Immunosensing Platforms Using Spontaneously Adsorbed Antibody Fragments on Gold

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This paper describes the construction and characterization of miniaturized antigenic immunosurfaces composed of spontaneously adsorbed Fab′-SH fragments on gold. Rabbit Fab′-SH fragments contain a free sulfhydryl group that forms a thiolate bond with a gold substrate as detailed by X-ray photoelectron spectroscopy. This approach creates surfaces of higher epitope density, a factor critical to the early detection of disease, than surfaces composed of adsorbed whole molecule IgG on gold. The viability and specificity of antigenic Fab′-SH immunosurfaces is demonstrated using atomic force microscopy and confocal fluorescence microscopy, and possible explanations for the larger epitope density are discussed.

Early diagnosis of disease is critical in determining patient outcome. Early detection using immunological methods, however, is often complicated because low-affinity antibodies dominate the first stages of the immune response. The detection of low-affinity antibodies, moreover, is intimately linked to epitope density.[1–3] Indeed, a report using ELISA found that even modest increases in epitope density result in a marked improvement in the ability to detect lower affinity antibodies.[1] Maximizing epitope density is then critical to early disease detection.

We recently described an immunoassay concept that exploits the topographic imaging capabilities of the atomic force microscope (AFM).[4] The assay is based on the height change that results from the specific binding of a ligand in solution to its surface-immobilized receptor. We demonstrated the capability of this technique using compositionally patterned surfaces created by combining photolithography and self-assembled monolayer technologies. To this end, micrometer-sized domains of a monolayer of a gold-supported coupling agent were surrounded by micrometer-sized grids of a strongly hydrophobic monolayer.
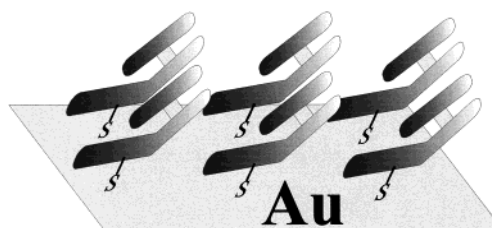

Figure 1. Idealized representation of Fab′-SH fragments chemisorbed to gold.

Thus, the coupling agent served as a means to tether IgG antigens in a spatially defined array, and the hydrophobic monolayer functioned as an internal reference plane for the height-based determination of specific binding. However, the large number of lysine residues, the target of the coupling agent, that are distributed throughout the IgG structure[5] resulted in a variety of orientational dispositions for immobilized IgG, a factor that influences the effective activity of immobilized antibodies.[5]

This paper investigates the use of spontaneously adsorbed Fab′-SH fragments for the construction of an antigenic immunosurface. The immunosurface is formed as a patterned array by using rabbit IgG Fab′ fragments, Fab′-SH, as a model test system. These fragments contain a free sulfhydryl group and were prepared by the controlled chemical reduction of rabbit F(ab′)$_2$. We postulated that if the free sulfhydryl group influences the immobilization process by its chemisorption to gold, the attachment may regulate the orientation of adsorbed Fab′-SH as well as minimize the decreases in activity due to denaturation. If so, the immobilization of fragmented antigens rather than whole molecule analogues could potentially yield an immunosurface with a higher epitope density. Figure 1 idealizes this concept.

Previous reports have investigated the use of Fab′-SH fragments and various linking chemistries that target the free sulfhydryl group to create immunosurfaces where the fragments act as the antibody rather than the antigen.[6–10] These efforts,

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however, have produced mixed results in their ability to bind more antigen when compared to surfaces created from whole molecule IgG or from Fab'-SH immobilized through its amine functionalities. To our knowledge, there have been no previous reports on the use of spontaneously adsorbed Fab'-SH fragments for construction of antigenic surfaces. This strategy, which does not rely on any chemical linkers to attach Fab'-SH to our chosen substrate, may also prove useful in elucidating the role of the linkers in systems where the fragments act as the capture antibody. Of note, there has been a brief report on an AFM characterization of Fab'-SH coated on gold, but use in an assay was not discussed.[11]

We report herein the formation of a patterned array of immunospecific Fab'-SH surfaces. We show by applying thin-layer cell spectroscopy that immunosurfaces constructed from the spontaneous adsorption of Fab'-SH on gold contain a higher epitope density when compared to those prepared by the adsorption of whole molecule analogues. Furthermore, the adsorbed fragments form viable immunosurfaces as is demonstrated using both AFM and fluorescence microscopy as readout methods. The potential merits of this strategy are briefly described.

