# Molecular Recognition between Genetically Engineered Streptavidin and Surface-Bound Biotin

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**Abstract:** This study examines the binding of wild-type streptavidin and streptavidin mutants to biotin-terminated self-assembled monolayers (SAMs) as a model of biomolecular recognition at solid−liquid interfaces. The types of streptavidin proteins employed in this work were wild-type, Tyr43Ala (Y43A), and Trp120Ala (W120A), which have biotin-binding affinities that span several orders of magnitude ($K_a$ varies from $\sim 10^{13}$ $M^{-1}$ for wild-type to $10^7$ $M^{-1}$ for W120A). Two types of biotin-terminated monolayers were examined: those formed by chemisorption of 11-mercaptoundecanoic-(8-biotinoylamido-3,6-dioxaoctyl) amide (**1**) and those formed from mixtures of 12-mercaptododecanoic-(8-biotinoylamido-3,6-dioxaoctyl) amide (**2**) and 11-mercapto undecanol (**3**). Our findings support two previously published studies that found that **1** forms monolayers on gold that are disordered, while **2** and mixtures of **2** and **3** form closely packed, well-organized SAMs. The kinetics of binding and desorption of wild-type streptavidin and the mutants to and from these monolayers were measured using surface plasmon resonance spectroscopy. Adsorption of the proteins was found to occur at a diffusion-limited rate and to saturate at different surface coverages depending on their biotin-binding affinity. On disordered monolayers formed from **1**, only a fraction of the bound mutants could be dissociated by exposure to free biotin. The fraction of undissociated mutants correlated with the biotin-binding affinity, suggesting that the formation of nonspecific interactions depends on the residence time of the protein on the surface. On mixed SAMs formed from **2** and **3**, complete dissociation of the proteins occurred upon exposure to free biotin in solution. The kinetics of desorption of streptavidin from mixed SAMs was analyzed using a model that included the possibility of bivalent protein binding to the SAM at high surface concentrations of biotin. It was found that rate constants of dissociation were larger for the dissociation of a streptavidin−biotin bond on the surface than in solution. On biotinylated SAMs, the kinetic constants of dissociation were dependent on the surface concentration of biotin. Slow dissociation rates at higher surface coverage result from attractive protein−protein interactions. The results demonstrate the importance of the preparation and the structure of the solid surface and the complexity of biomolecular recognition at solid−liquid interfaces. Molecular recognition is affected by interactions between the adsorbed proteins and the surface and also by interactions among adsorbed proteins. These conclusions have important implications for the development of reversible biosensors.

## Introduction

Streptavidin is a tetrameric protein which binds biotin with very high affinity ($K_a \sim 10^{13}$ $M^{-1}$) in one of the strongest receptor−ligand interactions found in nature.[1,2] This high binding affinity, the symmetry of the biotin-binding pockets which are positioned in pairs at opposite faces of the protein,[3] and the ease of functionalization of diverse biomolecules (e.g., antibodies, peptides, and nucleotides) with biotin make the streptavidin−biotin system extremely useful in a wide range of biotechnological applications such as in affinity separations,[4−7] in diagnostic assays,[6,7] and for "tagging" of molecules for imaging or delivery of therapeutics.[8−17]

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The creation of biotin-substituted self-assembled monolayers (SAMs) of alkanethiolates on gold has expanded the applications of streptavidin to the study of molecular recognition at the solid−liquid interface and to the development of biosensors.[18−22] A better understanding of how molecular recognition at a solid surface may differ from solution is necessary in order to optimize the immobilization of biomolecules for biotechnological applications. Binding events at a solid surface differ from those in solution in several respects: (a) the immobilized receptors or ligands may have a nonrandom orientation; (b) they are concentrated at the solid surface; (c) upon binding, the protein−ligand pair becomes entropically constrained at the surface; (d) nonspecific interactions of the protein and/or the ligand with the surface may influence binding; and (e) interactions among adsorbed molecules may also occur giving rise to cooperative effects. Minimization of nonspecific interactions is an important aspect of biosensor design, as they can generate false signals and may make binding of an analyte irreversible. Thus, it is important to characterize the binding events and the role that surface properties (chemical and physical) may have in such binding events. The well-characterized streptavidin−biotin-binding pair, the growing interest and versatility of SAMs for surface functionalization, and the need to better understand molecular recognition at the solid−liquid interface prompted this study.

Here we report on the binding and dissociation kinetics of wild-type and mutant streptavidin to biotin-terminated SAMs. The three streptavidin variants investigated differ markedly in their affinity for biotin. The respective affinities of the three types for biotin are as follows: wild-type, $K_a = 2.5 \times 10^{13}$ $M^{-1}$;[23] Y43A, $K_a = 3.7 \times 10^{11}$ $M^{-1}$;[24] and W120A, $K_a = 9.0 \times 10^6$ $M^{-1}$.[25] To investigate how the binding events at a solid surface differ from those in solution and how they are influenced by the characteristics of the surface, we have characterized the binding and dissociation events of these proteins on biotin-terminated SAMs using surface plasmon resonance spectroscopy (SPR). The study of the dissociation kinetics is especially insightful as to the molecular events taking place at the surface. Few studies have been published showing a different equilibrium dissociation constant ($K_d$) in solution versus binding at an interface, but none has systematically varied protein structure to vary the affinity of interaction. One example is the binding of carbonic anhydrase to benzenesulfonamide groups, which has been reported to proceed with higher affinity in solution than on SAMs presenting benzenesulfonamide groups at their surfaces.[26] These differences were proposed to occur because of steric hindrance or entropic repulsion between the bound protein and ethylene-glycol groups present on the mixed SAMs.[26]

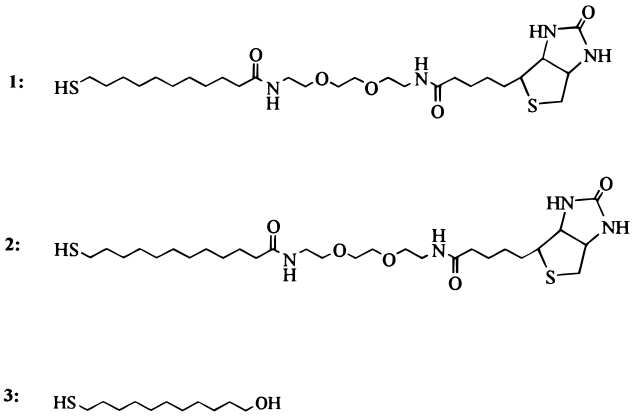


**Figure 1.** Chemical structures of 11-mercaptoundecanoic-(8-biotinoyl-amido-3,6-dioxaoctyl) amide (**1**), 12-mercaptododecanoic-(8-biotinoyl-amido-3,6-dioxaoctyl) amide (**2**), and 11-mercapto undecanol (**3**).

