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Reactive Surface Coatings Based on Polysilsesquioxanes: Controlled Functionalization for Specific Protein Immobilization

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The key designing in reliable biosensors is the preparation of thin films in which biomolecular functions may be immobilized and addressed in a controlled and reproducible manner. This requires the controlled preparation of specific binding sites on planar surfaces. Poly(methylsilsesquioxane)-poly(pentafluorophenyl acrylates) (PMSSQ-PFPA) are promising materials to produce stable and adherent thin reactive coatings on various substrates. Those reactive surface coatings could be applied onto various materials, for example, gold, polycarbonate (PC), poly(tetrafluoroethylene) (PTFE), and glass. By dipping those substrates in a solution of a desired amine, specific binding sites for protein adsorption could be immobilized on the surface. The versatile strategy allowed the attachment of various linkers, for example, biotin, L-thyroxine, and folic acid. The adsorption processes of streptavidin, pre-albumin, and folate-binding protein were monitored using surface plasmon resonance (SPR), Fourier transform infrared (FTIR) spectroscopy, fluorescence spectroscopy, and atomic force microscopy (AFM). The presented protein immobilization strategy, consisting of four steps (a) spin-coating of PMSSQ-PFPA hybrid polymer from tetrahydrofuran (THF) solution, (b) annealing at 130 °C for 2 h to induce thermal cross-linking of the PMSSQ part, (c) surface analogues reaction with different amino-functionalized specific binding sites for proteins, and (d) controlled assembly of proteins on the surface, may find various applications in future biosensor design.

Introduction

In recent years, there has been remarkable progress in the development of optical affinity biosensors and their applications in various areas.[1−8] An optical affinity biosensor consists of an optical transducer and a biological recognition element.[9] Various optical methods have been exploited in biosensors including fluorescent spectroscopy,[10] interferometry,[11,12] spectroscopy of guided modes in optical waveguides,[13,14] and surface plasmon resonance (SPR).[15,16] In the area of biomolecular recognition, SPR, as a surface-oriented method, has shown a great potential for affinity biosensors, allowing real-time analysis of biospecific interactions without the use of labeled molecules.[17,18]

A common bottleneck in the development of all next generation biosensors is the insufficient stability and reproducibility of their interfacial properties.[19,20] The selectivity of biosensors is usually obtained by utilizing biomolecular functions such as proteins, antibodies, enzymes, and so on. The key in designing reliable biosensors is the preparation of thin films on which these biomolecular functions may be immobilized and addressed in a controlled and reproducible manner.[21] This requires the controlled expression of biomolecules on planar surfaces.

Beside the immobilization of various biomolecular functions on thin films, the thin film itself has to adhere onto the desired substrate. For SPR analysis, gold or silver surfaces are used, and other optical methods require glass or silicon; furthermore, electrochemical detection methods require conductive substrates, such as platinum or carbon.[22] Recently, an increased interest in the combination of microfluidics and microarrays has led to the progressive replacement of common inorganic substrates by polymers, especially by polydimethylsiloxane (PDMS) or polycarbonate (PC).[23] Thus, various specific biomolecular functions have to be attached to a variety of underlying materials, covering metals, metal oxides, and polymers.

Various concepts have been introduced to covalently immobilize specific biomolecular functions onto surfaces, but most of these methods are limited to the attachment of one chemical function and to one given substrate: Surface attachment on gold or silicon/silicondioxid substrates is widely realized using self-assembled monolayers (SAMs) of thiol- or alkoxysilane-linkers, respectively.[24,25] Different SAMs can be used to anchor various ligands,[26] to anchor initiators capable to graft polymers[27] or

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peptides from the surface,[28] or to graft polymers,[29,30] dendrimers,[31] or proteins directly to the surface.[32] On polymeric substrates, protein immobilization was achieved by a grafting-from approach using redox-initiated polymerization,[33] by grafting biofunctionalized polymers to the surface using photo-cross-linkers,[34] or by utilizing molding and hydrophobic effects at the surface.[35] However, all the above-mentioned methods are limited to one substrate only and have to be developed for each new substrate.

To overcome limitations toward a specific biomolecular function, various protein immobilization strategies were introduced, using reactive moieties attached to the surface. Those reactive moieties selectively bind to corresponding groups carrying the specific biomolecular function.[36] Attachment of epoxides,[37] anhydrides,[38,39] aldehydes,[40] or reactive esters (NHS ester[41−43] or pentafluorophenyl ester[44−47]) on the surface could be used to immobilize amino-functionalized specific binding sites or proteins directly via the N-terminus. Besides amine chemistry to attach biomolecular functions to a surface, other methods were successfully used: Michael addition between maleimide and thiols,[48,49] Huisgen reaction between acetylene and azide,[50,51] and Diels−Alder reactions between dienes and dienophiles.[52,53]

All those methods could successfully been used to immobilize different biomolecular functions onto planar surfaces, but the main drawback is the limitation toward specific substrate materials to attach the reactive moiety and thus limits the applicable biosensor method.

A general approach to immobilize biomolecular functions on surfaces should consist of reactive moieties for later specific immobilization and furthermore of substrate-independent adhesive moieties.

Recently, we introduced inorganic−organic hybrid polymers, obtained by grafting organic monomers from functionalized poly (methylsilsesquioxanes) (PMSSQ).[54−56] The strong substrate-independent adhesion of these materials after spin-coating and curing arises from chemical bonding interfaces on hydroxylated surfaces, polymeric diffusion interlayers on polymeric surfaces, and mechanical interlocking interfaces due to high degree of internal cross-linking.[57,58] By grafting pentafluorophenyl acrylate (PFPA) from PMSSQ, reactive surface coating materials could be synthesized. Stable reactive coatings could be realized on various substrates (e.g., Si, glass, gold, PMMA, PDMS, steel, etc.). Surface-analogous reaction with various amines was used to specifically alter surface properties.[59]

Within the present study, we investigated the use of these PMSSQ-PFPA hybrid polymers as coating materials to attach various biomolecular functions to planar surfaces without applying complex coating techniques or additional surface treatments.

Experimental Section

Materials. All chemicals and solvents were commercially available (Acros Chemicals, ABCR) and used as received unless otherwise stated. All used proteins, fluorescent-labeled proteins, and fluorophores were commercially available from Sigma-Aldrich. 1,4-Dioxane and tetrahydrofuran (THF) were distilled from sodium/benzophenone under nitrogen.