EXPERIMENTAL SECTION

A. Gold Substrate Fabrication. Substrates were prepared using silicon wafers ((100) single crystals, Montco Silicon) or glass slides (Fisher). The silicon substrates were cleaned in an ultrasonic bath for 30 min in water and for 30 min in methanol. The glass substrates were cleaned in a dilute Micro (Cole-Parmer) solution and rinsed with deionized water and methanol. All substrates were then dried in a stream of high-purity argon (Air Products) and placed in a vacuum evaporator (Edwards High Vacuum Products). Next, the substrates were coated with 15 nm of chromium at 0.1 nm/s followed by 300 nm of gold (99.9%) at 0.3−0.4 nm/s. During coating, the pressure in the deposition chamber was $\sim 8 \times 10^{-6}$ Torr. The substrates were either used immediately upon removal from the evaporator or stored under dry nitrogen.

B. Monolayer Formation and Photopatterning (Steps 1 and 2). The preparation of the compositionally patterned monolayers paralleled the process previously described.[4] Step 1 involved the formation of a fluorinated monolayer by immersing the gold-coated substrates in a 10 mM solution of recrystallized $(SH(CH_2)_2$-$(CF_2)_7CF_3)$ for ~ 24 h.[12−14] These samples were then rinsed extensively with ethanol (Quantum, punctilious grade) and dried under a stream of argon.

Step 2 entailed the photopatterning process and followed the guidelines described in the literature.[15] The patterns were prepared by sandwiching carefully a copper transmission electron microscopy (TEM) grid (2000 mesh, hole size, 7.5 μm; bar size, 5.0 μm) (Electron Microscopy Sciences) between the monolayer-coated sample and a quartz plate. A 200-W, medium-pressure

mercury lamp (Oriel) was used as the light source. The beam was collimated, reflected off an air-cooled, dichroic mirror (220−260 nm), focused by a fused-silica lens, and passed through the TEM grid before impinging on the sample for ~ 20 min. During this process, the irradiated gold-bound thiolates are converted to various forms of oxygenated sulfur (e.g., RSO_3^-)[15−17] which are readily rinsed off the surface with most organic solvents.[17] Immediately following irradiation, the surfaces were rinsed extensively with deionized water and ethanol and dried under a stream of argon.

C. Preparation and Immobilization of Fab'-SH Fragments at Gold (Step 3). Fab'-SH fragments were obtained from the controlled chemical reduction of rabbit $F(ab')_2$, a truncated form of whole molecule IgG,[18] by using 2-mercaptoethylamine (2-MEA). This step cleaves the single disulfide bond which holds the two heavy chains of rabbit $F(ab')_2$ together to form two Fab'-SH fragments. We note that rabbit Fab'-SH fragments contain four disulfide bonds, some of which lie close to the antigen binding site. However, Fab'-SH fragments in which these disulfide bonds are reduced generally retain their structure and specific binding activity.[19] In addition, adjustment of the reaction conditions (e.g., concentration of 2-MEA and the duration and temperature of incubation) greatly minimizes the reduction of disulfide bonds near the antigen binding region.[20] We found that a 90-min incubation at 37 °C using 2-MEA at 50 mM was optimal for reducing rabbit $F(ab')_2$ at a concentration of 5 mg/mL to rabbit Fab'-SH with minimal cleavage of the disulfide bonds in the antigen binding region. Indeed, analysis of rabbit $F(ab')_2$ and its reduction product by matrix-assisted laser desorption/ionization mass spectrometry revealed the expected molecular weights of 89 189 $\pm$ 89 and 45 638 $\pm$ 46, respectively.