## Experimental Section

**Protein Expression.** The streptavidin mutants were expressed in *E. coli* in a T7 expression system. The details on the construction of the mutant gene sequences, protein production, and the determination of their binding affinities and dissociation kinetics in solution have been previously detailed.[23−25, 27]

**Buffers.** Dulbecco's phosphate buffered saline solution (PBS) (Sigma, St. Louis, MO) at a pH of 7.4 was used for the experiments described in this work. All of the protein-binding experiments were done at a concentration of streptavidin in PBS equal to 30 $\mu$g/mL (0.5 $\mu$M), and unless otherwise noted, binding to the surfaces was allowed to proceed for 1 h. Dissociation of streptavidin from the surfaces was accomplished by exposure to a 1 mM solution of d-biotin (Sigma, St. Louis, MO) in PBS at a pH of 7.4.

**SAM Precursors.** The compound 11-mercaptoundecanoic-(8-biotinoylamido-3,6-dioxaoctyl) amide (**1**) was synthesized following a synthetic route reported before[22] and was found to form disorganized biotin-terminated films upon adsorption to gold substrates.[18] In addition, 12-mercaptododecanoic-(8-biotinoylamido-3,6-dioxaoctyl) amide (**2**) was custom synthesized by Boehringher Mannheim (Penzberg Bayern, Germany). In contrast to compound **1**, thiol **2** has been found to form closely packed and well-organized biotin-terminated SAMs.[20] The compound 11-mercapto undecanol (**3**) was purchased from Sigma (St. Louis, MO) and was used as received. The chemical structure of these compounds is shown in Figure 1.

**Formation of SAMs.** Microscope glass slides (Baxter Scientific, #M6145) for surface analysis and SF10 glass slides (CVI Co.) for SPR were cleaned by immersing them in piranha solution (3/7 by volume of 30% $H_2O_2$ and $H_2SO_4$). *CAUTION: piranha solution reacts violently with most organic materials and must be handled with extreme care*. They were then thoroughly rinsed with DI water (specific resistance ~18 M Ω cm) and absolute grade ethanol and blown dry with a stream of nitrogen before mounting them on a rotating carousel in a vacuum chamber for gold deposition. An adhesion layer of 10 Å of chromium (rate of deposition = 0.5 Å/s) was deposited on the glass slides before depositing 560 Å of gold (rate of deposition = 1 Å/s) by evaporation of the metals at $10^{-7}$−$10^{-6}$ Torr. To form SAMs, we immersed gold deposited films overnight in $10^{-4}$ M solution of **1** in dimethyl sulfoxide (DMSO) (J. T. Baker) or in solutions of compounds **2** and **3** in degassed ethanol (total thiol concentration was $5 \times 10^{-4}$ M) for the formation of SAMs. Samples were pulled out of solution, sequentially rinsed with

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DMSO or ethanol, DI water, and finally with ethanol again, then dried with a stream of nitrogen, and used immediately.

**Surface Characterization.** The biotin-terminated SAMs were analyzed by ellipsometry, wettability, and X-ray photoelectron spectroscopy (XPS). The contact angles of the slowly advancing front of water drops were measured using a Ramé-Hart goniometer, Model 100 (Mountain Lakes, NJ). An average of six measurements was obtained (both sides of the drop on three different regions for each sample) and averaged.

X-ray photoelectron spectroscopy was conducted on an AXIS−HSi instrument from Kratos Analytical, Inc. (Ramsey, NY). An Al $K\alpha_{1,2}$ monochromatized X-ray source ($h\nu$ = 1486.7 eV) with an emission power of 150 W (15 kV and 10 mA) was used to stimulate photoelectron emission. The residual pressure in the analysis chamber was on the order of $10^{-9}$ Torr or lower during spectral acquisition. All spectra were acquired with a constant pass energy of 40 eV. The spectral envelopes were resolved into Gaussian peaks to fit the spectra, and the hydrocarbon C1s peak was referenced at 284.6 eV.

Ellipsometric measurements were performed with a spectroscopic ellipsometer (M-44 from J. A. Woolam Co., Inc., Lincoln, NE). Ellipsometric measurements were taken at 70° on thick gold substrates (2000 Å) deposited on silicon before and after SAM formation. The thicknesses of the monolayers was calculated assuming an index of refraction for the SAMs of 1.45.

**Surface Plasmon Resonance Spectroscopy (SPR).** Protein binding was monitored at room temperature using a custom-made double beam SPR instrument using the Kretschmann configuration and equipped with a reference element, rotating stages, and a flow cell. A description of our system has been published elsewhere.[28] SPR is a well-established technique for sensing analytes by monitoring changes in the plasmon dispersion relation at the interface of a thin metal film and a fluid. A fundamental description of this analytical technique can be found elsewhere.[29] In the data presented here, the amount of protein adsorbed to the surface is assumed to be linearly proportional to the change in the angle of maximum plasmon resonance ($\Delta\theta$).[26]

**Atomic Force Microscopy (AFM).** A Nanoscope III from Digital Instruments (Santa Barbara, CA) was used to image the adsorbed proteins on the biotinylated SAMs. A liquid cell was used to image the adsorbed wild-type streptavidin in buffer. The images were collected in tapping mode using silicon nitride microfabricated tips. Low-pass filtering of the images was used to enhance the features of the images.

## Results

**Surface Characterization.** Surface analysis of biotin-terminated SAMs was performed using X-ray photoelectron spectroscopy, ellipsometry, and wettability measurements. The results are consistent with the presence of thiolates of compounds **1** and **2** on gold. There was a marked difference between monolayers formed by chemisorption of biotin-terminated thiol **1** and those formed from **2**. The water contact angle for monolayers formed from **1** was 55° ± 3° in agreement with previous reports.[22] However, the wettability of a SAM formed from **2** was 42° ± 1°, which is higher than the 33° reported previously.[20] We note, however, that we measured the slowly advancing fronts of water droplets. If a measurement is performed right after the moving front stops advancing (sessile drop), a wettability of 33° is obtained. The difference in the contact angles between SAMs made from **1** and **2** is probably a result of the more disordered monolayer exposing some of the hydrophobic methylene groups to the surface (vide infra).

Ellipsometry showed that monolayers formed from **1** and **2** differ considerably in their thicknesses. Compound **1** produces films with an average thickness of 5 ± 3 Å as determined by ellipsometry. This agrees well with an SPR measurement of 7 Å reported elsewhere.[18] In contrast, SAMs created from **2** form monolayers with ellipsometric thicknesses of 35 ± 2 Å in agreement with the theoretical thickness of fully extended chains forming a closely packed monolayer (34 Å); this is also in agreement with SPR measurements reported elsewhere.[20] Formation of monolayers of **1** from different solvents (dimethyl formamide, chloroform, ethanol, and a 50/50 mixture of ethanol/chloroform) did not change significantly the observed ellipsometric thickness. Thus, the disordered monolayer is not a result of the quality of the solvent but is likely an intrinsic property of SAM precursor **1**.

