Fabrication and Imaging of Two-Dimensional Patterns of Proteins Adsorbed on Self-Assembled Monolayers by Scanning Electron Microscopy[1]

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Abstract: This paper describes methods for controlling both the concentration and spatial distribution of proteins adsorbed onto patterned, self-assembled monolayers (SAMs). Patterned SAMs were formed by the serial chemisorption of two or more ω-functionalized alkanethiols ($HS(CH_2)_nR$) on gold. Several techniques (microwriting, micromachining, stamping, and UV microlithography) were used to fabricate the patterned SAMs. The most useful systems of patterned SAMs for studying the adsorption of proteins are those in which spatially-defined areas that resist protein adsorption are formed from oligo(ethylene glycol)-terminated thiols (e.g., R = $(OCH_2CH_2)_6OH$) and other areas that allow protein adsorption are formed from thiols terminated by nonpolar (R = CH_3) and ionic (R = CO_2^-, PO_3H^-, 2-imidazolo) groups. Scanning electron microscopy (SEM) allows characterization of patterns of proteins adsorbed on SAMs of alkanethiolates. The adsorbed proteins, when correctly prepared, form layers that appear to be homogeneous by SEM. When the protein layers are prepared differently, images obtained by SEM clearly show heterogeneity and defects in the layer of adsorbed proteins. The ability to assay the uniformity of coverage of surfaces by adsorbed proteins using SEM will be useful in studies involving protein adsorption.

Introduction

Phenomena associated with the adsorption of proteins to solid, synthetic materials are important in many areas of biotechnology (for example, production, storage, and delivery of pharmaceutical proteins, purification of proteins by chromatography, design of biosensors and prosthetic devices, and production of supports for attached tissue culture).[4] The importance and ubiquity of these phenomena have stimulated many studies on protein adsorption.[5] Our previous studies have demonstrated the utility of self-assembled monolayers (SAMs) of alkanethiolates on gold in studies of molecular mechanisms of protein adsorption.[6,7] In particular, these studies indicated that SAMs formed by the chemisorption of oligo(ethylene glycol)-terminated alkanethiols ($HS(CH_2)_m(OCH_2CH_2)_nOH$) resist the adsorption of a variety of proteins (e.g., avidin,[6] hexokinase,[6] pyruvate kinase,[6,7] proteins from whole, adult chicken blood,[6] fibrinogen,[7] ribonuclease A,[7] lysozyme,[7] laminin,[8] and proteins from diluted, fetal bovine serum[8]).[9]

We report here several methods that can be used to pattern SAMs incorporating oligo(ethylene glycol)-terminated alkanethiolates to create surfaces in which spatially-defined areas resist the adsorption of proteins and areas terminated in other functional groups adsorb protein. A number of other papers have demonstrated the formation of patterns of proteins[10] or the formation of patterns of cells[11] (a phenomenon almost certainly preceded by the formation of patterns in proteins). These procedures have typically relied on photolithography. For example, Bhatia *et al.* demonstrated that photo-induced oxidation of thiol-terminated alkylsiloxane monolayers produced areas resistant to the adsorption of certain proteins, presumably because they present negatively-charged sulfonate groups.[12,13] This and other photolithographic approaches offer, however, only limited control over surface chemistry. In this work, we demonstrate four techniques (including both nonlithographic and lithographic procedures) for patterning SAMs of alkanethiolates on gold. These techniques permit precise control over the chemistry of the surface

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in contact with the protein solution. These techniques are based on the spatially-controlled derivatization of gold surfaces by microwriting[14,15] and by microstamping[16] and the removal of specific areas of pre-existing SAMs using micromachining[17] and UV photolithography.[18] These techniques, in aggregate, provide a simple and flexible methodology for controlling the adsorption of proteins on solid surfaces.

As part of this work, we have also been concerned with developing techniques for imaging layers of adsorbed proteins, with resolution in the 1-μm range (i.e., comparable to the dimensions of a mammalian cell). Here we establish the value of scanning electron microscopy (SEM) as a semiquantitative tool for imaging protein layers adsorbed on SAMs. We, and others, recently introduced SEM as a technique for imaging patterned SAMs on gold.[14,19] SEM also works well for imaging adsorbed layers of proteins on these SAMs. Secondary electron emission from metals through organic overlayers, and hence image contrast in SEM, is sensitive to the thickness and the composition of the organic overlayer.[20,21] SEM provides no information about the conformation or activity of proteins on a surface but it is sensitive to low protein coverages and can be used for relatively rapid (when compared to other characterization techniques such as X-ray photoelectron spectroscopy, XPS), semiquantitative characterization at high spatial resolution.

Results and Discussion

Imaging Proteins Adsorbed on SAMs by SEM. Figure 1a shows an SEM micrograph of a patterned SAM composed of distinct areas formed by the adsorption of $HS(CH_2)_{10}CH_3$, $HS(CH_2)_{15}CH_3$, and $HS(CH_2)_{11}(OCH_2CH_2)_6OH$ (denoted as $HS(CH_2)_{11}EG_6OH$) onto gold. The patterned SAM was formed by first writing a line (the light area in the micrograph) with a micropen loaded with $HS(CH_2)_{15}CH_3$ and then pinning reactive drops of solutions of $HS(CH_2)_{11}EG_6OH$ and $HS(CH_2)_{10}CH_3$ on either side of the line.[14] Figure 1b shows a micrograph of a similar patterned SAM after it had been exposed to a solution of pyruvate kinase (EC 2.7.1.40), removed from solution, washed, and dried.[7] The area of the SAM that corresponds to R = CH_3 appeared, by SEM, uniformly darker after it had been exposed to the solution of protein than it did before exposure to the solution. The brightness of the region with R = EG_6OH was not changed by exposure to protein solution.