Instrumentation. ^{1}H NMR spectra were recorded on a Bruker 300 MHz FT-NMR spectrometer, and ^{19}F-NMR spectra on a Bruker DRX 400 FT-NMR spectrometer. ^{29}Si CPMAS NMR spectra were measured on a Bruker DSX 400 MHz FT-NMR spectrometer (rotation: 5000 Hz, T = RT, 4 mm rotor). Chemical shifts (δ) were given in ppm relative to tetramethylsilane (TMS). FD mass spectra were measured using a Finnigan MAT 95 mass spectrometer. Gel permeation chromatography (GPC) was used to determine molecular weights and molecular weight distributions, M_w/M_n, of polymer samples. (THF used as solvent; polymer concentration, 2 mg/mL; column setup, MZ-Gel-SDplus 10^2, 10^4, and 10^6 Å^2; used detectors, refractive index, UV, and light scattering). Thermogravimetrical analysis was performed using a Perkin-Elmer Pyris 6 TGA instrument in nitrogen (10 mg of pure polymer in aluminum pan). Surface plasmon resonance (SPR) was measured on a home-built system, using a $\Theta/2\Theta$ setup, a 632 nm laser, and a photodiode. Glass slides were coated with 1.5 nm of chromium and 50 nm of gold by evaporation. SPR scans were fitted using WINSPALL.[60] Atomic force microscopy (AFM) measurements were performed using a Veeco Dimension 3100 instrument in tapping mode. IR spectra were recorded using a Nicolet 5 DXC FT-IR-spectrometer on ATR crystal. Film thicknesses were measured using an EL X-02C ellipsometer. Fluorescence spectra were recorded on a Perkin-Elmer Luminescence spectrometer LS50B. All chemical reactions were performed in argon atmosphere.

PMSSQ RAFT Macrochain Transfer Agent (PMSSQ-mCTA). The macroinitiator was synthesized as described previously.[55,59] ^{1}H NMR ($CDCl_3$) δ: 7.99 (br, 5H); 7.36 (br, 2H);

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5.80 (br, 1.9H); 4.55 (br, 2H); 3.48 (br, 1.1H); 2.71 (br, 2H); 0.99 (br, 2H); 0.17 (br, 69.1H). M_n = 4990 g/mol, PDI = 1.63.

PMSSQ-PFPA. PMSSQ-PFPA was synthesized as described previously.[59] ^{1}H NMR ($CDCl_3$) δ: 7.40−6.85 (br); 3.05 (br, 1H); 2.81 (br); 2.32−2.10 (br, 2H); 1.50−1.20 (br); 0.15 (br, 1H). ^{19}F NMR (CDCl3) δ: −153.52; −157.11; −162.50. ^{29}Si CPMAS NMR δ: −48.55 (T1); −57.92 (T2); −65.93 (T3). M_n = 32 000 g/mol, PDI = 1.72. Cross-linking conditions (determined by TGA): 130 °C, 2 h.

Biotin Pentafluorophenyl Ester. An amount of 1 g of biotin (4.1 mmol) was dissolved in 20 mL of dimethylformamide (DMF) and 0.76 mg of triethylamine (7.5 mmol). A total of 1.7 g of pentafluorophenyl trifluoroacetate (6.15 mmol) was added slowly; afterward, the reaction mixture was stirred for 30 min at room temperature. Residual pentafluorophenyl trifluoroacetate and the solvents were removed by reduced pressure. The crude product was purified by precipitation in diethylether. Yield: 1.51 g (3.66 mmol, 94%).

^{1}H NMR (DMSO-d_6) δ: 6.44 (s, 1H); 6.36 (s, 1H); 4.28 (t, ^3J = 5.2 Hz, 1H); 4.14 (t, ^3J = 5.2 Hz, 1H); 3.05 (m, 1H); 1.71−1.40 (m, 8H). ^{19}F NMR (CDCl3) δ: −153.02; −156.22; −162.10. FTIR: activated ester band at 1748 cm^{-1}. FD mass spectra (m/z): 183.5 (100%); 243.0 (99%); 410.1 (90%); 411.1 (15%) (calcd mass: 410.07 g/mol).

Biotin-2-aminoethane Amide (H_2N-Biotin). A total of 1.5 g of biotin pentafluorophenyl ester (3.66 mmol) was slowly added to a solution of 2.2 g of ethylene diamine (37 mmol) in 10 mL of DMF. The solution was stirred for 2 h at 0 °C. Afterward, the product was precipitated in diethylether, filtered, and washed with ether. Yield: 0.90 g (3.1 mmol, 85%).

^{1}H NMR (DMSO-d_6) δ: 6.50 (s, 1H); 6.36 (s, 1H); 4.29 (t, ^3J = 5.2 Hz, 1H); 4.12 (t, ^3J = 5.2 Hz, 1H); 3.07 (m, 2H); 2.58 (m, 2H); 2.48 (t, ^3J = 6.6 Hz, 2H); 2.06 (t, ^3J = 6.6 Hz, 2H); 1.46 (m, 2H); 1.29 (m, 2H). FTIR: amide band at 1646 cm^{-1}. FD mass spectra (m/z): 286.2 (100%); 287.2 (11%); 288.1 (6%) (calcd mass: 286.15 g/mol).

Bis(5-carboxypentyl) Disulfide Bis(pentafluorophenyl) Ester (PFP-Disulfide). Bis(5-carboxypentyl) disulfide bis(pentafluorophenyl) ester (PFP-disulfide) was synthesized as previously described.[61]

Bis(5-carboxypentyl) Disulfide Dithyroxine Amide (T4-Disulfide). A total of 502 mg (0.646 mmol) of thyroxine was dissolved in 20 mL of dry DMF. And then 0.4 mL of triethylamine (2.87 mmol) and 175 mg (0.280 mmol) of bis(5-carboxypentyl) disulfide bis(pentafluorophenyl) ester dissolved in 1 mL of dry DMF were added. Upon addition of the activated ester, the turbid mixture turned clear within seconds. The reaction was stirred for 7 h at room temperature. The solvent was removed, and the residue dried in high vacuum. Chloroform was added, and the mixture was filtered through a 0.2 μm membrane. After drying with $MgSO_4$, the solvent was removed.

^{1}H NMR (DMSO-d_6) δ: 7.59 (s, 2 H); 7.01 (s, 2 H); 4.70 (m, 1 H); 3.05 (m, 2 H); 2.58 (t, ^3J = 6.6 Hz, 2 H); 2.18 (t, ^3J = 6.4 Hz, 2 H); 1.57 (m, 4 H); 1.33 (m, 2 H). FTIR (cm^{-1}): 2940 w, 1680 m, 1510 s, 1250 w, 1450 w, 1000 s.

Bis-(2-aminoethane Amide) Folic Acid. An amount of 880 mg of folic acid (2 mmol) was dissolved in 20 mL of DMF and 0.76 mg of triethylamine (7.5 mmol). And then 1.7 g of pentafluorophenyl trifluoroacetate (6.15 mmol) was added slowly; afterward, the reaction mixture was stirred for 30 min at room temperature. Residual pentafluorophenyl trifluoroacetate and the solvents were removed by reduced pressure. The crude product was purified by precipitation in diethylether. The obtained pentafluorophenyl ester was slowly added to a solution of 2.2 g of ethylene diamine (37 mmol) in 10 mL of DMF. The solution was stirred for 2 h at 0 °C. Afterward, the product was precipitated in diethylether, filtered, and washed with ether. Yield: 720 mg (1.37 mmol, 68.5%).