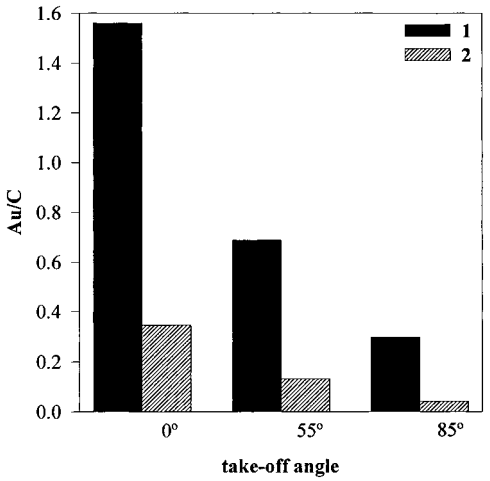


**Figure 2.** Typical angular-dependent XPS analyses of SAMs formed from pure **1** and pure **2**. Shown are the Au/C ratios as a function of takeoff angle (the angle between the axis of the XPS analyzer and the surface normal). The gold signal is attenuated as the takeoff angle is increased and larger attenuation is obtained on SAMs made from pure **2**, which is consistent with the presence of a thicker overlayer on the gold surface.

X-ray photoelectron spectroscopy analysis provided additional evidence that monolayers formed from compound **1** are not closely packed, while those formed from **2** consist of closely packed chains. This is shown in Figure 2 which shows the ratio of Au/C at take off angles of 0°, 55° and 85° (the takeoff angle is defined as the angle between the axis of the analyzer and the surface normal). The presence of an organic film adsorbed on a gold substrate results in an attenuation of the gold signal as the takeoff angle is increased. The gold signal is attenuated more in SAMs formed from **2** than on those formed from **1** (larger values of Au/C), which indicates a much thicker overlayer on substrates prepared by chemisorption of **2** than of **1**.

For mixed SAMs of **2** and **3**, XPS analysis was used to estimate the mole fraction of biotinylated thiolates ($\chi_2$) by taking the ratio of the N1s signal from a sample relative to that of a pure monolayer of **2**, that is,[33]

$$\chi_2 = \frac{\text{N1s(SAM of } \mathbf{2} \text{ and } \mathbf{3})}{\text{N1s(SAM of } \mathbf{2})} \qquad (1)$$

Figure 3 shows the relationship between the molar fraction of **2** in solution and that at the surface. Small values of $\chi_2$ at

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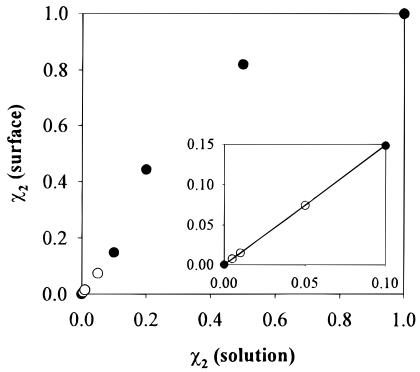


**Figure 3.** Relationship between the molar fraction of **2** ($\chi_2$) in solution versus $\chi_2$ at the surface (determined by XPS). Open circles denote values interpolated from the trend of $\chi_2$ in solution versus $\chi_2$ at the surface for those samples where the amount of nitrogen was below the limits of detection of XPS (inset). Open circles denote values of $\chi_2$ of 0.007, 0.015, and 0.07.

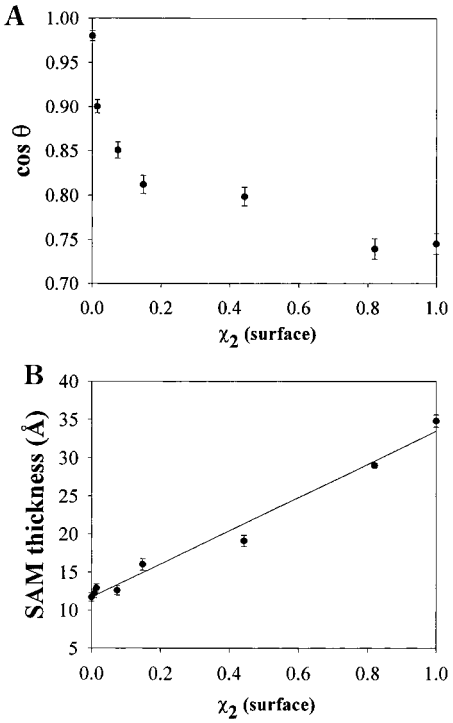


**Figure 4.** (A) Advancing water contact angles and (B) ellipsometric thicknesses of mixed SAMs of **2** and **3** versus molar fraction of **2**, $\chi_2$, at the surface.

the surface (open circles in Figure 3) that were below the limits of detection of XPS were obtained using linear interpolation of the values of $\chi_2$ at the surface versus solution for SAMs of pure **3** ($\chi_2 = 0$) and the sample with the lowest detectable amount of nitrogen ($\chi_2 = 0.15$). The data shows that **2** partitions preferentially on the surface. This may be a result of the lower solubility of **2** in ethanol. Water contact angle measurements and ellipsometric thicknesses of mixed SAMs of **2** and **3** as a function of biotin concentration at the surface ($\chi_2$) are presented in Figure 4, panels A and B, respectively. The trends observed are consistent with the presence of increasing amounts of thiolate **2** at the surface. The linear relationship between the ellipsometric thickness and molar fraction of **2** at the surface is an indication that SAMs of **2** and **3** are well-organized throughout the entire composition interval.

**Binding of Streptavidin to Biotin-Terminated SAMs.** SPR measurements showed that monolayers formed from pure **1**

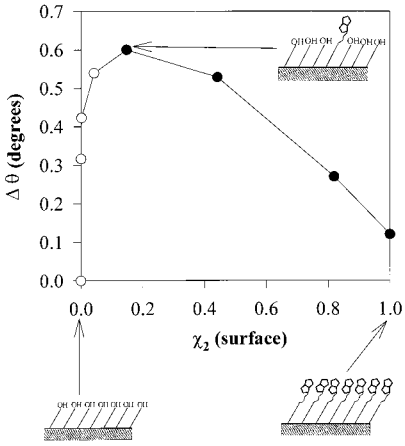


**Figure 5.** Binding of wild-type streptavidin (1 h adsorption) to mixed SAMs formed from the chemisorption of **2** and **3** on gold. The *y*-axis represents the shift in the angle of resonance ($\Delta\theta$) due to the adsorption of streptavidin. Open symbols denote estimated values of $\chi_2$ (see text).

bound more wild-type streptavidin at saturation (1 h adsorption) than SAMs formed from pure **2** ($\Delta\theta = 0.36$ and $\Delta\theta = 0.12$ for adsorption of wild-type streptavidin to SAMs formed from **1** and **2**, during 1 h, respectively). The low binding on the latter is thought to be a result of the close packing of biotin groups that hinder molecular recognition with the biotin-binding pockets of streptavidin.[20] Figure 5 reflects the level of wild-type streptavidin binding to mixed monolayers of **2** and **3** versus the molar fraction of **2** at the surface ($\chi_2$). At high concentrations of **2**, the crowding of biotin groups sterically hinders binding of streptavidin. When the biotin groups are optimally spaced (corresponding to a surface mole fraction of **2** of about 0.15), maximum binding of streptavidin occurs.[20]