The relative brightness of an area of the micrograph is related to the secondary electron emission from that area of the sample.[14,20] The decrease in brightness of an area of the surface after exposure to the solution of protein reflects the adsorption of a layer of protein.[14] Comparison of the regions having R = CH_3 and R = EG_6OH before and after exposure to the solution of protein indicates that protein has adsorbed on the former but not on the latter. XPS and ellipsometry confirmed the presence of a protein adlayer (~40 Å thick) on the region of the SAM with R = CH_3; no protein was detected by either technique on the region with R = EG_6OH.[22]

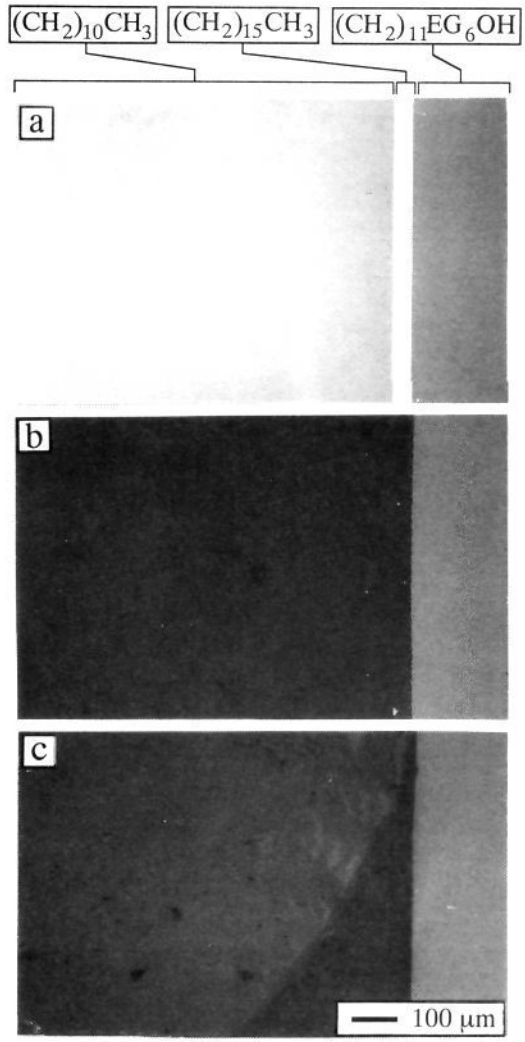

Figure 1. (a) SEM micrograph of a patterned SAM formed by the adsorption of $HS(CH_2)_{10}CH_3$, $HS(CH_2)_{15}CH_3$, and $HS(CH_2)_{11}EG_6OH$ onto gold. (b) SEM micrograph of a patterned SAM after exposure to an aqueous solution of pyruvate kinase (1 mg/mL in phosphate buffer, pH 7.5) for 1 min. (c) SEM micrograph of a patterned SAM after exposure to the same solution of pyruvate kinase through which O_2 had been bubbled for 15 min. The 100-μm scale bar applies to all micrographs.

Figure 1c shows the coverage observed by adsorption of protein from a solution of pyruvate kinase through which O_2 had been bubbled for 15 min at room temperature. This solution contained protein aggregates formed by oxidation and interfacial denaturation. The material adsorbed on the surface of the SAM is not uniform. The observed nonuniformities may reflect differences in the thickness and/or the density of the adsorbed protein layer; both properties affect the emission of secondary electrons from the surface.[20,21] There are two types of variations in the brightness of the region corresponding to R = CH_3. The first type of variation covers large areas of the region with R = CH_3 (e.g., dark, triangular region in the image) and is due to nonuniformities in the adsorbed protein layer. The second type of variation is splotches (1–50 μm in diameter) of adsorbed material that may be due to particles of protein gel. We found that, after several days, protein solutions tend to generate nonuniform adsorbed layers even on homogeneous (unpatterned) SAMs. Figure 1c demonstrates the utility of SEM in detecting nonuniformities in adsorbed protein.

Figure 2a illustrates imaging by SEM of patterned monolayers made from several types of ω-substituted alkanethiolates.[14] It also illustrates the versatility of the technique of pinning reactive drops of thiol solutions in preparing patterned SAMs with different

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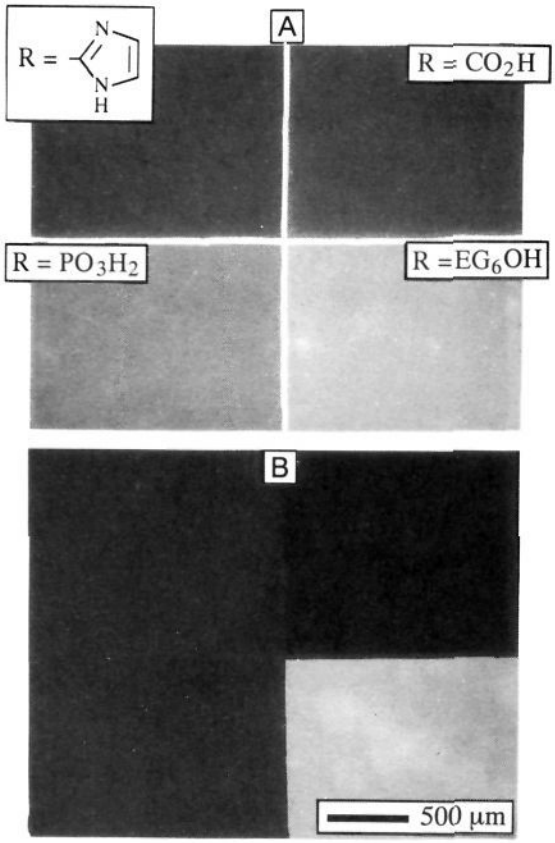

Figure 2. (a) SEM micrograph of a patterned SAM formed by the adsorption of $HS(CH_2)_{15}CH_3$, $HS(CH_2)_{10}N_2C_3H_3$, $HS(CH_2)_{11}CO_2H$, $HS(CH_2)_{11}PO_3H_2$, and $HS(CH_2)_{11}EG_6OH$ onto gold. To form the patterned SAM, two intersecting lines (light areas in the micrograph) were drawn on the bare gold using a micropen loaded with $HS(CH_2)_{15}CH_3$. Drops of ethanolic solutions (10 mM) of each of the thiols were then placed in the quadrants formed by the two intersecting lines. Drops of the thiol solution were allowed to spread to cover each quadrant and thus to react with the surface within the quadrant for 5 min. (b) SEM micrograph of the patterned SAM shown in (a) after it had been exposed to a solution of bovine carbonic anhydrase (1 mg/mL) in phosphate buffer (10 mM, pH 7.5) for 2 h.

surface chemistries. Using a micropen, we drew two intersecting lines of a SAM from $HS(CH_2)_{15}CH_3$ on a piece of bare gold. We then covered each of the quadrants formed by the two lines with an ethanolic solution (10 mM) of either $HS(CH_2)_{11}EG_6OH$, $HS(CH_2)_{10}N_2C_3H_3$ (2-(10-mercaptodecyl)imidazole), $HS(CH_2)_{15}CO_2H$, or $HS(CH_2)_{11}PO_3H$. In this way, we created a patterned surface that was composed of areas of functional groups that were polar, apolar, positively charged, or negatively charged. We believe that the ability provided by SAMs to make surfaces with different compositions and properties conveniently and consistently will be broadly useful in efforts to understand and to manipulate the interactions of proteins with solid surfaces.

