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Biofunctionalized, Ultrathin Coatings of Cross-Linked Star-Shaped Poly(ethylene oxide) Allow Reversible Folding of Immobilized Proteins

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Abstract: Dense, ultrathin networks of isocyanate terminated star-shaped poly(ethylene oxide) (PEO) molecules, cross-linked at their chain ends via urea groups, were shown to be extremely resistant to unspecific adsorption of proteins while at the same time suitable for easy biocompatible modification. Application by spin coating offers a simple procedure for the preparation of minimally interacting surfaces that are functionalized by suitable linker groups to immobilize proteins in their native conformations. These coatings form a versatile basis for biofunctional and biomimetic surfaces. We have demonstrated their advantageous properties by using single-molecule fluorescence microscopy to study immobilized proteins under destabilizing conditions. Biotinylated ribonuclease H (RNase H) was labeled with a fluorescence resonance energy transfer (FRET) pair of fluorescent dyes and attached to the surface by a biotin-streptavidin linkage. FRET analysis demonstrated completely reversible denaturation/renaturation behavior upon exposure of the surface-immobilized proteins to 6 M guanidinium chloride (GdmCl) followed by washing in buffer. A comparison with bovine serum albumin (BSA) coated surfaces and linear PEO brush surfaces yielded superior performance in terms of chemical stability, inertness and noninteracting nature of the star-polymer derived films.

Introduction

Chemically designed surface coatings that can prevent unspecific protein adsorption are essential for various biotechnological applications. Besides impeding biofouling, e.g., in membrane applications, nonadherent surface properties present a key condition for single molecule studies with immobilized proteins, for protein microarrays, and cell assays. To specifically bind proteins or to interact in a biomimetic way with living cells, surfaces have to be modified toward specific biological recognition,[1−4] whereas at the same time avoiding uncontrolled adsorption that could lead to denaturation of proteins or unwanted activation of biological processes. While protein immobilization itself is easy to accomplish, it is of utmost importance in studies of protein folding and function that the interaction between the protein under study and the surface environment is minimized. Otherwise, the results may not reflect intrinsic properties of the protein, but rather artifacts due to surface interactions. A number of surface preparations have been developed for the purpose of protein resistance, such as self-assembled monolayers on gold,[5−7] glass,[8] silicon,[9] titanium and titanium oxide,[10−13] polyelectrolyte multilayer films[1,14] and

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hydrogels.[15] Poly(ethylene oxide) (PEO) brushes have been especially recognized as biocompatible and resistant to protein adsorption[5,6,8,16−18] due to the hydrophilic but uncharged nature of the polymer. Still, these methods suffer either from insufficient protein repellence, preparation methods that are tedious and difficult to reproduce, or low surface functionality. Here, we focus on a versatile, easily applicable functional surface coating for immobilization of proteins, prepared with a dense, ultrathin network formed from star shaped PEO molecules, linked at their chain ends via urea groups. We address the question as to which extent destabilization of the natural folded conformation can be avoided by the proper choice of surface linkage. Denaturation and renaturation of a protein, RNase H, specifically linked via biotin/streptavidin to ultrathin, cross-linked star polymer layers was studied by attaching a donor−acceptor pair of fluorescent dye molecules at specific locations along the polypeptide chain so that the two dyes are in close proximity in the folded structure and further apart in the unfolded chain. The strong distance dependence of fluorescence resonance energy transfer (FRET) enables direct insights into the folding dynamics of the polypeptide chain.[19−22] Our cross-linked star PEO surfaces showed superior performance compared with surfaces coated with linear PEO chains or prephysisorbed proteins.