On SAMs made from compound **1** or mixtures of **2** and **3**, blocking the biotin-binding sites of the streptavidins (wild-type and mutants) by addition of a molar excess of biotin to the protein solution prevented binding to biotin-terminated surfaces. Adsorption and desorption of streptavidin to and from SAMs formed from **1** were compared to that from SAMs formed from mixtures of **2** and **3** at values of $\chi_2$ corresponding to (1) the optimum for binding of wild-type ($\chi_2 = 0.15$) and (2) the lowest biotin concentration investigated ($\chi_2 = 0.007$). Figure 6 shows the kinetics of binding of streptavidin to monolayers formed from pure **1**, and Figures 7 and 8 show the kinetics of binding of streptavidin to mixed monolayers formed from **2** and **3** at surface concentrations of biotin ($\chi_2$) of 0.15 and 0.007, respectively. In all cases, binding of unblocked streptavidin proceeded quickly and was diffusion-limited (initial amount bound versus $\sqrt{t}$ is linear),[35] consistent with findings reported for wild-type streptavidin binding onto biotin-terminated SAMs.[18,20]

**Dissociation of Surface-Bound Mutants.** Dissociation experiments were carried out with substrates formed from pure **1** and on mixed monolayers of **2** and **3** that had equal or less amounts of biotin than required for optimum binding. For all streptavidin types, measurable desorption of the surface-bound protein in buffer alone did not occur in a time frame comparable

(34) The saturation coverage of protein on a surface can be described by random sequential adsorption theory (RSA). According to RSA, this coverage is equivalent to only 55% of the projected protein area. Even after this limit is considered, the numbers indicate a number of biotin groups on the surface an order of magnitude larger than that required to form a monolayer.

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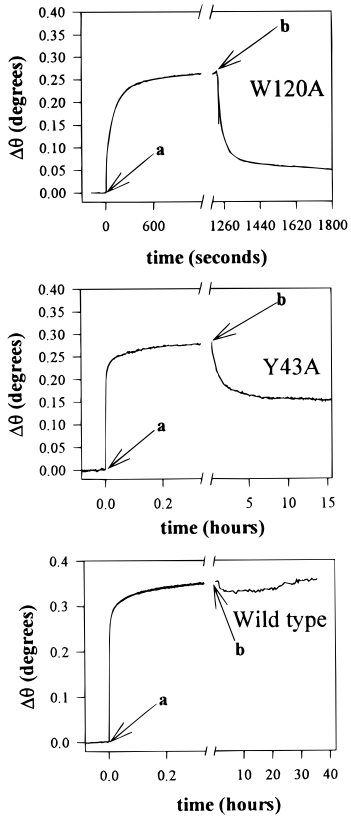


**Figure 6.** Binding of streptavidin mutants and competitive dissociation with biotin on biotin-terminated monolayers formed by the chemisorption on gold of precursor **1** as a function of time. The *y*-axis represents the shift in the position of the angle of minimum reflected intensity, $\Delta\theta$, as the molecules adsorb onto the surface. Point **a** represents the time where the protein is introduced in the liquid cell to start the adsorption process. Point **b** corresponds to the time when the protein solution was displaced by the addition of 1 mM biotin solution to competitively dissociate the adsorbed protein from the surface.

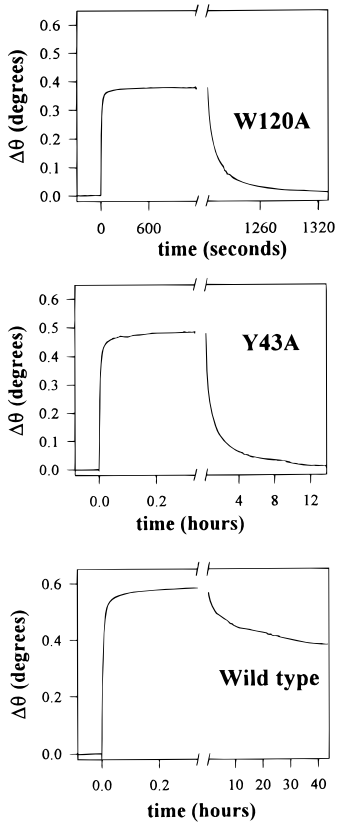


**Figure 7.** Binding of streptavidin mutants and competitive dissociation with biotin on mixed monolayers of biotin- and hydroxyl-terminated thiolates (from precursors **2** and **3**) as measured by SPR. These monolayers contain a molar fraction of biotin groups, $\chi_2 = 0.15$, that corresponds to the optimum $\chi_2$ for binding of wild-type streptavidin.

to the experiments described here. However, it was possible to desorb surface-bound protein molecules by competitive dissociation with free biotin in solution. This was achieved by quickly flushing the cell with a 1 mM solution of biotin. This resulted in fast replacement of the streptavidin solution with biotin solution that remained in the cell during the dissociation experiment.

Typical progression of SPR measurements of binding and desorption of the streptavidins to and from SAMs formed from **1** are shown in Figure 6. Upon introduction of streptavidin, binding occurred quickly (point **a** in Figure 6) at the diffusion-limited rate. After the protein solution was flushed out of the cell with a biotin solution in PBS (point **b** in Figure 6), the bound molecules began to desorb from the surface. The desorption kinetics in the presence of biotin showed large differences for the streptavidin mutants. Only a fraction of the proteins initially bound to monolayers made from pure **1** could be dissociated with free biotin in solution, whereas complete dissociation from SAMs made from **2** and **3** was achieved (vide infra).

**Atomic Force Microscopy.** Figure 9 shows AFM images of a biotinylated SAM formed from **2** and **3** ($\chi_2 = 0.007$) before and after adsorption of wild-type streptavidin. We estimate from the AFM images collected that about 30−50% of the surface is not covered by protein. This is consistent with the amount of streptavidin bound at this fraction of biotin ($\chi_2 = 0.007$).[34] The AFM images also suggest that the adsorption of streptavidin is nonuniform and that the proteins tend to cluster on the surface. More than 80% of the adsorbed proteins are found in clusters of three or more molecules. When the corrugations of the gold are accounted for when analyzing the topography of the protein clusters, there is little evidence for the presence of multilayers of proteins. This is in contrast to STM studies done with biotinylated monolayers that suggested extensive clustering of the bound streptavidin and protein multilayers that were reported to be up to eight layers thick.[22]

## Discussion

**Structure of SAMs.** Monolayers prepared from precursors **1** or **2** differed markedly in their degree of assembly as determined from ellipsometry and angular-dependent XPS. Assuming homogeneous organic overlayer films, a mean free path of C1s photoelectrons in a hydrocarbon film of 36 Å, and a mean free path of Au4f photoelectrons in a hydrocarbon film of 42 Å,[30] the estimated thicknesses obtained from the angular-dependent XPS are 6 and 26 Å for SAMs formed from **1** and **2**, respectively. This is consistent with the ellipsometric measurements reported above. The slight discrepancy in the numbers with ellipsometry may be a result of the assumed index of refraction in ellipsometry and/or the assumptions in the homogeneity of the monolayers and the photoelectron mean free paths.[30]