^{1}H NMR (DMSO-d_6) δ: 8.51 (s, 1H); 8.42 (s, 1H); 7.5−6.8 (m, 4H); 4.41 (m, 3H); 3.62 (m, 4H); 2.52 (m, 6H); 1.98 (m, 2H). FTIR: amide band at 1648 cm^{-1}. FD mass spectra (m/z): 527.4 (100%); 528.3 (14%) (calcd mass: 527.27 g/mol).

Perfluorophenyl 4-(2-(chlorodimethylsilyl)ethyl)benzoate. A total of 2 g (6.4 mmol) of pentafluorophenyl 4-vinylbenzoate, 1.21 g (13 mmol) of chlorodimethylsilane, 25 mg of platinum on charchoal, and 20 mL of toluene were placed in a 50 mL round-bottomed flask and stirred magnetically at 40 °C for 5 days. The crude reaction mixture was filtered over Celite, and the solvent was removed under reduced pressure. Yield: 2.1 g (5.1 mmol, 79.7%).

^{1}H NMR ($CDCl_3$) δ: 8.01 (m, 2H); 7.10 (m, 2H); 2.31 (m, 2H); 1.42 (m, 2H); 0.31 (s, 6H). FTIR: activated ester band at 1749 cm^{-1}.

Surface Coating and Modification. The PMSSQ-PFPA solution was spin-coated onto clean substrates (15 s, 4000 rpm). To induce the secondary cross-linking of the inorganic block, the samples were annealed at 130 °C for 2 h and afterward washed with THF for 30 min to remove any nonbonded material. To functionalize the surface afterward via a polymer analogous reaction, the samples were placed in a solution of the desired amine in water (10 wt %) at room temperature for 1 h. To remove the excess of the amine, the surface was washed several times with water.

Comparison Surfaces/Self-Assembled Monolayers on Glass. As comparison surfaces, self-assembled monolayers on glass were prepared using either perfluorophenyl 4-(2-(chlorodimethylsilyl)ethyl)benzoate (comparison 1) or 3-aminopropyltriethoxysilane (APTES) (comparison 2). The monolayer preparation was carried out as described in the literature.[62,63] The freshly cleaned glass substrate was immersed in a 10 mM solution of the adsorbate in dry toluene for 4 h. After the substrate was removed from the solution, it was rinsed with toluene (three times), dichloromethane (twice), ethanol (twice), and water (twice) to remove any physisorbed material. The advancing contact angle for comparison 1 was 92°, while for comparison 2 an advancing contact angle of 38° was measured.

Results and Discussion

Poly(methylsilsesquioxane)-poly(pentafluorophenyl acrylate) (PMSSQ-PFPA) hybrid polymers represent a promising class of coating materials, which offer easy access toward adherent reactive surface coatings onto a broad range of different substrates (e.g., Si, glass, gold, PMMA, PDMS, PC, etc.) (see Scheme 1). The quality of adhesion between a film and its substrate depends to a large extent on the microstructure of the interface layer that is being formed.[64] Usually, four types of interface layers can be formed during the coating process of polymers onto different substrates: (1) mechanically interlocked interfaces; (2) chemical bonding interface layers; (3) electric double layers; and (4) diffusion interface layers.[57,58] The PMSSQ hybrid polymers are able to utilize different interface phenomena to guarantee adhesion on a substrate. On hydroxylated surfaces, for example, glass or silicon, a chemical bonding interface layer is formed by condensation of surface OH groups with PMSSQ-OH groups. On polymeric surfaces, for example, PDMS, PC, and PMMA, diffusion interfaces are involved in the adherence. Gold-covered glass slides or poly(tetrafluoroethylene) (PTFE) surfaces exhibit a micro- to nanometer scale roughness on the surface, and due to cross-linking of the PMSSQ parts very rigid regions

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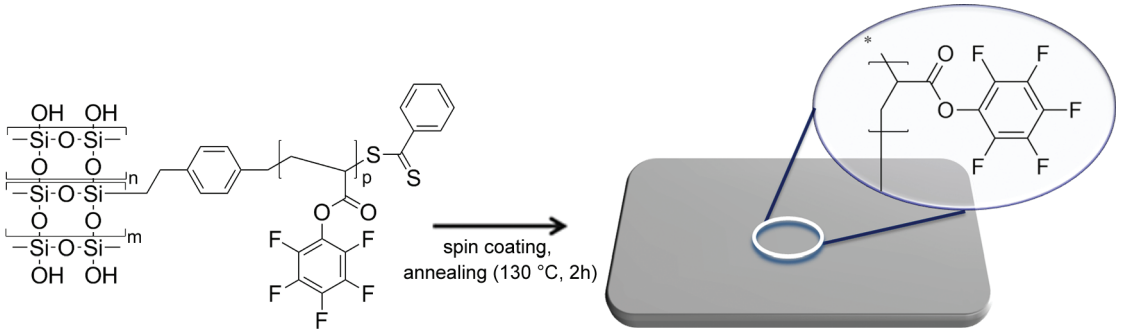
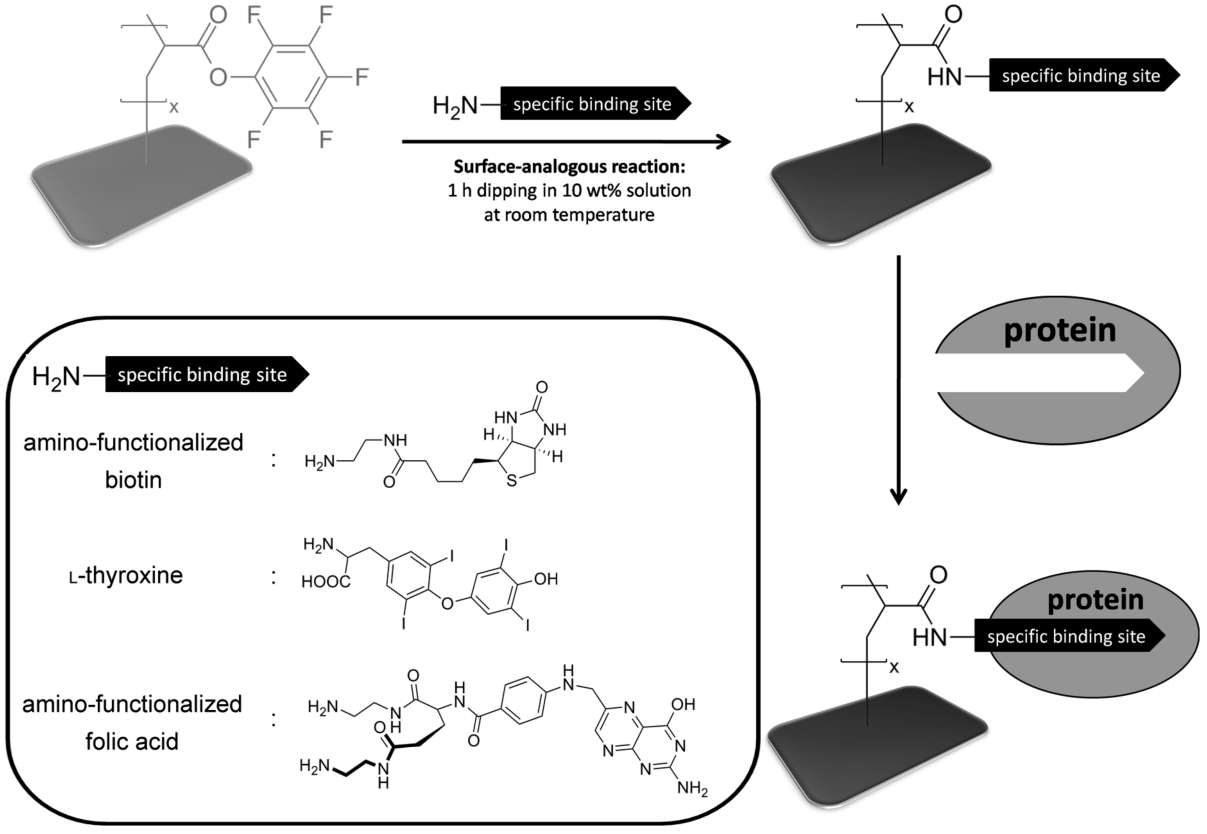
Scheme 1. Reactive Surface Coatings on Several Substrates Were Produced by Spin-Coating PMSSQ-PFPA from Solution and Annealing at 130 °C