Single-Molecule Studies of Protein Folding

To be biologically active, the nascent polypeptide chain folds into a specific three-dimensional structure after biosynthesis. Due to the many possible internal degrees of freedom of the polymer, a large number of microscopic pathways exist that connect the vast number of unfolded conformations with the much smaller ensemble of native, folded conformations.[23] Therefore, protein folding is an inherently heterogeneous process. In recent years, the concept of a funnel-shaped conformational energy landscape has become prevalent, in which the folding polypeptide chain is guided toward the thermodynamic free energy minimum, encountering barriers and experiencing a gradual loss of enthalpy and entropy.[24] Single-molecule studies can provide direct experimental evidence on the folding/unfolding pathways in the complex energy landscape of proteins. In recent years, FRET analysis has been applied in two-color confocal fluorescence studies on proteins diffusing freely in solution.[21,22,25] In this method, the observation time is limited to the time it takes for a molecule to diffuse through the detection volume, which is on the order of milliseconds. To gain information about slower processes, for example in studies of folding intermediates, it is necessary to immobilize the proteins. Various immobilization strategies have been reported for single-molecule spectroscopy, including trapping in porous polymer matrixes,[26,27] unspecific adsorption to surfaces[28,29] as well as specific adsorption via complex coordination of His-tagged proteins,[30,31] by using biotin/(strept-)avidin coupling[32,33] or charge interactions.[34,35] An immobilization technique, yielding minimal interaction with the environment, has been shown to be the trapping of proteins in surface-bound vesicles of ∼100 nm diameter, in which they can freely diffuse within a limited volume.[36,37] In this approach, however, it is not simple to control the solution conditions inside the vesicles in situ. The star polymer layers introduced here offer an easy and more versatile alternative since they are prepared by spin coating a solution of isocyanate terminated, six-arm star polymers from aqueous THF onto amino-functionalized substrates. They were examined for unspecific adsorption of RNase H and were found to be essentially nonadsorbing. In contrast to adsorbed biotinylated BSA films on hydrophilic glass and PEO brushes made with a small fraction (1%) of biotinylated PEO chains, unfolding/refolding of RNase H was completely reversible on the star polymer derived surfaces, and offered high chemical stability.

Experimental Section

Synthesis and Labeling of RNase H. Plasmid pJAL135C containing the gene of single cysteine mutant of RNase H was a generous gift from Prof. S. Kanaya (Osaka University, Osaka, Japan). The protein was overproduced in *Escherichia coli* HB101 and purified as described.[38,39] The RNase H molecules were subsequently labeled with Alexa Fluor 546-NHS (Molecular Probes) and biotin-NHS (Sigma-Aldrich St. Louis, MO). For FRET measurements, a mutant of RNase H was constructed that had cysteine residues at positions 3 and 135, the thiol side chains of which were labeled with the dye FRET pair (Alexa 546/Alexa 647) by multimode coupling.[40]

Preparation of Isocyanate-Terminated Star PEO. Hydroxyl-terminated star polymers with 80% ethylene oxide and 20% propylene oxide as the backbone (number average molecular weight 12 000 g/mol; polydispersity index 1.15) were functionalized through reaction with a 12-fold molar excess of isophorone diisocyanate (IPDI) in a solvent-free process at 50 °C for 5 d.[41] The excess of IPDI was

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removed by short path distillation. Size exclusion chromatography of the product (star PEO) proved that no dimer or trimer formation took place.

Surface Preparation and Specific Immobilization of RNase H. Cleaning, activation and aminofunctionalization of substrates was carried out under cleanroom conditions. Substrates were cleaned through sonication in acetone (Selectipur, Merck, Haar Germany), 18.2 MΩ Millipore water and 2-propanol (Selectipur, Merck) for one minute each. After activation by an oxygen plasma, the substrates were aminofunctionalized under an inert gas atmosphere for 2 h in a solution of 0.2 mL *N*-[3-(trimethoxysilyl)propyl] ethylenediamine (Sigma-Aldrich, 97%) in 50 mL dry toluene. Then the substrates were washed thoroughly and stored under dry toluene until further usage. For spin coating, the substrates were placed on the spin coater, covered by the star polymer solution and then accelerated within 5 s to 2500 rpm for 40 s. The resulting films were stored overnight at ambient atmosphere for cross-linking. For biotinylation, biocytin was dissolved in 9 mL of deionized water. This solution was mixed with the star polymer solution in 1 mL of THF. Films were then prepared as described above.

PEO surfaces were formed following cleaning and activation of glass substrates with a commercial aminosilane, Vectabond (Vector Laboratories, Burlingame, CA) according to the protocol recommended by the manufacturer. 100 mg/mL PEO solutions in 50 mM Na_2CO_3 buffer (pH 8.2) were prepared from mPEG-SPA, MW 5000 (Nektar Therapeutics, Huntsville, AL), or a mixture of Biotin-PEG-NHS, MW 3400 (Nektar Therapeutics) and mPEG-SPA, MW 5000 with 1% PEO-biotin by weight. PEO was reacted with the Vectabond amino-functionalized surface for 1 h in the dark. After completion of the reaction, samples were thoroughly washed with 18.2 MΩ Millipore water.