It is not clear why there is such a large difference in monolayer thickness for chemisorbed compounds **1** and **2**. As these precursors differ only by one methylene group in the alkyl chain, it is surprising that their degree of assembly when chemisorbed on gold differs so markedly. The poor packing of

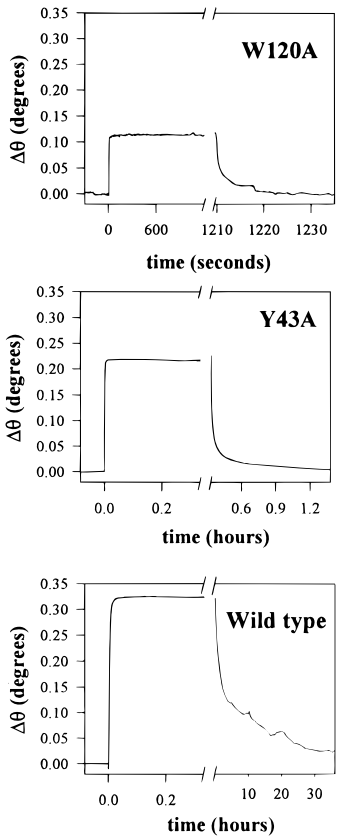


**Figure 8.** Binding of streptavidin mutants and competitive dissociation with biotin on mixed monolayers of biotin and hydroxyl-terminated thiols (**2** and **3**) as measured by SPR. These monolayers contain a molar fraction of biotin groups, $\chi_2 = 0.007$.

chains on monolayers made from **1** was originally believed to be due to the diamino dioxaoctyl (DADOO) hydrophilic spacer, which was thought to prevent a close packing of the thiolate of **1** on gold.[18] However, the same DADOO spacer is present in **2**, yet it produces SAMs with closely packed chains.[20] We can venture the hypothesis that, for an alkyl chain that is fully extended in an all trans configuration and tilted by about 30° with respect to the surface normal (as would be the case for perfectly assembled, closely packed alkyl chains), the rigid amide bond that couples the methylene groups to the DADOO moiety would be oriented approximately 30° with respect to the surface (60° with respect to the surface normal) for chemisorbed **1**, and that the rigid amide bond would be oriented at 60° with respect to the surface (30° with respect to the surface normal) for chemisorbed **2**.[31,32] Thus, close packing of chains may be hindered for **1** but not for **2**. Although this hypothesis might explain a less effective packing of **1** as compared to **2**, it may not fully account for the drastic difference in the extent of packing densities observed. An alternative hypothesis is that there may exist a threshold such that, below a certain number of methylene groups in the alkyl chains, disordered monolayers are obtained upon chemisorption of the precursors. Further synthesis and assembly of biotin-DADOO-substituted molecular precursors for SAM formation that have even/odd numbers of methylene groups will be necessary to test these hypotheses.

**Adsorption of Streptavidin to Biotinylated SAMs.** More streptavidin bound to monolayers made from pure **1** than to monolayers made from pure **2**. This was a result of steric hindrance due to the close packing of biotin on SAMs made from **2**. Biotin-blocked streptavidin did not adsorb to any of the SAMs, indicating that adsorption proceeded only through specific interactions, that is, only by molecular recognition of the biotin-binding sites of streptavidin and surface-immobilized biotin.

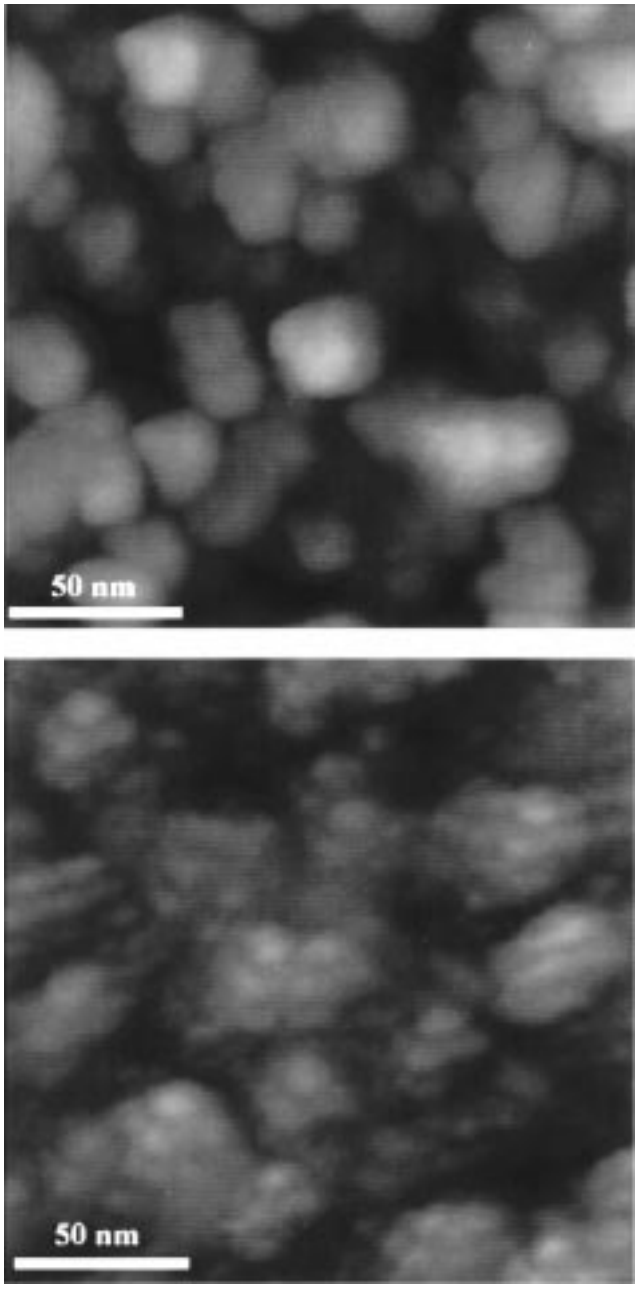


**Figure 9.** Atomic force micrographs of a biotinylated SAM formed from **2** and **3** ($\chi_2 = 0.007$) before (top) and after (bottom) adsorption of wild-type streptavidin. The bottom image was taken after exposure to protein solution for 1 h.