Figure 2b shows an SEM of the patterned SAM containing different functional groups after it had been exposed to a solution of carbonic anhydrase (EC 4.2.1.1; 1 mg/mL in phosphate buffer, pH 7.5, 2 h). The selective darkening of the image in regions having CH_3, imidazolo, PO_3H^-, and CO_2^- terminal groups, but not in regions having EG_6OH groups, demonstrates the adsorption of proteins on the former surfaces. It also establishes that SEM can be used to image films of proteins adsorbed on these several types of SAMs. We believe that SEM will be generally useful in rapidly assessing differential effects on protein adsorption due to changes in interfacial chemistry.

Figure 3 illustrates the value of SEM as a technique for imaging proteins that are intentionally patterned on a surface. To form a patterned SAM, a micropen containing $HS(CH_2)_{15}CH_3$ was

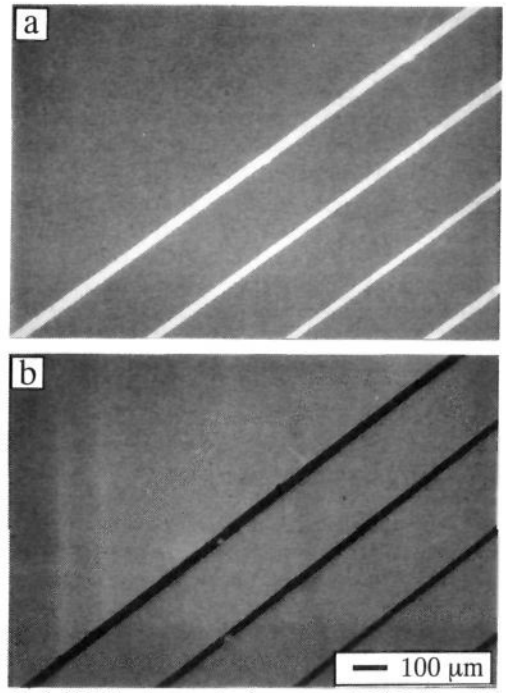

Figure 3. (a) SEM micrograph of a patterned SAM formed by first writing lines of a SAM (light areas) on a gold film with $HS(CH_2)_{15}CH_3$ and then covering the remaining surface with a SAM formed from $HS(CH_2)_{11}EG_6OH$. (b) SEM micrograph of a patterned SAM similar to that shown in (a) after it had been exposed to a solution of RNase A (1 mg/mL in phosphate buffer, pH 7.5, 2 h) that had been labeled by conjugation with eosin isothiocyanate. The 100-μm scale bar applies to both (a) and (b).

first used to write lines (light areas) of a SAM having $R = CH_3$. The gold film was then exposed to an ethanolic solution (1 mM) of $HS(CH_2)_{11}EG_6OH$ for 1 min to form the protein-resistant areas. Figure 3a shows a micrograph of the patterned SAM before the adsorption of proteins. Figure 3b shows the sample after adsorption of ribonuclease A (RNase A, EC 3.1.27.5) that had been labeled with a fluorophore (eosin isothiocyanate). The pattern of proteins detected readily by SEM was confirmed (albeit with low signal to noise) by fluorescence microscopy. SEM is a general technique for imaging protein adlayers on SAMs on gold that does not require conjugation of the proteins with dyes or other markers and that can be used to resolve features that cannot be resolved by optical microscopy.

Spatial Patterning of Adsorbed Proteins. Figure 3 shows that microwriting with alkanethiols can be used to pattern SAMs. Figure 4 illustrates three alternative techniques for making patterned SAMs: (i) micromachining of a gold surface to remove a pre-existing EG_6OH-terminated SAM, followed by the adsorption of a CH_3-terminated SAM,[17] (ii) partial photo-oxidation of a SAM formed from $HS(CH_2)_7CH_3$ followed by the adsorption of $HS(CH_2)_{11}EG_6OH$,[18] and (iii) formation of a methyl-terminated SAM by localized deposition of $HS(CH_2)_{15}CH_3$ using a poly(dimethylsiloxane) stamp, followed by the derivatization of the unreacted gold surface with $HS(CH_2)_{11}EG_6OH$.[16]

All four techniques—microwriting, micromachining, microstamping, and microlithography—can, in principle, be used to make patterns of SAMs and of proteins adsorbed on SAMs, with minimum dimensions less than 1 μm. In practice, patterns formed by UV lithography resulted in less contrast between the regions with and without adsorbed proteins than patterns formed by the other techniques (as imaged by SEM under similar conditions). This difference is probably due to lower protein adsorption on the methyl-terminated regions (relative to that observed in the methyl-terminated areas of the SAM in Figure 4a,c) because of partial

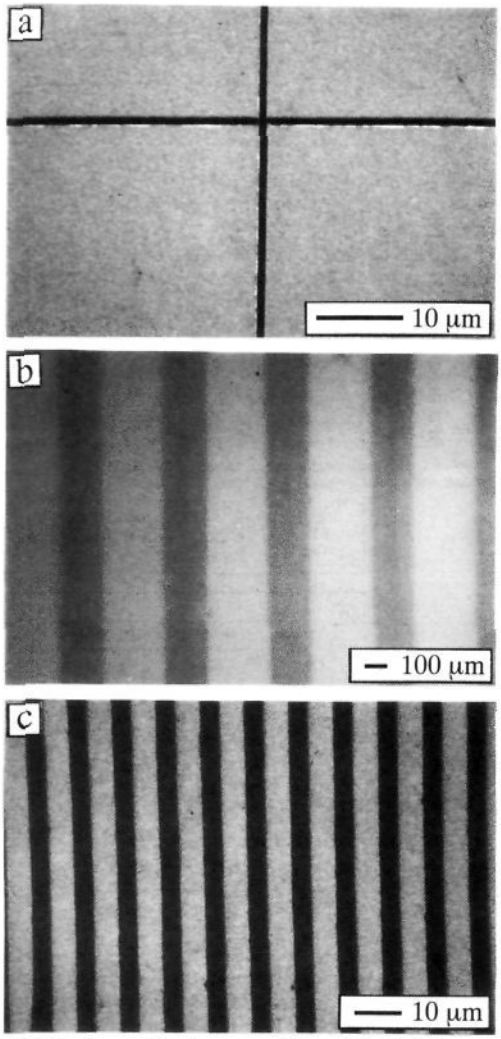

Figure 4. SEM micrographs of pyruvate kinase (adsorbed from a 1 mg/mL solution in 10 mM phosphate buffer, pH 7.5, for 30 min) adsorbed onto patterned SAMs formed by serial deposition of alkanethiols using different patterning techniques. (a) Micromachining: Patterning of the SAM was achieved by using a scalpel blade to scratch the surface of a gold film that had previously been treated with $HS(CH_2)_{11}EG_6OH$. A SAM was then formed from $HS(CH_2)_{15}CH_3$ on the freshly-exposed gold. The bright areas along the edges of the micromachined lines are present in the absence of SAMs and are due to the topography of the surface—metal shavings and sharp ridges—near the micromachined trough. (b) Optical lithography: A SAM formed from $HS(CH_2)_7CH_3$ was exposed to UV light through a metal mask in air for 0.5 h. The photo-oxidized reaction products were then removed by rinsing and displaced by reaction with $HS(CH_2)_{11}EG_6OH$ (10 mM in ethanol) for 10 s. (c) Stamping: A stamp molded from cross-linked poly(dimethylsiloxane) was used to pattern the surface of a gold film in specific areas with $HS(CH_2)_{15}CH_3$. The remainder of the surface was then derivatized by exposure to an ethanolic solution (10 mM) of $HS(CH_2)_{11}EG_6OH$ for 10 s.

displacement of the SAM formed from $HS(CH_2)_7CH_3$ by $HS(CH_2)_{11}EG_6OH$ during patterning and due to low edge resolution in the low-resolution lithographic procedure we used in these survey experiments.