To form BSA covered surfaces, fluorescent contaminations were removed from untreated glass coverslips by brief exposure to an open flame. The surface was incubated with a 1 mg/mL BSA-biotin (Sigma-Aldrich) solution in 0.1 M sodium phosphate buffer (pH 7.4) for 10 min, then washed with the same buffer and used immediately afterward.

Biotinylated BSA/PEO/star polymer surfaces were exposed to 10 μg/mL streptavidin (Sigma-Aldrich) in 0.1 M sodium phosphate buffer (pH 7.4) for 10 min. Afterward, the surfaces were incubated with ~100 pM RNase H solution in buffer A (20 mM Tris-HCl, 100 mM KCl, 10 mM $MgCl_2$, pH 7.4) for 10 min. Finally, excess protein was washed with buffer A.

Single Molecule Measurements. Single-molecule microscopy was performed by using a homemade laser scanning confocal fluorescence microscope with Ar^+/Kr^+ laser (modified model 164, Spectra Physics, Mountain View, CA) excitation. It is based on an inverted microscope (Axiovert 35, Zeiss, Göttingen, Germany) and has two separate detection channels for measurement of the emission in two spectral channels to enable FRET experiments. The setup is described in detail by Heyes et al.[40]

Results and Discussion

The cleaned and activated substrates were checked by scanning force microscopy (SFM, root-mean-square roughness ≤ 0.2 nm for 1 μm scans) and contact angle (below the detection limit with water). Ellipsometry measurements showed an increase in the SiO_x layer on the silicon of about 0.5 nm due to the activation step. The aminosilane layer exhibited a thickness between 1.1 and 1.5 nm and proved smooth when examined with SFM (root-mean-square roughness 0.6 nm for 1 μm scans).

Spin coating of the star polymers from aqueous THF solutions resulted in smooth, homogeneous films. The isocyanate terminated stars started to react with water already in solution. Some of the isocyanate groups converted into amines, which reacted with isocyanate groups to yield di-, tri-, and higher oligomers of stars (Figure 1). Consequently, during spin coating, oligomers

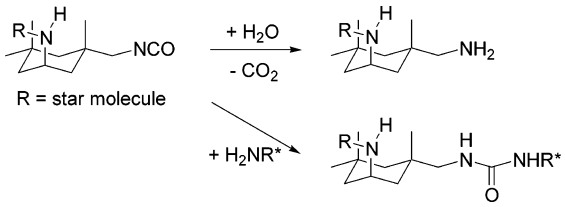

Figure 1. Scheme of the cross-linking reaction of the polymer.