Maximum binding of wild-type streptavidin to mixed monolayers of **2** and **3** occurs at $\chi_2 = 0.15$. With the assumption of an area of 21 Å$^2$ per adsorbed thiolate,[26] the molar fraction of biotin for maximum streptavidin binding ($\chi_2 = 0.15$) at the surface would correspond to 140 Å$^2$ per biotin moiety. By comparison to the projected area of the streptavidin molecule (~2500 Å$^2$),[3,34] this corresponds to an average of about 18 biotin groups per adsorbed streptavidin molecule. This is several times larger than necessary to bind a monolayer of streptavidin where each protein molecule attaches to the surface through two biotin bonds.[34] Previous studies of biotinylated SAMs did not quantitate the amount of biotin present on the surface ($\chi_2$) and, thus, did not make these inferences possible. If all of the biotin groups

on the surface were available for binding, a streptavidin molecule could readily bind two biotin groups while covering the remaining unbound biotin moieties. Upon further dilution, there would still be enough biotin groups to support binding of a monolayer of streptavidin. If this was the case, instead of the sharp maximum observed in Figure 5, a region where the maximum binding occurs would extend down to $\chi_2 \sim 0.01$ (the minimum surface concentration necessary to achieve a full monolayer[34] of streptavidin bound through one biotin group per protein molecule). What is observed instead is that streptavidin binding decreases as $\chi_2$ decreases from 0.15 to 0.01. The inaccessibility of some of the biotin groups could have several origins: (1) it could be due to nonrandom distribution of thiolates of **2** and **3** on the gold surface such that the close packing of some of the biotin moieties would hinder the binding of streptavidin; (2) some of the thiolate chains could be in an orientation that is not favorable for binding of streptavidin; or (3) because of the microscopic roughness of the gold surface prepared by the metal evaporation method, those biotin groups located between the microscopic crevices of our surfaces could be prevented from binding streptavidin. It is very likely that a combination of these three scenarios is responsible for the unavailability of some of the biotin groups.

The relative amounts of streptavidin mutants adsorbed onto biotin-terminated SAMs depend on their binding affinities for biotin. For the purpose of comparison, the results for Y43A and W120A will be discussed as the percentage relative to wild-type, the variant with the highest binding affinity for biotin. Monolayers of pure **1** bound about 90% of the Y43A mutant and 76% of the W120 mutant with respect to wild-type (Figure 6). Mixed SAMs of **2** and **3** at a surface concentration of biotin, $\chi_2 = 0.15$ (Figure 7), adsorbed about 94% Y43A and 67% W120A of the amount of wild-type. At a surface concentration of biotin of $\chi_2 = 0.007$ (Figure 8), only 71% of Y43A and 39% of W120A (with respect to wild-type) adsorbed. The decreased binding with respect to wild-type at the lower surface coverage of biotin may suggest that binding affinity depends on the surface concentration of biotin groups. The apparent higher binding affinity to SAMs with higher surface concentration of biotin is very likely a result of cooperativity (molecules attached through two biotin−streptavidin bonds and/or attractive interactions among adjacent adsorbed protein molecules; see below).

**Desorption of Streptavidin from Biotinylated SAMs.** Only a fraction of the adsorbed mutants desorbed from monolayers formed from precursor **1** upon exposure to free biotin in solution (Figure 6). About 80% of the bound W120A and 45% of the bound Y43A desorbed. No measurable desorption of the wild-type streptavidin was observed in a period of 2 days. The amount desorbed correlated negatively with the biotin-binding affinity and positively with the rate of dissociation of the mutants (i.e., W120A > Y43A > wild-type). The fact that a fraction of the bound molecules remains irreversibly attached, even for the lowest binding affinity mutant (W120A), suggests that irreversible nonspecific interactions can take place after the proteins bind to SAMs formed from **1**. As shown above, monolayers formed from **1** are poorly organized. Because the adsorption process only takes place through specific molecular recognition (blocked streptavidin does not bind to these SAMs), we believe that the poorly formed monolayers present a hydration layer that prevents nonspecific adsorption. We hypothesize, however, that the surface-bound protein molecules may induce changes in the state of the monolayer after binding that facilitate nonspecific interactions with the gold substrate or with hydrophobic moieties of the alkanethiolate. In other words, the bound protein molecules can interact nonspecifically only after they are "locked" to the surface. As the slower desorption rates correspond to longer residence times on the surface, we hypothesize that the streptavidin that stays attached to the surface for a longer period of time is more likely to undergo nonspecific association. This would explain the correlation between the observed rates of dissociation and the amount dissociated from the surface.

These results demonstrate the importance of an adequate design and characterization of the surface architecture when immobilizing biomolecules for biotechnological applications. This conclusion is timely in that several other research groups are currently investigating similar systems for the creation of biospecific surface architectures. For example, a detailed surface characterization of biotinylated SAMs made with a different biotinylated thiol precursor by Nelson et al. has recently shown that highly disordered SAMs are obtained with the pure biotinylated thiol employed.[41] It is hypothesized in that work that the degree of disorder may arise on pure biotinylated SAMs because of the presence of bulky amide groups, the asymmetric biotin moiety, and the flexible ether linkages of the SAM precursor. The same work showed that more ordered films are obtained by forming mixed monolayers with other thiols[41] and thus also illustrates the need for further understanding of steric effects that might hinder the formation of well-organized SAMs.

Nonspecific interactions between streptavidin and the surface did not occur on mixed SAMs of precursors **2** and **3**. As mentioned before, surface analysis of the molecular assemblies made from **2** indicated better packing of the molecules comprising these SAMs. On SAMs formed from pure **3**, the close packing of hydroxyl groups prevents nonspecific adsorption of streptavidin (Figure 4).[20] Thus, on mixed SAMs of **2** and **3**, specific molecular recognition without nonspecific interactions is achieved because the biotin functionalities protrude from a matrix of hydroxyl groups that prevent the bound streptavidins from interacting with the gold substrate or hydrophobic methylene groups of the alkyl chains. For mixed SAMs of **2** and **3**, complete desorption of streptavidin molecules by exposure to biotin solution was achieved for the W120A and the Y43A mutants (Figures 7 and 8). For wild-type streptavidin, complete dissociation was not achieved after 2 days for $\chi_2 = 0.15$, but it was achieved for $\chi_2 = 0.007$. Incomplete dissociation from SAMs with $\chi_2 = 0.15$ could be due to the extremely slow dissociation kinetics of the wild-type streptavidin−biotin-binding pair and cooperative effects (see below).[36,37] We believe that complete desorption of wild-type from this monolayer could be possible over a longer period of time. These results lend support to our hypothesis that, on pure biotin-terminated SAMs prepared from precursor **1**, nonspecific interactions occurred after the binding process.