Quantitative Analysis of Adsorbed Organic Layers with SEM. Figure 5A is an SEM micrograph of a patterned SAM partitioned into six regions, each containing a mixed SAM[23] with different mole fractions of $HS(CH_2)_{15}CH_3$ and $HS(CH_2)_{11}EG_6OH$. The patterned SAM shown in Figure 5A was formed by first drawing three intersecting lines using a micropen loaded with $HS(CH_2)_{15}CH_3$,[14,15] Each of the six wedge-shaped areas between these lines was filled with a different ethanolic solution that contained a

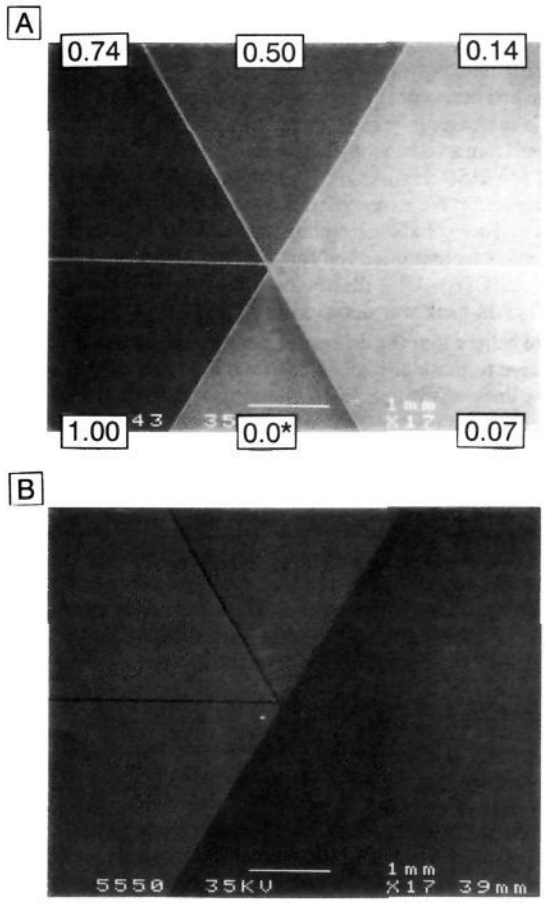

Figure 5. (A) SEM micrograph of patterned SAMs formed by first writing three intersecting lines from $HS(CH_2)_{15}CH_3$ and then adsorbing SAMs from mixtures of $HS(CH_2)_{15}CH_3$ and $HS(CH_2)_{11}EG_6OH$. The lighter lines forming the borders between regions 0.0 and 1.0 correspond to monolayers formed by the adsorption of neat $HS(CH_2)_{15}CH_3$ by microwriting. The six areas (denoted by their value of $\chi^{surface}_{EG_6OH}$) are composed of mixed monolayers formed by the pinning of drops of ethanolic solutions (10 mM total thiol) that differed in the relative concentrations of $HS(CH_2)_{15}CH_3$ and $HS(CH_2)_{11}EG_6OH$. Region 0.0 may contain non-alkanethiolate, oxygenated contaminants (please see text). (B) SEM micrograph of the patterned SAM shown in the top micrograph after exposure of the sample to a solution of pyruvate kinase (1 mg/mL in phosphate buffer, 10 min).

mixture of $HS(CH_2)_{15}CH_3$ and $HS(CH_2)_{11}EG_6OH$ at a total concentration of 10 mM. Because the lines formed from $HS(CH_2)_{15}CH_3$ are lyophobic to ethanol, each solution was bounded in the wedge-shaped area in which it was placed. Each region was therefore derivatized by a solution with specified mole fractions of $HS(CH_2)_{15}CH_3$ and $HS(CH_2)_{11}EG_6OH$.

This procedure resulted in a pattern of areas in which the mole fraction of alkanethiolates with $R = EG_6OH$ ranged from 1.0 to 0.0. We estimated the relative concentration of the different alkanethiolates by acquiring XPS spectra of spots ($\sim$600 μm in diameter) within each of the six areas. The mole fraction of alkanethiolates with $R = EG_6OH$ ($\chi^{surface}_{EG_6OH}$) in each region was calculated by dividing the intensity of the O 1s photoelectron peak obtained from that region by the intensity of the O 1s peak obtained from the region exposed to a solution of alkanethiols that contained only $HS(CH_2)_{11}EG_6OH$. For simplicity, we denote the regions by the value of $\chi^{surface}_{EG_6OH}$ obtaind for that region.

(23) We use the term "mixed SAMs" to refer to a SAM formed by coadsorption from a solution containing mixtures of more than one type of ω-functionalized alkanethiol: Bain, C. D.; Whitesides, G. M. *J. Am. Chem. Soc.* **1988**, *110*, 6560–6561. The issue of the homogeneity of these systems is addressed by: Folkers, J. P.; Laibinis, P. E.; Whitesides, G. M.; Deutch, J. *J. Phys. Chem.*, submitted for publication.

"Region 0.14" refers to the region on the patterned SAM that contained a mixed SAM with $\chi^{surface}_{EG_6OH} = 0.14$.

With the exception of region 0.0, there is a progressive decrease in the brightness of the regions as the mole fraction of R = EG_6OH is increased. In region 0.0, the area of the patterned SAM formed from an ethanolic solution that contained only $HS(CH_2)_{15}CH_3$ (no $HS(CH_2)_{11}EG_6OH$), the image appears darker than anticipated. We expected the pixel intensity for an area that consisted only of alkanethiolate formed from $HS(CH_2)_{15}CH_3$ to be similar to that observed for the lines of SAMs formed from neat $HS(CH_2)_{15}CH_3$ that bound the wedge-shaped areas.[14] A small O 1s peak was detected in region 0.0 by XPS.