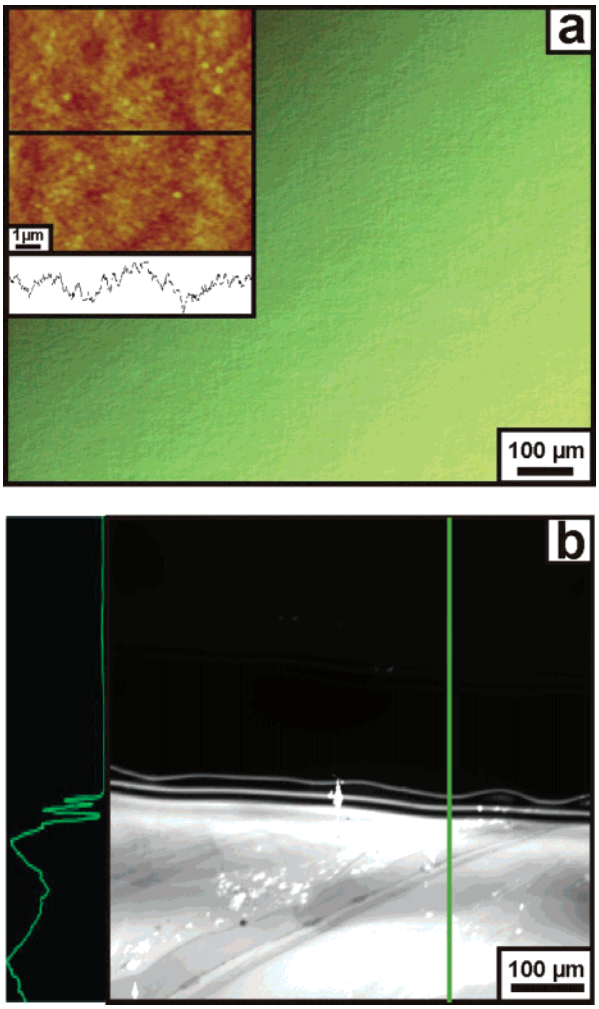

Figure 2. Images of star polymer derived surfaces. (a) optical microscopy (DIC) and scanning force microscopy (height image; profile height is 2 nm) highlight the smoothness of the polymer films. (b) fluorescence microscopy image of a star polymer covered substrate half-dipped into a polystyrene solution. Labeled streptavidin adsorbs unspecifically on polystyrene but not on the star polymer. Fluorescence intensities are ~1000 s^{-1} for the star polymer covered area (equal to background intensity) and >15 000 s^{-1} for the polystyrene covered area.

coexisted with monomers containing amine groups and unmodified monomers. Unreacted isocyanate groups can bind covalently to the amine groups on the aminosilanized substrate surface. Because of partial hydrolysis and cross-linking in solution, the deposited layer then reacts quickly to a highly cross-linked network. With the star polymer concentration chosen for these experiments (1 mg/mL), a film thickness of 5 ± 0.5 nm was measured by ellipsometry on silicon substrates corresponding to at most three monolayers. The contact angle with water was determined by sessile drop measurements (advancing contact angle) as 52° ± 3° (Θ_{adv}) and by captive bubble measurements after storing the samples for 12 h in deionized water (receding

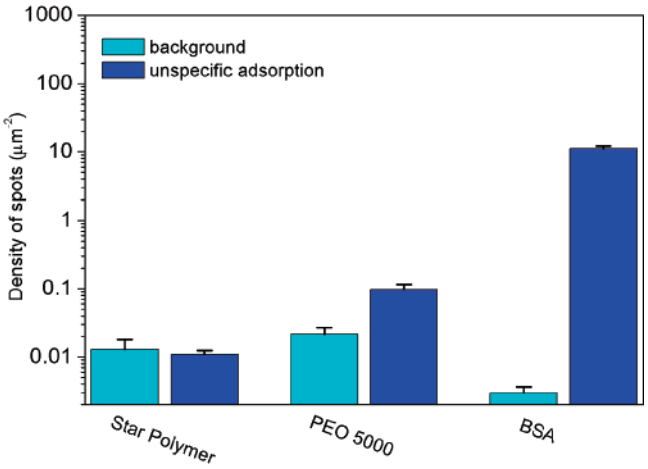

Figure 3. Analysis of the density of fluorescent spots on freshly prepared surfaces and after exposure to RNase H molecules labeled with a single fluorophore. Significantly higher levels of unspecific adsorption were observed on BSA than on PEO surfaces. The star polymer surfaces showed negligible unspecific adsorption.

contact angle) as 45° ± 3° (Θ_{rec}). Figure 2a shows the exquisite smoothness of the film, as examined by optical microscopy and SFM.

To examine the resistance of the star polymer films to unspecific protein adsorption, the star polymer covered substrates were dipped halfway into a polystyrene solution in toluene. Polystyrene surfaces are known for strong unspecific adsorption of proteins.[38] The so-created half polystyrene covered samples were immersed into protein solutions (streptavidin and avidin, labeled with fluorescent dyes) in different buffer systems (pH 5, pH 7.4, and pH 9.5). The proteins adsorbed onto the polystyrene, but not at all onto the star polymer coating (Figure 2b). In a control experiment, unspecific protein adsorption on plain, aminosilanized wafers was shown to be high (not shown). Therefore, prevention of unspecific protein adsorption on the star polymer surfaces was demonstrated from pH 5 to 9.5—the pH range important for functional biomolecules.

Unspecific adsorption was studied at the single-molecule level with unbiotinylated star-polymer surfaces and, for comparison, with physisorbed biotinylated BSA surfaces and with unbiotinylated PEO brush surfaces. All three samples were simultaneously incubated with the same ~15 nM solution of single-dye labeled RNase H in buffer A for 10 min and then thoroughly washed with buffer A. Because streptavidin was absent from the solution, specific binding to the biotinylated BSA was excluded. Control experiments of the surface cleanliness were performed to ensure that the observed fluorescent spots were from labeled RNase H and not from contamination. The amount of unspecific adsorption was calculated from the density of spots (single molecules) on each of the surfaces. Typically, twenty images (18 × 18 μm) of each sample were examined to obtain statistical significance. The data in Figure 3 show that the BSA surfaces were the cleanest in terms of background fluorescence, but they also showed the highest unspecific adsorption. The star polymer derived surfaces and PEO surfaces were slightly more contaminated, but showed markedly lower amounts of unspecifically adsorbed RNase H proteins. In fact, the density of spots did not increase at all from the background density on the star polymer layers, underscoring their highly adsorption-resistant nature.