**Modeling of Streptavidin Desorption.** The desorption of streptavidin from biotinylated SAMs (Figures 6−8) does not follow simple first-order kinetics (that is, a monoexponential process) as would be expected for simple 1:1 interactions between ligand and receptor.[38,39] Deviations from simple first-

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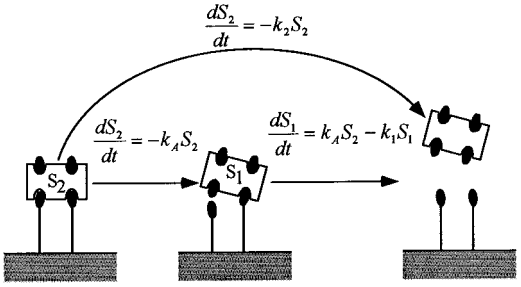
**Scheme 1**. Dissociation Processes of Streptavidin from Biotin-Terminated SAMs[a]




[a] Two populations of bound molecules may exist: species $S_1$ that are attached through one biotin-binding site and species $S_2$ that are attached through two biotin-binding sites. Desorption of $S_2$ occurs by sequential dissociation of streptavidin−biotin bonds or simultaneous dissociation of two streptavidin−biotin bonds.

order kinetics are commonly found in the dissociation of bound proteins from surfaces, and several hypotheses have been proposed to explain their origin.[39,40] These include effects of (1) heterogeneity of immobilized ligand sites,[39] which creates a nonuniform population of ligands with varying affinities for the protein, and (2) rebinding due to mass transport effects.[40] Crowding effects could be present even at low surface concentrations of biotin if nonrandom distribution of the thiolates exists on the surface. We rule out substantial rebinding of streptavidin to the SAMs because dissociation of the bound streptavidins was accomplished using 1 mM solution of biotin, which is much higher than the $K_d$'s of the streptavidin variants and, thus, likely results in the blocking of biotin-binding pockets once the protein has dissociated. Crowding effects due to nonrandom mixing and formation of phase separated domains of alkanethiolates have been suggested to exist on mixed SAMs presenting benzenesulfonamide groups on gold.[26] However, these effects do not seem to influence first-order dissociation kinetics of carbonic anhydrase from SAMs.[26] As binding of carbonic anhydrase to benzenesulfonamide groups is monovalent,[26] and its dissociation can be modeled by simple first-order kinetics,[38] we assume that nonrandom distribution of the alkanethiolates does not influence departure from a monoexponential process to a large extent. Our initial hypothesis was that bivalent binding could account for the observed double-exponential behavior.

***(1) Effects of Bivalent Binding.*** To model the dissociation kinetics of streptavidin from biotin-functionalized monolayers, we assume that streptavidin molecules bind to the surface through only one or two of their biotin-binding pockets. It is safe to assume a maximum of two binding sites because the biotin-binding pockets are located in pairs at opposite sides of the streptavidin tetramer[3] and because the streptavidin molecule is bigger than the dimensions of a fully extended chain of **1** or **2**. Under Scheme 1, before exposure to the solution of free biotin, there are two populations of streptavidin molecules bound to the surface; one that is bound through only one biotin group (with a concentration of $[S_1]_0$) and another that is bound through two biotin groups (with a concentration of $[S_2]_0$). Molecules bound through one biotin group can desorb from the surface by dissociation of a single biotin−streptavidin bond with a first-order kinetic rate constant $k_1$, whereas those bound to the surface through two biotin groups have to desorb by either the simultaneous or sequential dissociation of two biotin−streptavidin bonds. The kinetic rate constant for simultaneous dissociation of two biotin−streptavidin bonds is given by $k_2$, whereas the first-order kinetic rate constant for dissociation of one biotin−streptavidin bond when the molecule remains attached to the surface is given by $k_A$. Under this scheme, the expression describing the total amount of streptavidin on the surface during the dissociation process is given by the following equation that contains two exponential terms:

$$[S] = \left\{[S_1]_0 - \frac{k_A}{k_1 - k_A - k_2}[S_2]_0\right\}\exp[-k_1 t] + \frac{k_1 - k_2}{k_1 - k_A - k_2}[S_2]_0\exp[-(k_A + k_2)t] \quad (2)$$

In this equation, $k_1$ would correspond to the exponential term giving the fastest rate constant and represents the kinetic constant of dissociation of a biotin−streptavidin bond at the surface. Here we assume that the rate constants (and hence, the corresponding activation energies) are different for the dissociation of streptavidin−biotin bonds when the molecule is attached to the surface through one or two biotin-binding sites ($k_1$ and $k_A$). In other words, the rate of biotin−streptavidin bond dissociation would be different when it leads to desorption than when it does not lead to desorption from the surface of an adsorbed streptavidin.

In the absence of interactions among neighboring adsorbed molecules, desorption would be expected to follow single first-order kinetics when the concentration of biotin groups at the surface is low enough (as long as gold thiolates of **2** and **3** are randomly distributed on the surface) such that the majority of bound molecules would be attached through only one biotin-binding site. It turns out that even with SAMs where $\chi_2 \sim 0.007$, the desorption process still deviates from first-order dissociation. At this molar fraction, the corresponding average area per biotin group is 3000 Å$^2$, which is larger than the projected area of a streptavidin molecule. If all of the biotin groups were uniformly distributed on the surface, this would correspond, on average, to one biotin−streptavidin bond per adsorbed protein molecule and thus first-order dissociation process would be expected. The fact that at $\chi_2 = 0.007$ the desorption process is double-exponential suggests nonrandom distribution of biotin groups on the surface. Additionally, at this concentration of biotin on the surface, the amount of wild-type adsorbed should be about 30% less than a monolayer,[34] yet Figure 5 shows about 50% of the maximum amount bound; that is, not all of the biotin groups are available for binding of streptavidin. The presence of biotin groups on the surface with different availability for binding to streptavidin could imply different binding affinities for streptavidin and explain the departure from monoexponential dissociation kinetics. However, benzenesulfonamide-terminated SAMs also present only a fraction of groups available for binding to carbonic anhydrase.[26] Still, monoexponential dissociation kinetics are observed on these SAMs at different coverages of benzenesulfonamide groups. We note that binding of carbonic anhydrase to benzenesulfonamide is monovalent and that attractive interactions were not observed in that system.[26] Thus, we infer indirectly from that work that the only effect of nonrandom mixing of the adsorbed thiolates would be in the availability of the biotin groups and not on departure from single first-order kinetics.

***(2) Effects of Cooperative Interactions between Adsorbed Proteins.*** Faster desorption is observed on SAMs with lower surface concentration of biotin (cf. Figures 7 and 8). We postulate that attractive interactions among neighboring adsorbed proteins slow the dissociation process. At high surface concentrations of streptavidin on the surface, the fraction of adsorbed molecules experiencing near neighbor interactions would be larger than at low surface coverages. We note that the slower

**Table 1.** Apparent Kinetic Constants of Dissociation, $k_1^{app}$, for Streptavidin Mutants Bound to Biotin-Terminated SAMs[a]

| | | $k_1^{app}$ $(s)^{-1}$ | | |
|---|---|---|---|---|
| | $k_{off}$ $(s^{-1})$ | | SAMs formed from **2** and **3** | |
| streptavidin type | solution[24,27] | SAMs formed from **1** | $\chi_2 = 0.15$ | $\chi_2 = 0.007$ |
| wild-type | $(3.3 \pm 0.1) \times 10^{-6}$ | | $(1.37 \pm 0.08) \times 10^{-4}$ | $(3.31 \pm 0.16) \times 10^{-4}$ |
| Y43A | $(5.7 \pm 0.1) \times 10^{-4}$ | $(4.16 \pm 0.17) \times 10^{-4}$ | $(2.05 \pm 0.04) \times 10^{-3}$ | $(7.25 \pm 0.40) \times 10^{-2}$ |
| W120A | *b* | $(5.40 \pm 0.08) \times 10^{-2}$ | $0.336 \pm 0.019$ | $2.44 \pm 0.24$ |

[a] The rate constants of dissociation of streptavidin−biotin in solution are smaller than on the surface and were obtained from refs 24 and 27. [b] Dissociation of biotin from W120A is too fast (<60 s) for an accurate determination of $k_{off}$.

desorption at higher surface concentrations of biotin is not entirely due to a larger population of streptavidin molecules bound to the surface through two biotin groups. According to Scheme 1 and eq 1, the effect of different populations of surface-bound streptavidin ($[S_1]_0$ and $[S_2]_0$) would be in the relative contribution of each exponential term and not in the rate constants ($k_1$, $k_2$, and $k_A$). As shown below, $k_1$ is significantly affected by the surface coverage, $\chi_2$, of biotin on these SAMs.