We believe that the decreased average brightness of region 0.0 (relative to those of regions 0.07 and 0.14 and to that observed in the lines separating the regions) reflects the process used to form the monolayer in region 0.0. The drop of solution that was used to derivatize this region initially covered it completely but then receded spontaneously after ~10 s as a partial monolayer formed.[24] In all other regions, the drop remained in contact with the solution for the full time allowed (5 min). This spontaneous retraction of the drop edge resulted in a shorter time of contact between the solution containing the thiol and the surface of gold in region 0.0 than in the others. We believe that a consequence of the reduced reaction time is the partial population of the gold surface with adventitious contaminants that are not alkanethiolates.[25]

The relationship between relative pixel intensity and the composition of the mixed SAMs is summarized quantitatively in the top curve in Figure 6a. The value of $\chi^{surface}_{EG_6OH}$ for each of the areas in the patterned SAM (shown on the abscissa) is a direct measure of the mole fraction of alkanethiolates with R = EG_6OH in each region. Taking the intensity of pixels in the lines as representative of a SAM of pure alkanethiolates with R = CH_3, we observe a monotonic decrease in the average brightness of pixels in the different regions as $\chi^{surface}_{EG_6OH}$ is increased from 0.0 to 1.0.

This result validates the use of SEM as a semiquantitative technique for analysis of mixed monolayers. SEM may be the analytical technique of choice in applications where a short data acquisition time is required (in this example, a few minutes compared to a few hours for XPS) or where a high-resolution spatial mapping of the composition of mixed monolayers is desired (a circumstance under which use of XPS is difficult or impossible). The differences in the mechanism of sensitivity of SEM to the composition of the SAM and adlayers on it—scattering of secondary electrons by adsorbates on the gold—and of other analytical techniques (XPS, for example, which relies on photoemission of electrons from adsorbates) may make SEM particularly useful in the identification of adventitious contaminants on or in SAMs.[25] The limitation of SEM as a quantitative technique for the analysis of organic overlayers is its insensitivity to functional group character. As with regions 0.50 and 0.0, two different organic overlayers may appear similarly bright when imaged by SEM, although they may have very different chemical compositions.

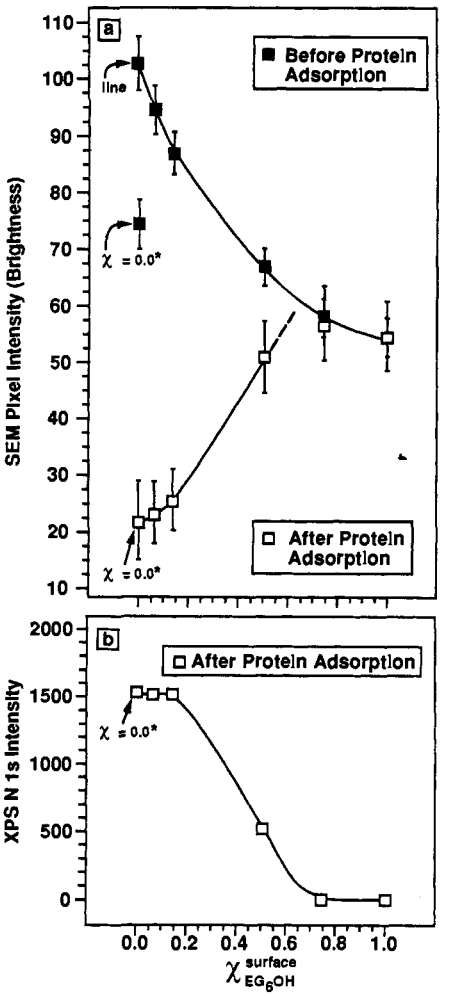

Figure 6. SEM pixel intensities and XPS N 1s peak intensities in the different areas of patterned, mixed SAMs formed from $HS(CH_2)_{15}CH_3$ and $HS(CH_2)_{11}EG_6OH$ (see Figure 5) before and after the adsorption of pyruvate kinase. (a) SEM pixel intensities of the different regions of the patterned, mixed SAMs before protein adsorption (see Figure 5A) and after protein adsorption (see Figure 5B) as a function of $\chi^{surface}_{EG_6OH}$, the estimated mole fraction of alkanethiolates with R = EG_6OH. $\chi^{surface}_{EG_6OH}$ was calculated by dividing the O 1s peak intensities measured by XPS in each region (before the adsorption of protein) by the intensity of the O 1s peak obtained for the SAM formed from a solution containing only $HS(CH_2)_{11}EG_6OH$ (region 1.00 in Figure 5A). The data point denoted as "line" was obtained by measurement of the average pixel intensity within the lines (corresponding to SAMs formed from $HS(CH_2)_{15}CH_3$ by microwriting) between regions 0.0 and 1.0. The patterned SAM was exposed to pyruvate kinase (1 mg/mL) in phosphate buffer (10 mM, pH 7.5) for 10 min. The SEM pixel intensities are presented in relative, arbitrary units. Higher values correspond to a brighter image. Pixel intensities were offset so that the average intensity of region 1.0 was the same before and after protein adsorption. (b) XPS N 1s peak intensities from analysis of spots (~600 μm in diameter) in each region of the patterned SAMs after they had been exposed to the solution of pyruvate kinase. The lines through the data are provided as guides for the eye.

Figure 5B shows an SEM micrograph of the patterned SAM shown in Figure 5A after it had been exposed to a solution of pyruvate kinase (1 mg/mL in phosphate buffer) for 10 min. The darkening of areas corresponding to regions 0.0–0.50 relative to their respective brightnesses in Figure 5A suggests the adsorption of pyruvate kinase on these regions, but not on regions 1.00 and 0.74. These latter regions have similar average pixel intensities before and after exposure to the solution of protein. On the basis of the data from SEM, we conclude that the minimum value of $\chi^{surface}_{EG_6OH}$ required for these mixed SAMs to resist adsorption of pyruvate kinase under these conditions is between 0.74 and 0.5. For comparison, an independent determination of this number

(24) In contrast to the case of mixed monolayers with R = CH_3 and R = EG_6OH components we investigated, ethanol does not wet a SAM formed from just $HS(CH_2)_{15}CH_3$. The advancing contact angle for ethanol on these SAMs is ~45°; the receding contact angle is ~30°.