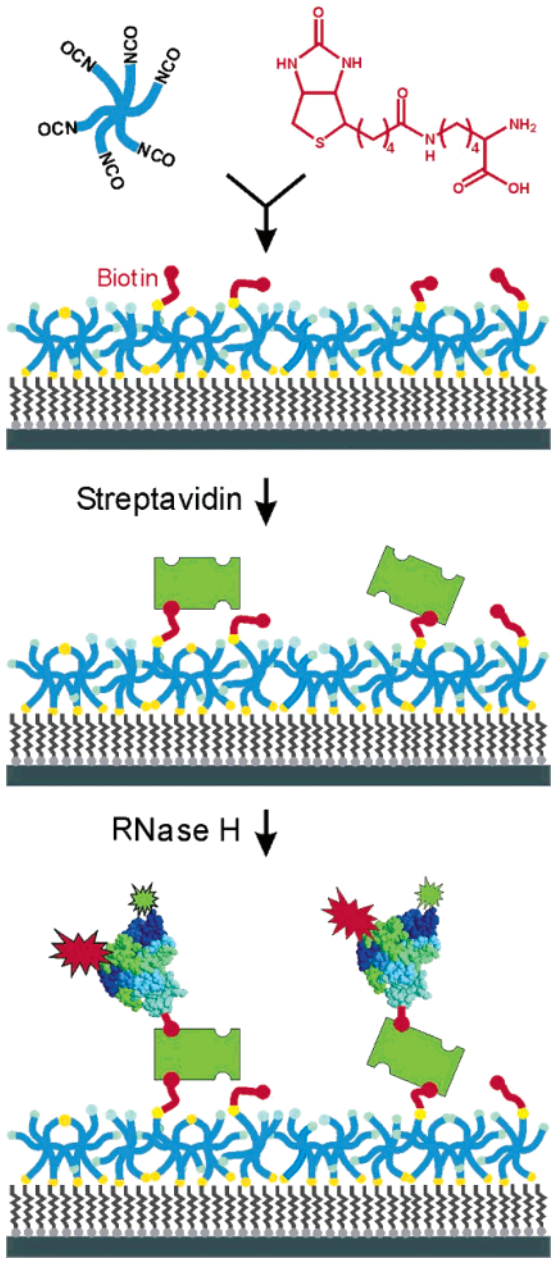

Figure 4. Preparation of biotinylated, star pre-polymer derived layers with subsequent specific binding of streptavidin and RNase H.

The star polymers were further modified with biotin anchors for specific attachment of RNase H molecules to the surface (Figure 4). To this end, biocytin was reacted with the isocyanate-terminated stars just prior to spin coating, yielding surfaces that were statistically decorated with biotin groups. These coatings were subsequently exposed to streptavidin (10 μg/mL in phosphate buffer for 10 min). Streptavidin is tetravalent to biotin, and biotinylated RNase H in buffer A was attached to the surface via the vacant binding sites on the already bound streptavidin.

Denaturation experiments were performed with RNase H molecules immobilized on the star polymer surface. To monitor the conformation of the polypeptide chain, a FRET pair of dye molecules was attached at positions 3 and 135 of the RNase H sequence. These locations were chosen such that of the dye molecules are in close proximity in the native conformation and significantly further apart in the denatured state. The FRET efficiency of each individual molecule can be calculated from