Table 1 lists the rate constant from the fastest exponential component obtained from fitting the dissociation data of Figures 6−8 to eq 2 as a conservative estimate of the rate constants for dissociation of streptavidin and a surface-bound biotin (Scheme 1). Because of the presence of attractive interactions among adsorbed molecules and with the surface, these values are very likely to be an underestimation of the true rate constants for dissociation of the biotin−streptavidin bond at the surface ($k_1$). Thus, they are referred to as apparent dissociation constants, $k_1^{app}$, in Table 1. Nevertheless, the apparent dissociation rate constants listed ($k_1^{app}$) are larger than the biotin−streptavidin dissociation rate constants measured for dissociation in solution which have been measured to be a single-exponential first-order process.[23,24] This indicates that, even in the presence of cooperative effects, dissociation from surface-bound biotin proceeds with faster rates on a surface than in solution. The factors that may lead to faster dissociation are: (1) unfavorable steric constraints introduced by tethering and (2) immobilization of the biotin moieties that restricts their mobility. Unfavorable steric constraints due to the tethering of biotin arise because this imposes a steric constraint in the binding of streptavidin to biotin. Restricted mobility of the biotin−streptavidin pair at the surface imposes an entropic constraint, which implies a lower energy barrier for dissociation from the surface. That is, desorption from a surface is favored because dissociation leads to a bigger change in entropy than in solution.

In the presence of positive cooperativity, protein−surface and protein−protein interactions should decrease the rate of dissociation. Table 1 shows that the apparent kinetic constants of dissociation, $k_1^{app}$, of the streptavidin mutants are greatly affected by interactions of the mutants with the surface and/or with neighboring adsorbed molecules. On monolayers prepared by chemisorption of the pure biotin-terminated thiol **1**, the dissociation kinetic constants are significantly smaller than on mixed biotin/hydroxyl-terminated SAMs formed from **2** and **3**. It is our hypothesis that nonspecific interactions on the poorly formed biotin-terminated monolayers slow the dissociation process. Significant differences in the kinetic constants of dissociation were also observed for different coverages of biotin moieties on SAMs made from **2** and **3**. The dissociation process was faster for low surface concentration of biotin moieties at the surface. This is consistent with our picture of attractive interactions among adsorbed streptavidin molecules on SAMs when there is a high surface concentration of biotin groups. Such interactions decrease when the surface-bound molecules are spaced further apart as is the case for lower surface coverage.

Positive cooperative effects among avidin molecules adsorbed to biotinylated Langmuir-Blodget (L−B) films on the surface of evanescent fiber optic sensors have been reported by Reichert and co-workers.[37] Measurements of avidin binding to biotinylated L−B films indicated that the extent of cooperativity can depend on (1) the concentration of surface-bound biotin and (2) the availability of the biotin groups at the surface.[37] This is in agreement with our observation that, at higher concentrations of surface-bound biotin, attractive interactions slow the desorption of streptavidin. Larger intermolecular cooperative effects have been reported for streptavidin molecules bound to biotinylated L−B films at the air−water interface, the latter leading to the formation of 2-D crystals of streptavidin.[42]

Ringsdorf et al. conducted scanning tunneling microscopy (STM) observations of streptavidin molecules bound to mixed monolayers of hydroxy- (**3**) and biotin-terminated alkanethiolates (**1**) on gold.[22] They found that, even on dilute biotin-terminated SAMs, adsorption is not homogeneous and does not lead to true monolayers.[22] Their STM images suggested that streptavidin adsorption was not uniform and that aggregates of streptavidin molecules existed on these surfaces that were, usually, two molecules thick but in some cases up to eight molecules thick. Using AFM (Figure 9), we have also observed nonuniform adsorption of proteins on mixed SAMs of **2** and **3**. However, when the topography of biotinylated SAMs on gold with and without streptavidin is compared, it is not clear that multiple layers of proteins exist, in contrast to STM results presented before.[22] Still, these observations are consistent with our hypothesis that attractive interactions among adjacent adsorbed protein molecules may exist (because of the two-dimensional clustering of adsorbed proteins) that slow the dissociation process.

## Conclusions

We have shown with the streptavidin−biotin-binding system that molecular recognition processes at surfaces differ in complexity from those in solution. In particular, we found that several factors can significantly affect the kinetics and equilibrium of biomolecular recognition reactions at surfaces. These factors include: (1) nonspecific interactions of the adsorbed proteins with the substrate (which are strongly dependent on the state of the monolayer), (2) bivalent binding, (3) interactions between adsorbed proteins, and (4) effects associated with tethering of the ligands. We found that a small difference in the chemical structure of the SAM precursor may have an enormous impact on the degree of assembly of the monolayer. Thus, a thorough characterization of such surfaces is necessary in order to understand the events leading to adsorption,

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desorption, and nonspecific interactions of proteins on SAMs that contain immobilized biomolecules. An important observation is that reversible adsorption was only observed on well-organized monolayers; on poorly ordered monolayers, time-dependent nonspecific association may occur even when initial binding is driven strictly by specific molecular recognition. This resulted in incomplete desorption of the bound streptavidin molecules and may be a result of interactions between the adsorbed proteins and the gold substrate or the hydrophobic moieties of the poorly organized monolayers. This is an important factor to consider if reversible binding is an issue (e.g., to achieve reversible biosensing).

These observations illustrate the advantages of studying molecular recognition at solid−liquid interfaces with a system that has different binding affinity for the immobilized ligands. The study of wild-type streptavidin alone would not have given much information about the time-dependent nonspecific association observed on monolayers made from **1**. This is because the correlation between the amount of protein irreversibly attached to the surface and the binding affinity and rate of dissociation could not be established using only one protein. In addition, complete dissociation of wild-type would have been difficult to achieve because of its extremely slow rate of dissociation from biotin. The latter illustrates the advantage of systematically varying the binding affinity of the protein for the ligand to determine the time dependence of nonspecific interactions at the surface. Our results suggest a higher complexity when molecular recognition occurs at a solid−liquid interface than in solution due to interactions between the surface and the bound analytes and/or among adsorbed molecules at the surface.

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