Scheme 2. Spin-Coating PMSSQ-PFPA Hybrid Polymers onto a Given Substrates Resulted in Thin Reactive Surface Coatings; after Surface-Analogous Reaction with Amino-Functionalized Specific Binding Sites, Proteins Were Assembled on Top



were formed within the coated film, leading to a mechanical interlocking.

By spin-coating onto a given substrate, annealing at 130 °C for 2 h, and conversion by a wet chemical surface-analogous functional step with various amines, surface properties can be controlled precisely, as explained in detail in ref 59.

In the present study, we want to apply PMSSQ-PFPA surface coatings as a powerful tool to attach various proteins to a given planar substrate. The presented protein immobilization strategy consists of four steps: (a) spin-coating of PMSSQ-PFPA hybrid polymer from solution, (b) annealing at 130 °C for 2 h to induce thermal cross-linking of the PMSSQ part, (c) surface-analogous reaction with different amino-functionalized molecules that provide specific binding sites for proteins, and (d) controlled assembly of proteins on the surface (see Scheme 2). For real-time analysis of biosepecific interactions, especially for the controlled assembly of proteins, we used SPR. Different binding sites were immobilized to assemble different proteins on the surface, demonstrating that PMSSQ-PFPA surface coatings are useful tools for a versatile protein-immobilization strategy.

Biotinylated Surfaces. As a first example, we choose biotin as specific binding site and streptavidin as protein to immobilize on the surface. Streptavidin is a basic tertrameric protein isolated from *Streptomyces avidinii* with a theoretical diameter of 5.6 nm. It binds tightly to biotin, with a binding constant of $K_d \approx 10^{-15}$ M.[65] Due to the ability of streptavidin to bind to four biotins, a streptavidin covered surface can be used as immobilization support for further biomolecular functionalization, using, for example, biotinylated DNA,[60] biotinylated enzymes,[66] or biotinylated antibodies.[67]

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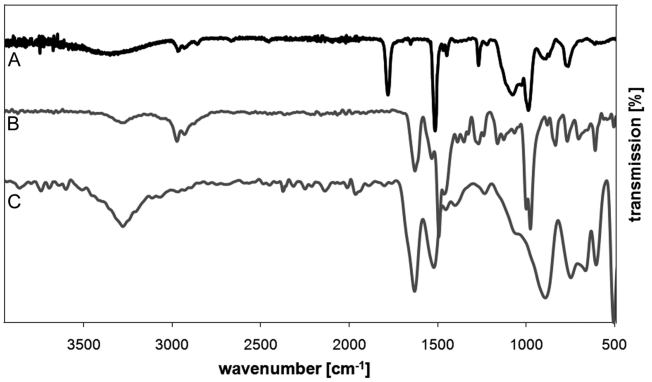

Figure 1. (A) FTIR spectrum of PMSSQ-PFPA coated on glass slides (active ester band at 1772 cm^{-1}). (B) FTIR spectrum of PMSSQ-PFPA coating on glass after conversion in 10 wt % H_2N-biotin solution in water for 1 h (amide bands at 1638and 1530 cm^{-1}). (C) FTIR spectrum of streptavidin in conjugation with the biotinylated surface coating of (B) (additional amine bands at 3300 cm^{-1}, additional amide bands at 1635 and 1530 cm^{-1} from the polypeptide).

Amino-functionalized biotin was synthesized by activation of the carboxyl group as a pentafluorophenyl ester using pentafluorophenyl trifluoroacetate, followed by reaction with an excess of ethylene diamine. The PMSSQ-PFPA active ester surface coating on top of a gold-covered glass slide was obtained by spin-coating from 0.04 wt % solution of PMSSQ-PFPA in THF and annealing at 130 °C for 2 h (film thickness, 3.8 nm; SPR scans fitted by Winspall). The surface-analogous reaction of amino-functionalized biotin (H_2N-biotin) with the pentafluorophenyl esters at the surface was carried out by dipping the coated substrate into a 10 wt % solution of H_2N-biotin in water for 60 min (see Scheme 2). To determine the conversion of the film, similar experiments were performed after spin-coating PMSSQ-PFPA from a 10 wt % solution on to a glass slide, yielding a comparably thicker film, which could be analyzed by Fourier transform infrared (FTIR) spectroscopy (see Figure 1). Prior to conversion, the band at 1772 cm^{-1} indicated the presence of the activated ester group (Figure 1A), while after conversion with H_2N-biotin the ester band vanished and the typical amide bands at 1638 and 1530 cm^{-1} were found (Figure 1B). The covalent attachment of H_2N-biotin onto the thin reactive coating could be monitored using SPR. PMSSQ-PFPA coated gold-covered glass slides were allowed to react in situ with a solution of 0.1 mg/mL H_2N-biotin in water. The measured SPR kinetic is shown in the Supporting Information Figure S2. A thickness increase of 0.5 nm was calculated for the binding of H_2N-biotin onto the reactive coating (fitting using Winspall, assuming constant refractive indices).

After successful attachment of the specific binding site, streptavidin was allowed to adsorb onto the biotinylated coating. A solution of 0.1 mg/mL streptavidin in phosphate-buffered saline solution (PBS) was injected into the SPR setup. The measured SPR kinetic is shown in Figure 2A. The adsorption was completed in less than 20 min. After complete adsorption, the SPR cell was rinsed with PBS several times to remove any nonspecifically bound protein. The thickness increase was fitted from SPR scans before and after protein injection/PBS rinsing and calculated as 5.2 nm (SPR scans in the Supporting Information, Figure S3). This matches with the theoretical diameter of streptavidin of 5.6 nm, indicating the formation of a streptavidin monolayer on top of the biotinylated PMSSQ-PFPA coating.