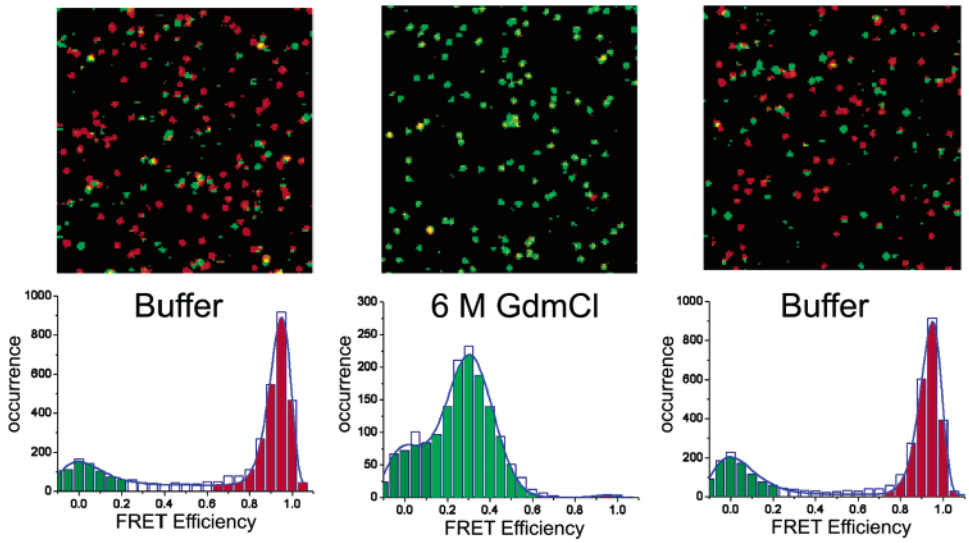

Figure 5. Two-color (red/green) images (top) and histograms of the number of molecules as a function of their FRET efficiencies (bottom) of RNase H bound to star polymer derived surfaces. One complete denaturation/renaturation cycle is shown, starting from the initial preparation in buffer solution (left) via the denatured state in 6 M GdmCl (middle) back to buffer solution (right). The changes in the distributions of FRET efficiencies indicate that the protein molecules unfold (bright green, $E \approx 0.3$) and refold (red, $E \approx 1$) completely reversibly. The peak at zero FRET efficiency (dark green) is due to RNase H molecules without a, or with an already bleached, red dye molecule.

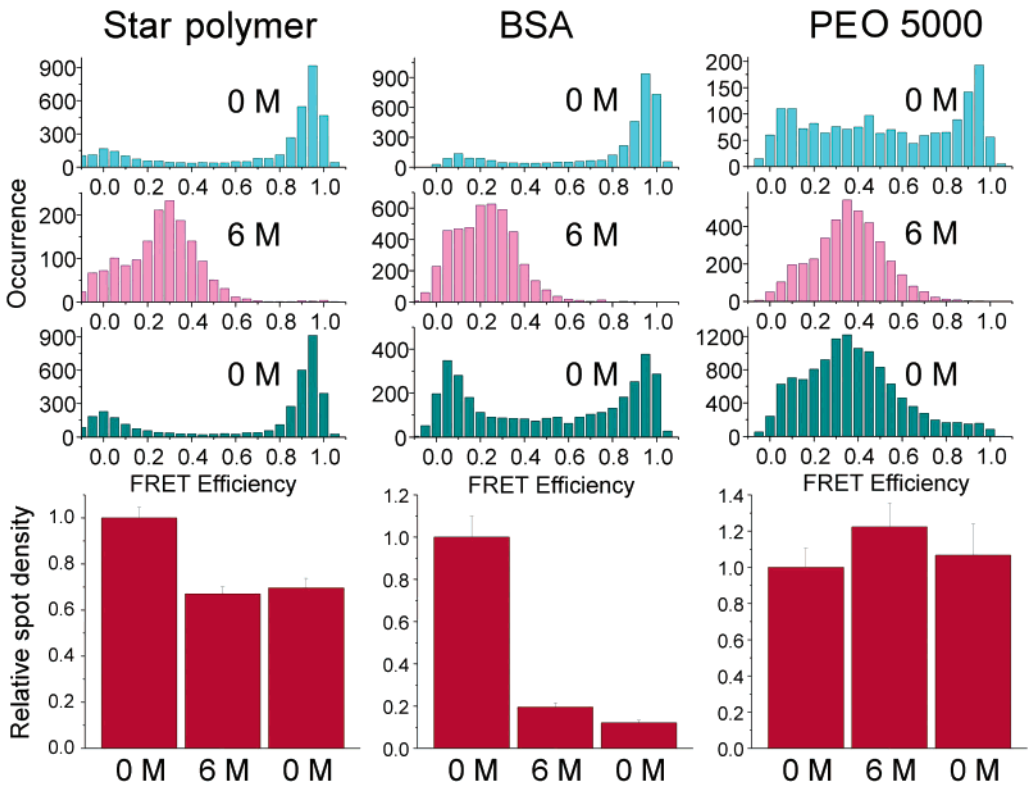

Figure 6. FRET efficiency histograms (top) and relative protein densities on the surface (bottom) during one denaturation/renaturation cycle (0 M, 6 M and finally 0 M GdmCl) of RNase H bound to a star polymer derived surface (left column), adsorbed BSA surface (middle column) and PEO 5000 polymer brush surface (right column). On PEO 5000 brush surfaces, protein denaturation is essentially irreversible. On BSA, labeled RNase H disappears from the surface under denaturing conditions, presumably together with the BSA layer. In contrast, RNase H can be unfolded and refolded completely reversibly on star polymer derived surfaces. Moreover, the spot density on the star polymer surface does not change after treatment with denaturant.