(25) We have previously observed (using secondary ion mass spectrometry) the presence of species that are not alkanethiolates (e.g., $CH_3(CH_2)_{15}SO_3^-$) present on the surface of gold that has been reacted with solutions of $CH_3(CH_2)_{15}SH$ for short periods of time. The presence of such species in region 0.0 may be responsible for its relative contrast in SEM. Although these species were present at concentrations on the surface below the detection limit of routine XPS analysis, they were easily detected by SEM: Lopez, G. P.; Biebuyck, H. A.; Frisbie, C. D.; Whitesides, G. M. Unpublished data. Other researchers have detected alkanesulfonate species in SAMs after they have been exposed to air: Li, Y.; Huang, J.; McIver, R. T.; Hemminger, J. C. *J. Am. Chem. Soc.* **1992**, *114*, 2428–2432. Tarlov, M. J.; Newman, J. G. *Langmuir* **1992**, *8*, 1398–1405.

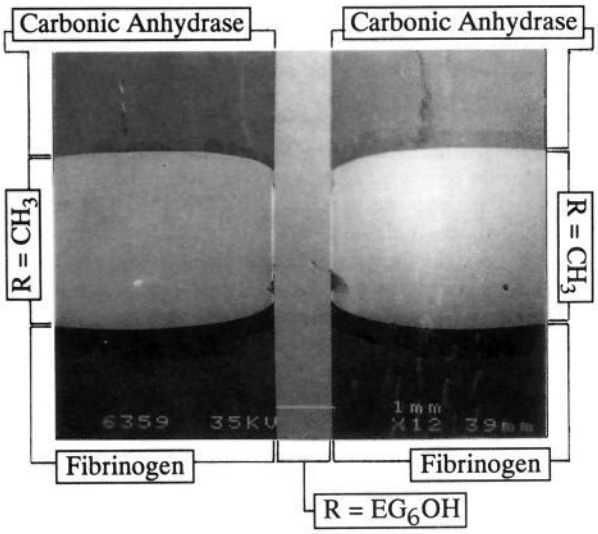

Figure 7. SEM micrograph of a patterned SAM composed of areas formed from $HS(CH_2)_{15}CH_3$, $HS(CH_2)_{10}CH_3$, and $HS(CH_2)_{11}EG_6OH$ that had been exposed in different areas to solutions of fibrinogen (bottom) and carbonic anhydrase (top). Areas formed from $HS(CH_2)_{10}CH_3$ (left and right) are separated from a central area formed from $HS(CH_2)_{11}EG_6OH$ by two lines (~100 μm wide) formed from $HS(CH_2)_{15}CH_3$. Fibrinogen was adsorbed first by dipping one side of the sample into an aqueous solution (0.1 mg/mL in phosphate buffer, pH 7.4) of fibrinogen for 30 min. After rinsing and drying, carbonic anhydrase was adsorbed by dipping the other end of the sample into a solution of protein (1.0 mg/mL in phosphate buffer, pH 7.4) for 30 min.

by ellipsometry suggested a value of 0.6 for mixed monolayers formed from $HS(CH_2)_{11}EG_6OH$ and $HS(CH_2)_{10}CH_3$.[7]

The bottom curve in Figure 6a shows quantitatively the relative, average pixel intensities of the regions of the surface after exposure to the solution of protein. Figure 6b also shows the N 1s peak areas from XPS spectra taken from each of the regions. Since pyruvate kinase was the only nitrogen-containing species on the surface, we used the intensity of the N 1s peak as a measure of the quantity of protein adsorbed on each of the regions. XPS confirmed that regions 0.0–0.50 contained adsorbed protein, while regions 0.74 and 1.00 did not. These data suggest that SEM can be used as a semiquantitative measure of the amount of adsorbed protein, provided the nature of the protein and of the underlying SAM is known.

Comparative Imaging of Different Adsorbed Proteins. Figures 5 and 6 demonstrate the value of SEM for the semiquantitative characterization of protein adlayers. Both the thickness of an adsorbed protein adlayer at saturation coverage (which may be related to the molecular weight and shape of the protein[6]) and the extent to which a protein adsorbs influence the relative intensities of the SEM image of layers of different proteins. Figure 7 illustrates the contrast between adlayers of two proteins, fibrinogen and carbonic anhydrase. The larger protein, fibrinogen (MW ~340 000), forms an adlayer that is darker by SEM than that of carbonic anhydrase (MW ~30 000). The curved pattern of protein left on the surface of the patterned SAM in both cases reflects the shape of the meniscus of the contact line between the solution of protein, the SAM, and the air. The figure also shows clear evidence of nonuniformities in the protein layers (especially around the solution/surface/air line). This sensitivity to heterogeneity should be very useful in characterizing adsorbed layers of proteins.

The ability to distinguish protein adlayers on the basis of their relative appearance may be useful in interpreting complex patterns of proteins and in probing processes (e.g., displacement, elution, and conjugation) involving protein adlayers and solutions of different proteins or other surfactants. Figure 8 illustrates the relative intensities for patterned adlayers of three different

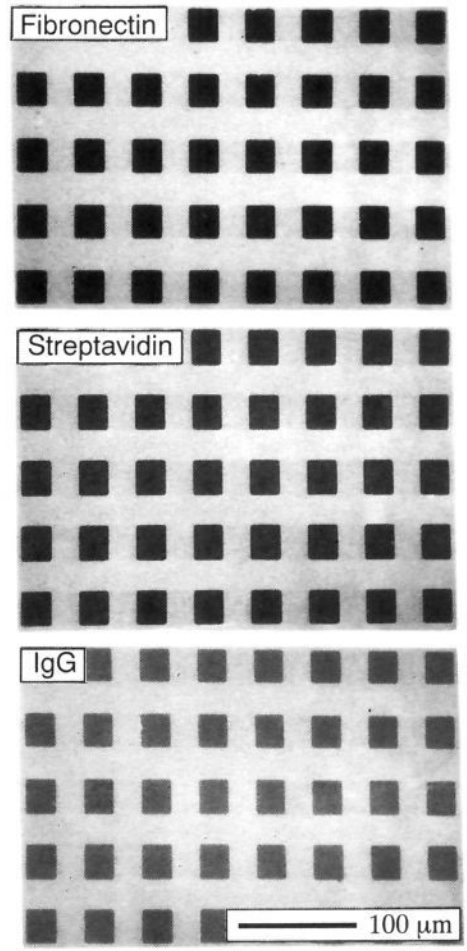

Figure 8. SEM micrographs of adsorbed proteins spatially patterned on a surface by adsorption onto patterned SAMs (formed using a rubber stamp) from $HS(CH_2)_{15}CH_3$ and $HS(CH_2)_{11}EG_6OH$: (a) fibronectin; (b) streptavidin; (c) bovine IgG. Each protein was adsorbed from a 0.1 mg/mL solution in phosphate buffer (pH 7.4) for 2 h. The 100-μm scale bar in (c) also applies to (a) and (b).

proteins—fibronectin, streptavidin, and immunoglobulin G (IgG)—that may be useful in controlling molecular recognition at the interfaces of synthetic solids and biological systems.