As the coating procedure is independent of the underlying substrate, different characterization techniques (even if requiring different underlying materials) could be applied. Thus, we also determined the thicknesses by ellipsometry, using exactly the same coating procedure. The obtained thicknesses were 4.1 nm for the biotinylated coating and 9.0 nm in total after streptavidin assembly. Thus, the streptavidin layer thickness of 4.9 nm, determined by ellipsometry, matches very well the thickness of 5.2 nm, obtained from SPR scans, proving the general applicability of PMSSQ-PFPA coatings.

To compare the desired specific adsorption of streptavidin on the biotinylated surface with complete nonspecific physisorption of streptavidin on hydrophobic or hydrophilic surfaces, similar adsorption experiments were performed on other surfaces. PMSSQ-PFPA coatings on gold-covered glass slides were functionalized with octylamine, yielding a hydrophobic surface, and amino-functionalized poly(ethylene glycol) (H_2N-PEG, $M_n = 550$ g/mol), yielding a hydrophilic surface. The SPR kinetics of the streptavidin adsorption on both surfaces in comparison to the biotinylated surface is shown in the Supporting Information, Figure S4. As expected, after rinsing, no physisorption could be detected on the PEGylated surface, whereas physisorption was prominent on the octylamine-functionalized coating. The maximum thickness increase was 0.65 nm, and after PBS rinsing 0.1 nm thickness remained, indicating that the physisorption on hydrophobic surfaces is only due to the hydrophobic effect of proteins (nonspecific denaturation on the surface) and therefore differs dramatically from the controlled specific adsorption on biotinylated surfaces.

Further characterization of the obtained streptavidin surface was performed by AFM. As the PMSSQ-PFPA coating is independent of the nature of the substrate,[54,55,59] silicon wafers were used for AFM investigations. The same streptavidin immobilization strategy, as explained above, was used. After applying the PMSSQ-PFPA coating, surface-analogous reaction with H_2N-biotin was performed and streptavidin was allowed to assemble from solution. The obtained AFM topography image is shown in Figure 2B. A smooth streptavidin surface was observed, and the root-mean-square (rms) roughness was 0.834 nm on an area of 1 μm^2, indicating again a monolayer formation of streptavidin on top of the coating. These smooth streptavidin layers, obtained in a simple combination of spin-coating and self-assembly, may represent an ideal platform for further biosensor application using biotinylated biomolecules.

The conjugation of streptavidin onto the biotinylated coating on glass could also be identified by surface FTIR spectroscopy (see Figure 1C). Additional amine bands at 3300 cm^{-1} and peptide bands at 1635 and 1530 cm^{-1} appeared. Further, FTIR characterization of the immobilization strategy was also applied on PC and is shown in the Supporting Information Figure S5.

To compare the streptavidin binding of the presented surface-biotinylation method with other common glass-biotinylation protocols, we prepared two comparison samples. Well established glass derivatization methods use self-assembled monolayers[68,69] of either amino-functionalized alkoxysilanes (usually APTES)[70] or chlorosilanes with activated moieties.[71,72] Comparison sample 1 was produced by formation of a SAM of perfluorophenyl

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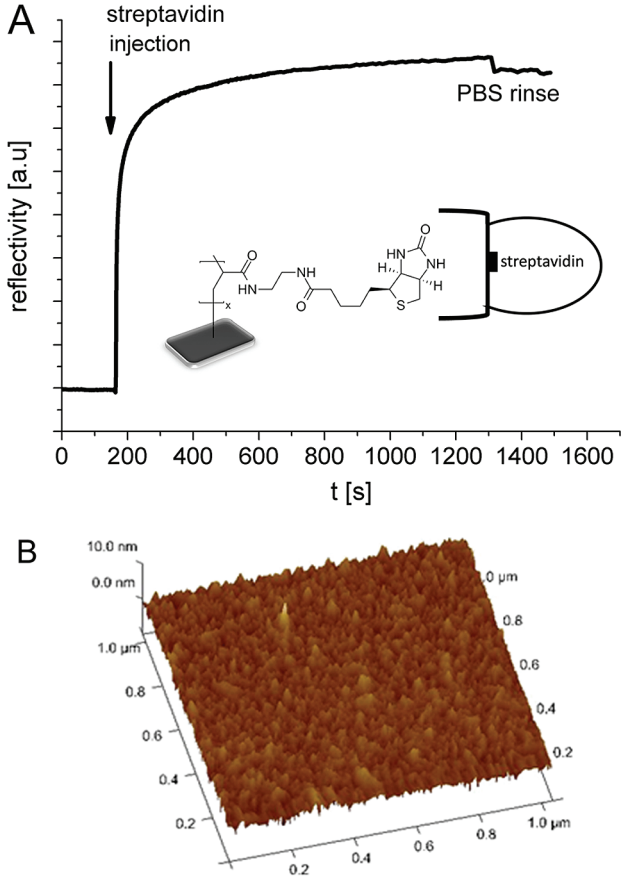

Figure 2. (A) SPR kinetic of streptavidin adsorption on a PMSSQ-PFPA coated gold-covered glass slide converted with H_2N-biotin. Calculated thickness increase after several PBS rinsing steps was 5.23 nm. (B) AFM height image after streptavidin adsorption on the biotinylated reactive coating on a silicon wafer (image rms: 0.834).

4-(2-(chlorodimethylsilyl)ethyl)benzoate on a glass slide, and afterward the activated ester was functionalized with amino-biotin, yielding the desired monolayer of biotin moieties on the glass slide. Comparison sample 2 was produced by SAM formation of APTES onto glass, followed by attachment of biotin moieties using biotin pentafluorophenyl ester.

Comparison samples 1 and 2 and a PMSSQ-PFPA coated glass slide, functionalized with amino-biotin as explained above, were incubated in a solution of fluorescent-labeled streptavidin (streptavidin-CY3, 0.1 mg protein/mL in PBS) for 1 h. Fluorescence emission spectra of all three samples were measured and are shown in Figure 3. All three biotinylation procedures showed comparable results, as nearly the same fluorescence intensity was observed for all three cases, indicating similar streptavidin conjugation to the biotin-functionalized surfaces with comparable binding capacities on the interface. Further streptavidin binding experiments were performed using Streptavidin Fluorescent Polymer, Ultrasensitive. The comparison samples 1 and 2 (glass substrate) and PMSSQ-PFPA coated substrates, functionalized with biotin-amine (Si, glass, PDMS, PC, PMMA, PTFE), were incubated in a PBS/BSA solution containing 0.005 mg protein/mL. The fluorescent emission spectra showed similar emission independent from the functionalization method (see the Supporting Information, Figure S6). In case of the PMSSQ-PFPA coated substrates, functionalized with amino-biotin, almost no effect of the underlying material was observed, proving again the versatile applicability of the PMSSQ-PFPA coating, independent from the