the intensities of photons in the red (acceptor) and green (donor) channels, I_A and I_D, respectively

$$E = \frac{I_A}{I_A + \gamma I_D}$$

The correction factor γ accounts for the difference in detection efficiency between the two channels. The FRET efficiency varies with the inverse sixth power of the dye-to-dye separation and is thus exquisitely sensitive to distance variations due to structural changes of the protein. Denaturation and subsequent renaturation of surface-bound RNase H was performed by variation of the concentration of guanidinium chloride (GdmCl) as a denaturing agent. Figure 5 (top) shows scan images taken

under different solvent conditions with excitation at 514 nm. Each spot represents an individual RNase H molecule. In the initial scan, performed in buffer, the majority of spots emitted photons predominantly into the red spectral channel (sensitive only to emission from the red dye), reflecting a large FRET efficiency ($E \approx 1$), and thus a close proximity of the dyes in the folded molecules. Upon exchange of the buffer with buffer containing 6 M GdmCl, most photons emitted from the spots were detected in the green spectral channel (sensitive to emission from the green dye) because of the lower FRET efficiency ($E \approx 0.3$) arising from the larger (average) dye-to-dye separation in the unfolded state. After re-exchange of the denaturant with buffer A, the spots turned red again, implying that the proteins refolded into the compact, high-FRET conformation. A small population of green spots prior to denaturation and a slightly increased number of green spots after renaturation represented molecules without red acceptor dye, because either it was lacking in the first place or it was photobleached during the experiment. These qualitative results were confirmed by a thorough quantitative analysis of the FRET efficiencies in Figure 5 (bottom), which shows complete refolding on the star polymer surfaces.

Figure 6 shows the comparison of the experiment presented in Figure 5 for the three surfaces examined. Together with the FRET data, we have also plotted the measured spot densities, normalized to unity for the first scan (in buffer). For the star polymer surfaces and the PEO brushes, the spot densities are relatively unaffected by the harsh chemical treatment with 6 M GdmCl. PEO brushes, however, are seen to completely prevent renaturation of the proteins after unfolding. Even in the first scan (in buffer), many molecules show low FRET efficiencies on linear PEO brush surfaces, due to partial or complete unfolding of the protein molecules. Most likely, the extended polypeptide chains interact and intermingle with the long PEO chains so that they cannot refold into their native conformation. Alternatively, bound RNase H may penetrate the flexible PEO brushes and interact with the underlying aminosilane. In any case, this process is apparently prevented by the extensively cross-linked star polymers. On the BSA surfaces, a substantial fraction of RNase H refolds, as seen from the recurrence of the maximum at high FRET efficiencies. Yet, the broad pedestal at intermediate FRET efficiencies suggests that some fraction of the molecules did not refold properly. Moreover, measurement of the spot (protein) density on the surfaces revealed that the protein concentration on BSA surfaces was reduced significantly under 6 M GdmCl. This observation implies that a large fraction of RNase H proteins was removed by treatment with GdmCl, most likely together with the BSA layer physisorbed to the glass.

Conclusions

Ultrathin, smooth layers from isocyanate terminated star polymers on glass substrates were shown to be extremely resistant to unspecific adsorption of proteins while at the same time suitable for easy chemical modification. Application by spin coating offers a simple procedure for the preparation of minimally interacting surfaces that are functionalized by suitable linker groups to immobilize proteins in their active natural conformation. These coatings form a versatile platform for biofunctional and biomimetic surfaces and single-molecule fluorescence microscopy studies on immobilized proteins. In single-molecule denaturation/renaturation experiments with RNase H molecules specifically attached to the star polymers, complete reversibility of this process was observed, implying minimal interaction between the protein and the surface. A comparison with adsorbed BSA and PEO brush surfaces clearly demonstrated the superior quality of the star polymer layers.

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