The progress of the reduction was followed by analyzing a small aliquot of the reaction mixture, which was first blocked with 5 μL of 700 mM iodoacetamide in 1.5 M Tris-HCl buffer (pH 8.5) to convert the free sulfhydryl to an amine-terminated thioether appendage and thus prevent the oxidation back to the disulfide, by nondenaturing sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). Upon completion of the reduction, the reaction mixture was transferred to a centrifuge tube containing a cellulose acetate filter with a nominal molecular weight limit of 5000 (Sigma) and spun at 6000 rpm to remove excess 2-MEA, which stopped the reduction. Aliquots of phosphate buffer (PB; pH 6) were added to the sample during centrifugation to avoid precipitation of Fab'-SH and to minimize its adsorption on the filter. Centrifugation continued until the desired volume (~ 50 μL) was achieved. The patterning process was completed by injecting the solution of the Fab'-SH fragments into the AFM liquid cell, whereby Fab'-SH chemisorbs onto the exposed, square-shaped regions of the gold substrate which was mounted to the cell.

D. Instrumentation. (i) Atomic Force Microscopy. AFM experiments were performed on a Nanoscope IIIa AFM (Digital

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Instruments) equipped with a 150-μm scanner. Oxide-sharpened Si$_3$N$_4$ cantilevers were used for both contact and tapping mode imaging (Nanoprobes, Digital Instruments). The normal bending force constant of the cantilevers was 0.06 N/m. When tapping mode imaging was employed, the set point amplitude of the cantilever was maximized relative to its free space amplitude to minimize the imaging force applied to the surface. In situ imaging was conducted either in 50 mM phosphate-buffered saline (PBS; pH 6) or in "binding buffer" (100 mM Tris, 100 mM MgCl$_2$, 50 mM NaCl, pH 7.6, and 1% (v/v) Tween 80). Tween 80 was used to minimize complications from nonspecific adsorption.[4] All images are presented without any subsequent data processing.

(ii) Confocal Fluorescence Microscopy. Fluorescence images were acquired using an Odyssey confocal scanning laser microscope (Noran Instruments) in combination with an Axiovert 135 inverted microscope (Zeiss). For all fluorescence experiments, 16 confocal fluorescence images were collected and averaged using 488-nm excitation, a 515-nm low-pass barrier filter (rejection at 488 nm, 4×10^{-4}), and a 25-μm slit width.

(iii) X-ray Photoelectron Spectroscopy (XPS). A Physical Electronics Industries 5500 surface system was used for XPS characterizations. This system is equipped with a hemispherical analyzer, toroidal monochromator, and multichannel detector. A pass energy of 29.35 eV was used with a resolution of ~0.3 eV. Monochromatic Al Kα radiation (1486.6 eV) at 300 W was used for excitation. Photoelectrons were collected at 45° from the surface normal, with a 36-min acquisition time for the whole molecule IgG and Fab′-SH modified surfaces, 12 min for the alkanethiol- and alkanedithiol-modified surfaces, and 53 min for the thioether-modified version of our Fab′-SH surface. The Au(4f$_{7/2}$) emission band served as an internal reference for binding energies. The base pressure of the XPS chamber was less than 9×10^{-10} Torr during all analyses, and the sampling area was ~2 mm^2. Spectral deconvolution used Gaussian profiles with a peak width constraint of 0.9−1.3 eV and a relative area ratio of 2:1 ((2p$_{3/2}$)/(2p$_{1/2}$)).[21]

As with the samples used for thin-layer cell spectroscopy (see below), samples of whole molecule IgG were prepared by adsorbing the antibody from PBS onto an uncoated gold surface and then storing the sample overnight in a humidified chamber at room temperature. Samples of Fab′-SH and iodoacetamide-treated Fab′-SH were prepared in PB in an analogous manner. Samples of 1,9-nonanedithiol and 1-decanethiol were prepared by overnight immersion of gold substrates in ~1 mM ethanolic solutions. Prior to XPS characterization, all samples were rinsed in deionized water or ethanol and dried in an argon stream.