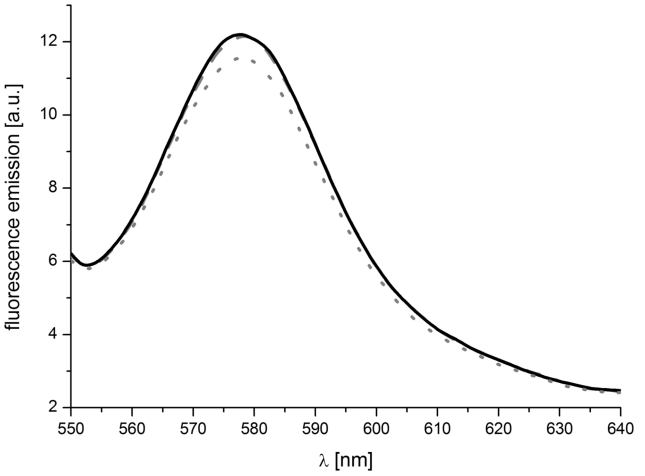

Figure 3. Fluorescence emission spectra of streptavidin-CY3 immobilized on different glass surfaces (excitation at 520 nm; slits, 15 nm). Black solid line: Streptavidin-CY3 immobilized on a glass slide coated with PMSSQ-PFPA after conversion with biotin-amine. Gray dashed line: Comparison 1, SAM of perfluorophenyl 4-(2-(chlorodimethylsilyl)ethyl)benzoate functionalized with biotin-amine in conjugation with streptavidin-CY3. Gray dotted line: Comparison 2, SAM of APTES on glass, functionalized with biotin pentafluorophenyl ester after assembly of streptavidin-CY3.

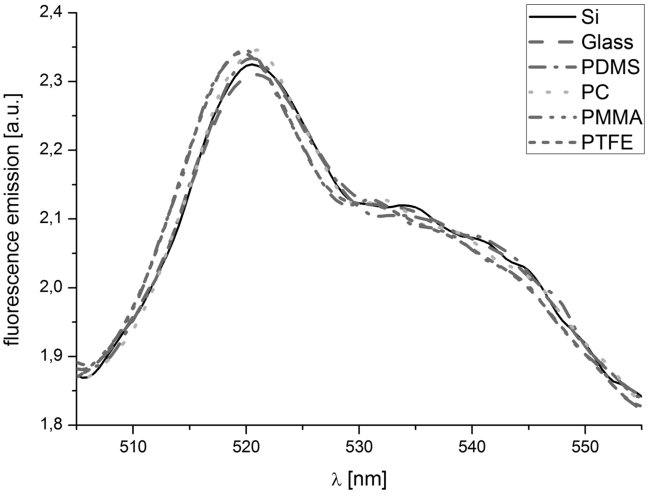

Figure 4. Fluorescence emission spectra of biotin-5-fluorescein conjugate bounded to streptavidin, immobilized on various underlying substrates using the presented reactive coating concept (excitation at 440 nm; slits, 15 nm).

material. Compared to the complicated procedure to obtain defined functional monolayers on glass, PMSSQ-PFPA coatings, produced by spin-coating, showed similar ability to immobilize proteins on the surface.

To demonstrate that the remaining binding sites of streptavidin, immobilized by the presented strategy, are available for further binding of biotin-labeled molecules, we allowed biotin-5-fluorescein to assemble at the free binding sites. Different streptavidin-functionalized substrates were placed into a solution of 0.5 mg biotin-5-fluorescein/mL PBS for 1 h. The measured fluorescent emission spectra are shown in Figure 4. The typical fluorescein emission spectra could be observed on any underlying substrate with again nearly the same intensity, proving the capability of further biotin conjugation to any streptavidin-functionalized surface with an equal number of binding site per surface area.

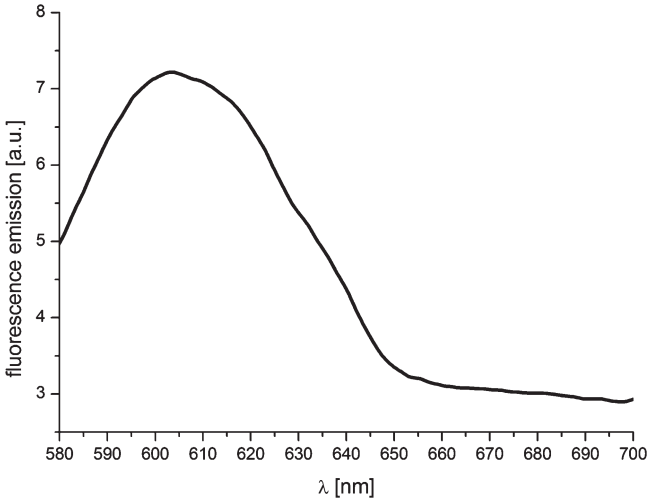

Figure 5. Fluorescence emission spectrum of albumin blue 580 (excitation at 550 nm; slits, 15 nm). PMSSQ-PFPA was coated onto a PC substrate, converted with biotin-amine, streptavidin was immobilized on top, and biotin-functionalized albumin was allowed to assemble. Afterward, the wafer was placed in a solution of albumin blue 580 in PBS.

Besides the binding of small biotin-labeled molecules, also biotin-labeled proteins could be attached to the streptavidin-functionalized surface. Streptavidin was immobilized on PC using the presented strategy. Afterward, the sample was incubated in a solution of 0.1 mg/mL biotin-labeled albumin in PBS. After several rinsing steps, the immobilized albumin was detected by adsorption of albumin blue 580, a specific fluorescent dye to determine albumin (functionalization of the well rinsed wafer in 0.5 mg/mL solution in PBS, containing isopropyl alcohol).[73,74] The fluorescence emission spectrum is shown in Figure 5 (emission max at 600 nm). A similar experiment was performed using nonfunctionalized albumin. No fluorescence could be detected using the same excitation (excitation wavelength 550 nm), indicating that the attachment is due to the streptavidin−biotin interaction between the streptavidin-functionalized surface and the biotin-labeled albumin.

To demonstrate the specific binding of the presented biotinylation process, biotin-functionalized surface coatings were also incubated in a solution of biotin-labeled albumin and pure BSA, respectively. After incubation in the albumin blue 580 solution and rinsing with PBS, no fluorescence could be observed in both cases. Further binding experiments on the biotinylated surface coating were performed by SPR. Neither albumin nor pre-albumin showed a binding to the biotin-functionalized gold surface, indicating specific binding of streptavidin to the PMSSQ-PFPA coating after biotin-amine functionalization (SPR kinetics shown in the Supporting Information, Figure S7).

