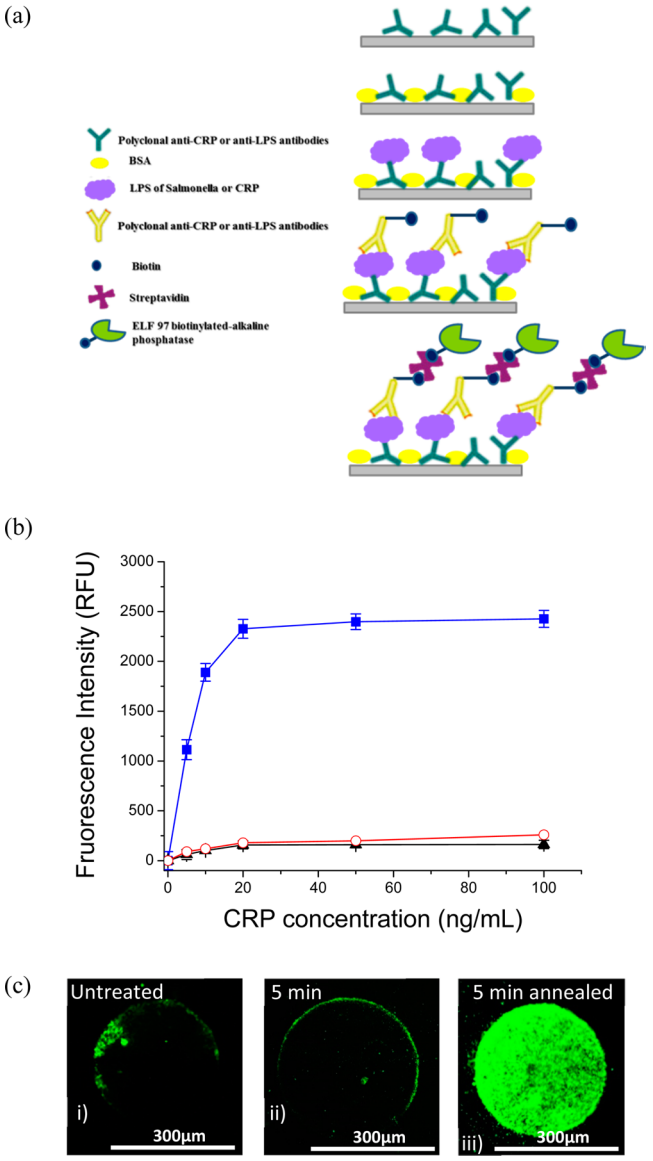

Figure 5. (a) Schematic depicting the steps of sandwich assay for the detection of CRP and salmonella LPS. (b) CRP calibration curves from assays performed on untreated (▲), O_2-plasma-treated in reactor 1 (○), and O_2-plasma-treated and thermally annealed surfaces (■). (c) Fluorescence images of spots corresponding to the 50 ng/mL CRP calibrator when the immunoassay was performed on (i) untreated, (ii) O_2-plasma-nanotextured, and (iii) O_2-plasma-nanotextured and thermally annealed PMMA surfaces.

In Figure 6, fluorescence images are provided from spots of anti-salmonella LPS antibody when the immunoassay was performed on (a) untreated, (b, left) 1-min-plasma-treated, (b, right) 1-min-plasma-treated and annealed, (c, left) 10-min-plasma-treated, and (c, right) 10-min-plasma-treated and annealed PMMA surfaces in reactor 2. The images show that a higher amount of anti-salmonella LPS antibody is immobilized on the micronanotextured surfaces, both stabilized and nonstabilized, compared to the untreated surfaces. Nevertheless, the stabilized surfaces provide higher spot homogeneity and spot shape regularity because of the stabilized contact angle compared to the spots created on surfaces nanotextured but not stabilized by thermal annealing. Spots on nonstabilized surfaces seem to "spread-out" because of surface

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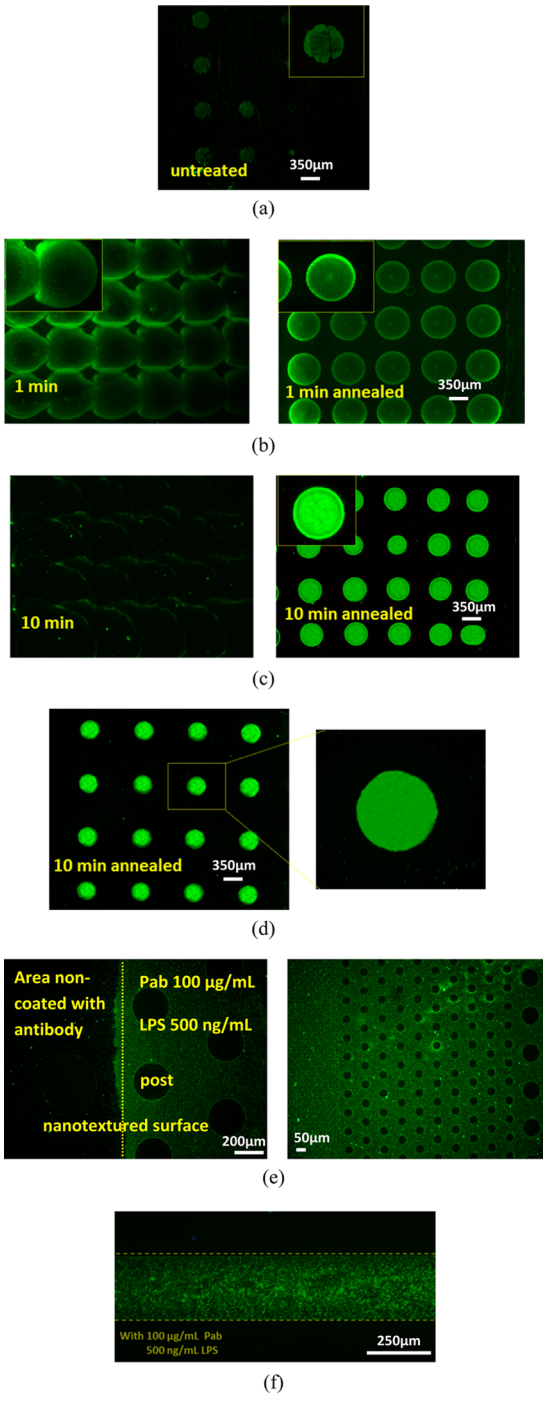