Heterogeneities in Adlayers on SAMs. Figure 9 shows several additional examples of heterogeneities in coverage that can arise from the adsorption of macromolecules (e.g., poly(lysine, glutamic acid)) and complex mixtures of proteins (diluted, heat-inactivated, fetal bovine serum). The micrographs in Figure 9 provide further evidence that SEM is able to detect nonuniformities in coverage by proteins and other macromolecules. It is not clear at what point in the protocol for sample handling the heterogeneities arise.[26]

Protein adsorption has been studied using many *ex situ* analytical methods—radioquantitation,[27] ellipsometry,[7] and XPS[7,28]—that assume uniform coverage by the protein for quantitation. Recently, static SIMS was also used to study

(26) We believe that the circular heterogeneities on the areas corresponding to R = CH_3 are due to the uneven drying of the surface after the adsorption of proteins and poly(amino acids). No adsorbed proteins were detected by XPS or ellipsometry on areas with R = EG_6OH when they were exposed to diluted, heat-inactivated, fetal bovine serum.

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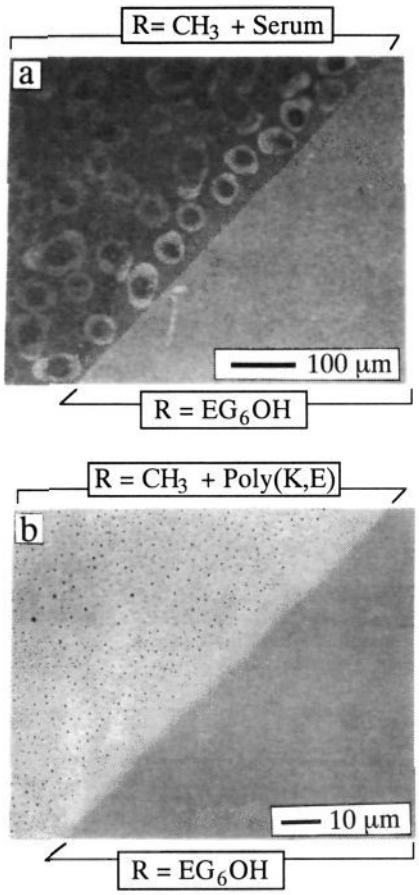

Figure 9. (a) SEM micrograph of a patterned SAM formed from $HS(CH_2)_{15}CH_3$ and $HS(CH_2)_{11}EG_6OH$ that had been exposed to an aqueous solution of fetal bovine serum (10% by volume serum in 10 mM phosphate buffer, pH 7.4) for 2 h. (b) SEM micrograph of a patterned SAM formed from $HS(CH_2)_{15}CH_3$ and $HS(CH_2)_{11}EG_6OH$ that had been exposed to an aqueous solution (1 mg/mL in 10 mM phosphate buffer, pH 7.4) of poly(lysine, glutamic acid)(poly(K,E), 6:4, MW 30 kDa, random copolymer) for 1 h.

adsorbed proteins.[29] Imaging of adsorbed proteins by SEM will complement these techniques and will be useful, in general, in the development of experimental protocols for studying protein adsorption.

Conclusion

We have demonstrated that patterned SAMs incorporating areas formed by the adsorption of oligo(ethylene glycol)-terminated alkanethiols control the two-dimensional distribution of proteins adsorbed to solid, synthetic surfaces. Several techniques are useful in preparing these SAMs and allow the patterning of proteins. Microwriting and pinning of reactive drops of thiol solutions are convenient, versatile techniques that result in patterned surfaces with well-defined chemistry and with features having dimensions from 10 μm to 10 cm. Stamping and UV microlithography are somewhat more complicated procedures that result in surfaces with compositions that are less well defined, because these techniques rely on the successive exposure of the sample to solutions of different thiols. These techniques are, however, very useful for generating surfaces with complex patterns in their composition and structure. They can, in principle, be used to pattern surfaces over dimensions as small as 1 μm. Micromachining is a convenient, nonlithographic technique that is capable of making simple patterns in surfaces with features as small as 0.1 μm.[17]

(29) Mantus, D. S.; Ratner, B. D.; Carlson, B. A.; Moulder, J. F. *Anal. Chem.*, in press.

SEM is a general method for imaging patterns of macromolecules adsorbed on SAMs of alkanethiolates on gold. Other methods for imaging protein adlayers—fluorescence microscopy, SIMS, autoradiography—can be experimentally inconvenient or can require chemical modification of the protein. We have demonstrated that SEM can be a semiquantitative imaging technique that maps the distribution of proteins adsorbed to the surface of a SAM and that SEM is useful in the detection of artifacts in the study of the adsorption of proteins to solid materials.

We believe the primary factor that determines the relative emission of secondary electrons from, and hence the relative contrast of, different areas of a surface patterned with proteins is the degree to which the adsorbed layer attenuates electrons generated in the underlying gold.[14] We expect both the thickness and the density of the adsorbed protein layer to determine the relative degree of attenuation of electrons.[20,21] The ultimate spatial resolution of SEM as a technique for imaging films of adsorbed macromolecules remains to be determined. We do not expect to be able to obtain information on the relative conformation or orientation of individual protein molecules adsorbed on surfaces by this technique.

Experimental Section

Materials. Dry N_2 was bubbled through absolute ethanol (Quantum Chemical Corp.) to remove O_2 from it. $HS(CH_2)_{11}(OCH_2CH_2)_6OH$[7] and $HS(CH_2)_{15}CO_2H$[30] were available from previous studies. $HS(CH_2)_{10}CH_3$ (Aldrich) was distilled prior to use. $HS(CH_2)_{15}CH_3$ (Aldrich) was purified by chromatography through silica gel prior to use. $HS(CH_2)_{15}PO_3H_2$ and 2-(10-mercaptodecyl)imidazole ($HS(CH_2)_{11}N_2C_3H_3$) were available from previous studies.[31] All proteins except pyruvate kinase (Biozyme Laboratories) and heat-inactivated fetal bovine serum (GIBCO, BRL) were obtained from Sigma Chemicals. Eosin-labeled RNase A was prepared by gently stirring 10 mg of RNase A in 2 mL of sodium borate buffer (0.1 M) with 0.2 mL of an eosin isothiocyanate (EITC, Sigma) stock solution (2.6 mg in 1 mL of borate buffer) in the dark.[32] Gel permeation chromatography (Bio-Gel P6, medium) was used to separate the unbound fluorescent label from labeled protein. Using UV spectrophotometry, the average number of labels per protein was determined to be 0.5 (EITC, ϵ_{278} = 16 000 M^{-1} cm^{-1}; RNase A, ϵ_{278} = 9420 M^{-1} cm^{-1}; EITC-protein, ϵ_{528} = 73 100 M^{-1} cm^{-1}).[33]

Preparation of Substrates. Gold films (~2000 Å thick) were prepared by electron-beam evaporation of gold (Materials Research Corp., Orangeburg, NY; 99.999%) onto single-crystal silicon (100) test wafers (Silicon Sense, Nashua, NH; 100 mm in diameter, ~500 μm thick) that had been precoated with a film of titanium (Johnson Mathey, 99.99%; ~50 Å thick) that acted as an adhesion promoter between the silicon oxide and the gold. The silicon wafers coated with gold were fractured into square slides (~2 cm × 2 cm) and used in the formation of the various types of patterned SAMs.