Thyroxine-Functionalized Surfaces. Thyroid hormones, such as L-thyroxine (T4), are essential for the modulation of the cellular metabolic rate and for the development and differentiation of several tissues.[75,76] The thyroid gland produces and releases T4. Once T4 enters the bloodstream, it is bound to transport proteins (thyroxine binding globulin (TBG), transthyretin (TTR), thyroxine binding pre-albumin (PA)).[77] Due to the interaction of the T4 transport system with polyhalogenated aromatic hydrocarbons (PHAHs), which are used as flame retardants,[78,79] the demand of biosensors that are selective for T4 transport proteins increased recently.[80]

As T4 consists of an amino acid, well separated from the halogenated aromatic part, an amino group for attachment to the PMSSQ-PFPA surface is already present. Thus, after surface-analogous reaction of T4 with the reactive polymer coating on a gold substrate (dipping process as explained above, adding 1 equiv of triethylamine to deprotonate the carboxylic acid group), pre-albumin (PA, 0.1 mg/mL) was allowed to adsorb onto the surface in situ. The obtained SPR kinetic is shown in Figure 6A. After PBS rinsing, a thickness increase of 2.55 nm was found. Complete adsorption took approximately 2 h, indicating that T4−PA binding is less effective than biotin−streptavidin binding, as expected. Again, the same PA-binding strategy was applied to silicon wafers to perform AFM measurements. A smooth and homogeneous PA surface could be observed in AFM topography (rms of 0.492 nm on 1 μm^2, Figure 6B).

To compare the obtained SPR kinetic results of the presented PMSSQ-PFPA protein immobilization strategy to the widely used disulfide-attachment[81] of biomolecular functions to gold surfaces,[82] we prepared bis(5-carboxypentyl) disulfide dithyroxine amide (T4-disulfide). The SAM formation of T4-disulfide could directly be performed in the SPR setup, using 0.1 mg/mL T4-disulfide in ethanol. After ethanol rinsing, a thickness increase of 1.66 nm was observed. Allowing PA to adsorb on top increased the thickness by 2.60 nm (assuming constant refractive index) (see Supporting Information, Figure S8). Both protein immobilization strategies provide concurrent results of thickness increase and adsorption kinetics.

The determination of the affinity of T4 transport proteins toward other polyhalogenated aromatic hydrocarbons (PHAHs) is an important issue, as explained above. To demonstrate that the presented strategy is applicable as a biosensor, comparing binding affinities between PHAHs-PA versus T4-PA, we chose pentabromophenol as the PHAH example. A PMSSQ-PFPA coated gold-covered glass slide was functionalized with T4 as explained above. We prepared a solution of 0.1 mg/mL PA in PBS and added 0.5 mg of pentabromophenol, and the solution was allowed to stand for 60 min. Afterward, PA adsorption onto the prepared T4 surface was measured by SPR. The obtained kinetic differed dramatically from the adsorption kinetic of PA without pentabromophenol (Figure 6A). No specific binding of PA onto the T4 surface could be detected anymore, which can be explained by an effective blocking of the PA binding motive by pentabromophenol. Similar experiments were carried out with other phenols using identical concentrations and setup. Addition of phenol, 4-fluorophenol or bisphenol A showed no effect on the PA adsorption, whereas addition of pentachlorophenol eliminated specific adsorption similar to pentabromophenol, which is in agreement with other T4-binding studies.[80,83−85]

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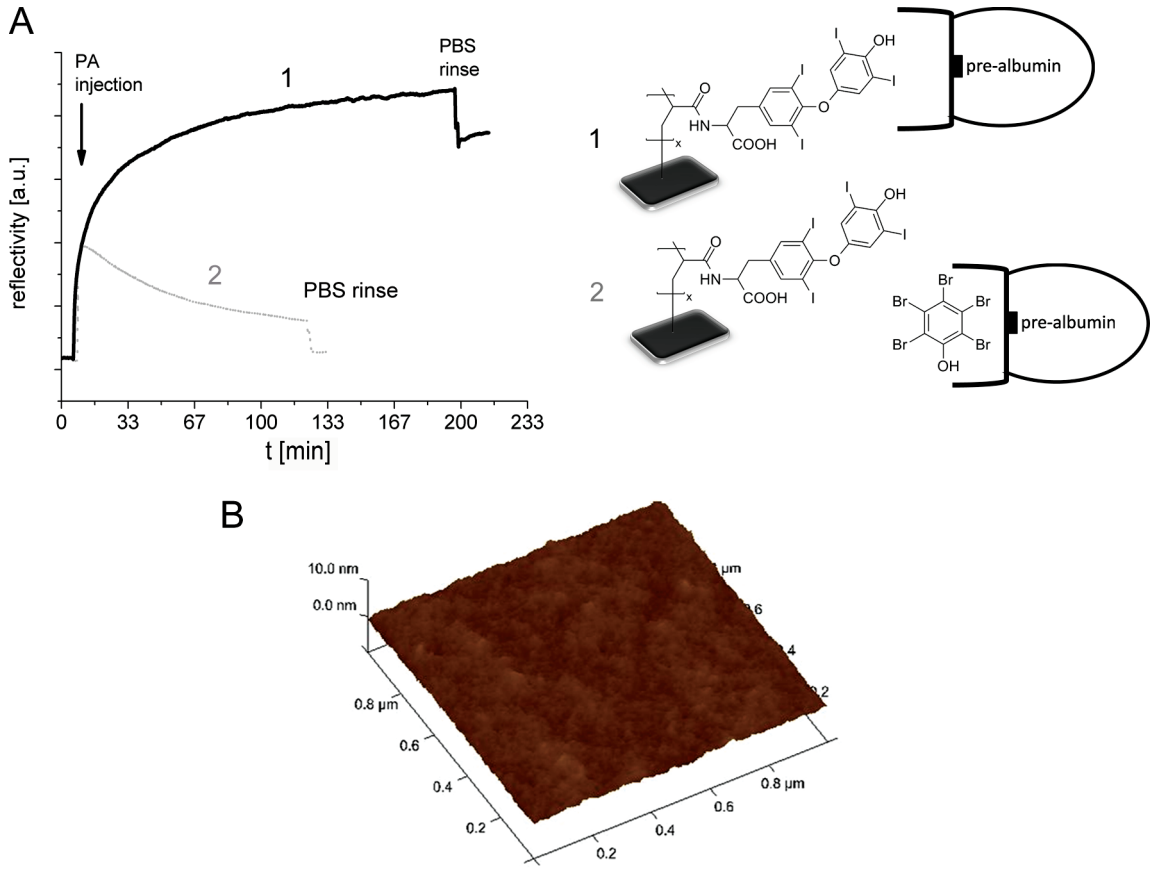

Figure 6. (A) After surface-analogous reaction of L-thyroxine (T4) with the reactive coating pre-albumin was adsorbed (SPR kinetic 1). After several PBS rinsing steps, the thickness increase was 2.55 nm. SPR kinetic 2: Pre-albumin (PA) adsorption on the L-thyroxine (T4)-functionalized PMSSQ-based surface coating after adding pentabromophenol. (B) Pre-albumin adsorbed onto a similarly prepared silicon wafer. AFM topography shows a smooth homogeneous surface with image rms of 0.492 nm.