Figure 6. Fluorescence images of spots corresponding to a 500 ng/mL salmonella LPS solution from microarrays created on (a) untreated and (b) 1-min- and (c) 10-min-O_2-plasma-micronanotextured PMMA surfaces in reactor 2 with (right column) and without (left column) stabilization with thermal annealing. (d) Fluorescence images of spots corresponding to a 500 ng/mL salmonella LPS solution from microarrays created on 1-year-old 10-min-O_2-plasma-nanotextured and annealed PMMA surfaces. (e) Fluorescence images from a microchannel coated with an anti-LPS polyclonal antibody after reaction with a 500 ng/mL LPS solution and detection, as illustrated schematically in Figure 5a. (f) Fluorescence images from a microchannel onto which the anti-LPS polyclonal antibody was immobilized through activation of the surface with EDC/NHS followed by reaction with a 500 ng/mL LPS solution and detection, as illustrated schematically in Figure 5a.

hydrophilicity. Quantitative evaluation of the sensitivity enhancement is provided in the SI (Figure S7). Finally, in Figure 6d, images are shown from a microarray created through immobilization of polyclonal anti-LPS antibody on PMMA surfaces 1 year after O_2 plasma nanotexturing and thermal annealing, indicating the high stability of thus-modified polymeric surfaces.

Furthermore, as shown in Figure 6e,f, immunoassays could be performed not only on planar micronanotextured surfaces, i.e., in microarray format, but also in a microfluidic format. In particular, in Figure 6e, we demonstrate immobilization of an anti-salmonella LPS antibody onto PMMA microchannels with posts created by photolithography and plasma etching, for which a roughened bottom layer is observed.[61] After plasma etching, the microchannels were stabilized and used 3 months later. Immobilization of the antibody was performed by covering part of the microchannel with the antibody solution and overnight incubation. The microchannel area nonincubated with the antibody solution appears dark, whereas the areas covered with antibody appear bright green apart from the posts, which were protected by photoresist during plasma etching.

Alternatively, the antibody can be covalently attached to the micronanotextured and annealed surfaces after activation of its carboxyl groups through reaction with EDC/NHS. This is demonstrated in Figure 6f, where a linear section of the microchannel without posts was used to immobilize the polyclonal anti-LPS antibody. As can be seen, the fluorescence intensities of the two images of direct LPS antibody immobilization or immobilization after EDC/NHS activation are similar, indicating that the nanotextured surfaces can be employed as substrates for immunoassays in either planar (microarrays) or microchannel format to improve the detection sensitivity of the analytes.

4. CONCLUSIONS

In conclusion, we presented a method to create substrates with stable-in-time chemical functionality and high-surface-area micro- and nano topography on which biomolecules, including proteins and antibodies, can be covalently bound with a high density per unit surface. To achieve this goal, we used plasma micronanotexturing, followed by a short thermal annealing step, which induces accelerated hydrophobic recovery while preserving important chemically functionality created by the plasma. We probed the chemical functionality of the PMMA nanotextured surfaces showing stable carbonyl and carboxyl groups over a period of 1 year. We also demonstrated the coexistence of covalent binding of protein molecules on the surface (for as short as 30 s plasma treatment) together with physical adsorption, as evidenced by the remarkable resistance to harsh washings with SDS and other detergents. Finally, we demonstrated 2 orders of magnitude more sensitive immunoassays on such surfaces in microarray and microfluidic format. In particular, two examples of immunoassays for the determination of CRP and salmonella LPS were demonstrated. The advantages of the proposed surface functionalization approach include simplicity, speed, and stability over time, compared to other functionalization protocols, where the surfaces must be used shortly after plasma treatment because they are prone to aging and gradual loss of chemical functionality.

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■ ASSOCIATED CONTENT

Supporting Information

Additional information about micronanotextured PMMA surfaces produced in reactor 2 (RIE, reactive ion etcher), EPR measurements, reproducibility, high-density covalent immobilization of b-BSA protein, and PCA for SIMS data. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b01754.

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Notes

The authors declare no competing financial interest.

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