Formation of Monolayers. (a) Writing with Micropens[14,15] (Figures 1–3, 5, 7, and 9). Slides were placed flat on a stage movable by rotation of a micrometer. A pen tip (Staedler, 757 030) was filled with $HS(CH_2)_{15}CH_3$ and suspended above the gold slide. The slide was raised and a meniscus was allowed to form between $HS(CH_2)_{15}CH_3$ on the pen tip and the surface of the gold. Translation of the slide relative to the pen using the micrometer controls resulted in the formation of a line of a SAM. The width of the line was controlled by maintaining a meniscus (viewed through a microscope) of approximately constant volume as the line was formed. After formation of this line of SAM from $HS(CH_2)_{15}CH_3$, the slide was rinsed with heptane and then with ethanol. The slide was dried under dry N_2. For Figures 5 and 9, the remainder of the surface was derivatized by reaction with $HS(CH_2)_{11}EG_6OH$ by dipping the slide in a 1 mM ethanolic solution of the thiol for 1 min.

(b) Pinning of Reactive Thiol Solutions[14] (Figures 1, 2, 5, and 7). To form the SAMs on either side of a line formed from $HS(CH_2)_{15}CH_3$, regions adjacent to the line were reacted by exposure to ethanolic solutions

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(10 mM) of the desired alkanethiol(s). Each solution was added in drops from a Pasteur pipet on one side of the line with R = CH_3. Because of the lyophobicity of the line, the solutions of alkanethiols did not cross the line with R = CH_3.[24] For each type of SAM, except those which formed lyophobic surfaces, the solution was allowed to react with the gold surface for at least 5 min before rinsing the slide with heptane and ethanol as described above. XPS suggested that SAMs formed in this manner have compositions similar to those formed as described previously.[7]

(c) Micromachining[17] (Figure 4a). A SAM was formed on the surface of a gold film by placing the slide in a 1 mM solution of $HS(CH_2)_{11}EG_6OH$ for 2 h. The slide was then placed on a movable stage, and the surface of the gold was scratched by the application of a load (~3 mN) through a scalpel tip as described elsewhere.[17] This procedure does not expose the underlying silicon interface and results in the formation of a groove in the gold film and the exposure of unreacted gold surface.[17] The exposed gold surface was derivatized by reaction with an ethanolic solution (1 mM) of $HS(CH_2)_{15}CH_3$ for 1 min.

(d) UV Photolithography[18] (Figure 4b). A SAM was formed on the surface of a gold film by placing the slide in a 1 mM solution of $HS(CH_2)_7CH_3$ for 2 h. A metal mask was then clipped to the top of the slide, and the surface was irradiated in ambient atmosphere using a water-cooled, medium-pressure mercury lamp (lamp PC 451-050, Model 7830 power supply, Ace Glass Co.) held 2 cm from the surface of the sample for 30 min. The mask was removed from the slide, and the slide was rinsed with heptane and then ethanol and dried with a stream of dry nitrogen. The slide was then placed in a 10 mM ethanolic solution of $HS(CH_2)_{11}EG_6OH$ for 10 s and rinsed and dried again.

(e) Rubber Stamping[16] (Figures 4c and 8). Each of these patterned SAMs was prepared by first forming a pattern of $HS(CH_2)_{15}CH_3$ on gold using a rubber stamp made of poly(dimethylsiloxane). The procedure is described elsewhere.[16] The remaining, underivatized surface of the gold was derivatized by placing the patterned slide in a solution containing $HS(CH_2)_{11}EG_6OH$ (10 mM in ethanol) for 10 s. The sample was rinsed with heptane and with ethanol and dried under a stream of dry nitrogen.

(f) Protein Adsorption. Buffer solutions were prepared from potassium dihydrogen phosphate (0.01 M) and titrated to pH 7.5 with sodium hydroxide (0.1 M). A modification of the general protocol described previously was used for effecting adsorption of proteins to the SAMs and rinsing of the adsorbed layers of protein.[7] Briefly, this protocol involved the immersion of SAMs in solutions of the protein of interest in phosphate buffer at room temperature. After the desired time of immersion, the SAM was removed from the solution, rinsed with distilled deionized water, and dried under a stream of nitrogen. To pattern an adsorbed protein, we found that it was desirable to use a modification of the protocol of Horbett[27] and first to place the patterned SAM in buffer and then to add concentrated protein solution so that the final, total concentration of protein in the solution in contact with the SAM was that desired for adsorption (e.g., 1 mg/mL).[27] This procedure eliminated the exposure of the SAM to the interface of the solution of protein and air. After the appropriate adsorption time, the solution of protein was displaced with at least five equivalent volumes of distilled deionized water.[27] The samples were further rinsed directly with distilled deionized water and dried under a stream of nitrogen.

Instrumentation. Images were acquired in the secondary electron detection mode of a JEOL JSM-6400 scanning microscope operating with a chamber pressure of $\sim 5 \times 10^{-6}$ Torr, using a 35-kV primary electron beam with a current of 1–2 nA. The electron detector was operated with a collection voltage of +300 V. For acquisition of the micrographs, the electron beam was slowly scanned over the image area for a total exposure time of 80 s. For analysis of image pixel intensities, a videotape recorder (Mitsubishi) was used to record images directly from the microscope. Individual frames from the videotape were then captured using a frame grabber (8 bits/pixel, Mediagrabber, RasterOps Inc.) and analyzed using software available from the National Institutes of Health (Image, version 1.49).

XPS was carried out using an SSX-100 spectrometer (Surface Science Instruments) using monochromatic Al Kα X-rays as described previously.[34] Ellipsometric measurements were made with a Rudolf Research Type 43603-200E ellipsometer as described previously.[34] The thicknesses of the adsorbed layers of proteins were calculated with a planar, three-layer (ambient–protein–SAM), isotropic model, with assumed refractive indices of 1.00 and 1.45 for the ambient and the protein, respectively.[7] Fluorescence imaging was done on a Leitz Laborlux S microscope.

Acknowledgment. We thank John P. Folkers, Nicholas L. Abbott, and Rahul Singhvi for providing technical assistance.

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