Thus, T4-functionalized PMSSQ-PFPA coatings offer a convenient method for further PHAH-PA interaction screenings.

As the T4-functionalized PMSSQ-PFPA coating should be specific for PA adsorption (or other T4 transport proteins), other proteins should not show a stable assembly on this surface. Using the SPR setup, we allowed streptavidin and albumin to assemble on the surface from PBS solution. Both proteins showed no specific binding and were completely removed by PBS rinsing steps (see the Supporting Information, Figure S9). Furthermore, fluorescence emission spectroscopy was performed after incubating a T4-functionalized PC wafer in albumin solution, rinsing several times with PBS, incubating in albumin blue 580 solution, and rinsing again. No fluorescence could be detected (excitation 550 nm).

Folic-Acid-Functionalized Surfaces. Folic acid is a water-soluble vitamin of the B-complex group. It has emerged as an optimal targeting ligand for selective delivery of attached imaging and therapeutical agents to cancer tissues and sites of inflammation. Mammals are incapable of synthesizing folic acid and must obtain it from their dietary intake. Cells use folic acid in the synthesis of the DNA bases thymine and the purines.[86] The cell internalizes folic acid with the help of folate receptor proteins on the cell surface. To study binding effects between folic acid and folate receptor proteins, specific biosensors have been designed, for example, folic acid was immobilized on cantilever sensors, expressing the pterine end toward folate receptors.[87] Besides specific binding from folic acid to folate receptors on cell surfaces, folic acid is also known to bind to folate-binding proteins from bovine milk (FBP).[88–90] Folic-acid-functionalized biosensors were also used to study the interaction between folic acid and FBP, to determine binding affinity between folic acid and its receptors.[91–93]

To attach folic acid as specific binding site to a PMSSQ-PFPA coating, the carboxylic acid functionalities of the glutamic acid moiety of folic acid were modified with ethylene diamine, similar to the biotin functionalization explained above (the aromatic amino group present is not capable to substitute the pentafluorophenol moiety, due to less nucleophilicity). Like in other folic acid biosensors, mentioned above, the pterine end is expressed at the interface. FBP is adsorbed from 0.1 mg/mL PBS solution. The adsorption process was monitored by SPR, and the kinetic is shown in Figure 7A. Complete adsorption took less than 30 min, indicating a strong interaction between the pterine end as FBP. The thickness increase was determined from SPR scans to be 1.1 nm (assuming constant refractive index). AFM topography showed a smooth protein layer with a rms value (1 μm^2) of 0.624 nm, indicating a defect-free homogeneous immobilization of FBP on the pterine expressing surface (Figure 7B).

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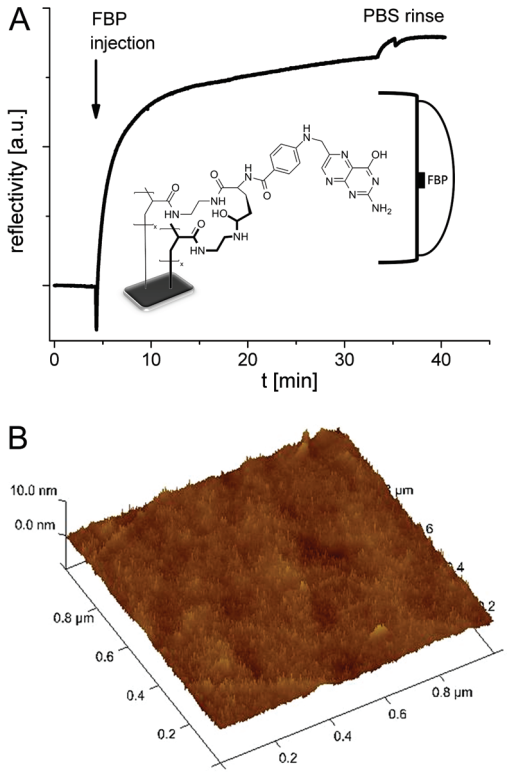

Figure 7. (A) After conversion of the reactive coating, either on gold or on silicon surfaces, with amino-functionalized folic acid, folate-binding protein from bovine milk was adsorbed (thickness increase 1.1 nm). (B) AFM topography showed a homogeneous protein-functionalized surface (image rms 0.624 nm).

To demonstrate specific binding between the pterine moiety and FBP, we performed similar adsorption experiments using albumin and streptavidin. Both did not assemble specifically on to the surface and could be rinsed off using PBS solution (see the Supporting Information, Figure S10).

Our approach to functionalize surfaces with different binding motives may be helpful to determine further protein-binding characteristics of FBP and its influence on the protein immobilization mechanism in the future.

Conclusions

Poly(methylsilsesquioxane)-poly(pentafluorophenyl acrylate) hybrid materials could successfully be used to create very thin reactive surface coatings on gold-covered glass slides, capable as an immobilization platform for SPR investigations. The strategy to assemble proteins on top contains a simple surface-analogous conversion step to bind specific binding sites on the surface. Every specific binding site, which carries an aliphatic amine or which can be functionalized with an aliphatic amino group, can be reacted with the thin active ester coating. Using this approach, biotin, L-thyroxine, and folic acid could successfully be linked to planar surfaces. Streptavidin, pre-albumin, and folate-binding protein could be immobilized, respectively. The assembly process could be monitored by SPR in situ.

The variety of attachable specific biolinkers is one main feature of the described PMSSQ-PFPA thin reactive coatings. Another feature is the substrate-independent adherence to the underlying substrate. Thus, the presented protein-immobilization protocol could also be used to functionalize Si, PC, PMMA, PDMS, and PTFE with proteins. The successful protein conjugation could be characterized using AFM, FTIR, fluorescence spectroscopy, and ellipsometry. This simple method to obtain smooth homogeneous protein layers may be useful for further biosensor applications, especially for streptavidin layers allowing further binding of biotinylated molecules.

In comparison to mostly complex protein immobilization strategies, our generally applicable functionalization method of substrate-independent reactive polymer coatings allows a specific attachment of proteins that may find versatile applications in different biosensor setups.

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Supporting Information Available: AFM topographies of PMSSQ-PFPA coatings on Au, PC, PDMS, and PMMA, SPR kinetic of H_2N-biotin attachment on the reactive coating, SPR scans of the streptavidin adsorption, SPR kinetic of streptavidin adsorption on different surfaces, FTIR spectrum of streptavidin immobilized on PC, fluorescence spectra of streptavidin fluorescent polymer on various substrates, SPR kinetics of albumin and pre-albumin on a biotinylated surface, SPR kinetic of T4-disulfide SAM formation and further pre-albumin adsorption, SPR kinetics of streptavidin and albumin on a T4-functionalized surface, and SPR kinetics of albumin and streptavidin on a folic-acid-functionalized surface. This material is available free of charge via the Internet at http://pubs.acs.org.