(iv) Thin-Layer Spectroscopy. A long optical path length thin-layer spectroscopy cell was constructed as previously described.[22] These cells, by virtue of their long optical path length and large gold surface area to solution volume ratio, provide ample sensitivity to monitor quantitatively a wide range of interfacial processes, including adsorption, desorption, and electrocatalysis.[23−26] Using
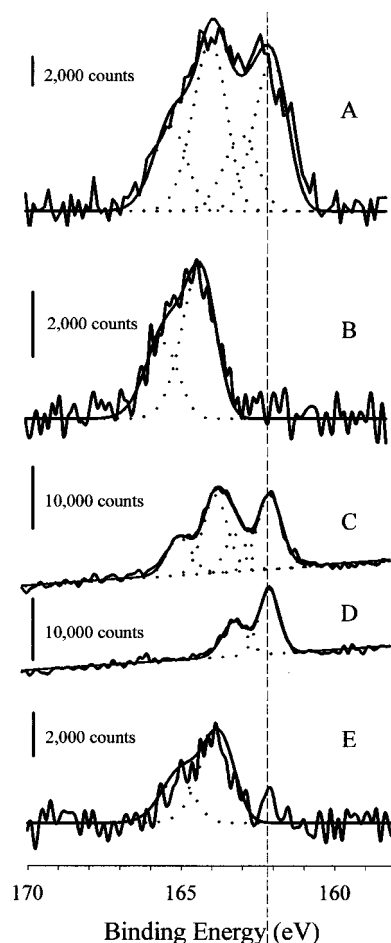
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Figure 2. XPS data in the S(2p) binding regions for rabbit Fab′-SH (A), whole molecule rabbit IgG (B), and iodoacetamide-treated rabbit Fab′-SH (E) modified gold substrates. The spectra for 1,9-nonanedithiol (C) and 1-decanethiol (D) on gold are included for comparison. Spectra A and B were acquired in 36 min, spectra C and D in 12 min, and spectrum E in 53 min.

these cells, experiments were conducted to determine whether the adsorption of Fab′-SH resulted in an immunosurface with a greater effective epitope density than found with whole molecule antibodies. The assessment was accomplished by monitoring the loss of both specific and nonspecific antibodies tagged with fluorescein isothiocyanate (FITC) upon injection into the cell with substrates coated with either whole molecule IgG or Fab′-SH. The exposed substrate area of the TLC was 2.54 cm^2. The spectroscopic data were collected using a Hewlett-Packard 8452A diode array spectrophotometer.

E. Reagents. Lyophilized polyclonal rabbit F(ab′)$_2$ (Organon Teknica and ICN Biomedical) and lyophilized polyclonal goat IgG and rabbit IgG (Sigma) were reconstituted according to vendor specifications. Affinity-isolated, antigen-specific, FITC-conjugated polyclonal goat anti-rabbit IgG and rabbit anti-goat IgG, 2-MEA, and iodoacetamide were purchased from Sigma and used as received. 1-Decanethiol and 1,9-nonanedithiol were purchased from Aldrich and used as received. Stock solutions of 2-MEA were prepared in PB. The vendor-specified extinction coefficients for the FITC-labeled goat anti-rabbit IgG and rabbit anti-goat IgG at 495 nm were 0.15 and 0.2 cm^{-1} (mg/mL)$^{-1}$, respectively.

RESULTS AND DISCUSSION

A. XPS Characterizations. To assess whether the immobilization of Fab′-SH on gold results in the formation of a gold-bound thiolate, characterizations in the S(2p) binding energy region were conducted using XPS. The S(2p) region is characterized by a doublet (i.e., $2p_{3/2}$ and $2p_{1/2}$) between 160 and 180 eV that arises from spin−orbit coupling.[27] For most thiols and disulfides, this region is dominated by the more intense $2p_{3/2}$ band that lies between 163 and 165 eV and is ∼1 eV lower in energy than the $2p_{1/2}$ band. In contrast, monolayers derived by the chemisorption of thiols and disulfides at gold have a binding energy for each component of the 2p doublet that is ∼1 eV lower than those of their precursors.

The results of these characterizations are presented in Figure 2. Figure 2A is an XPS spectrum for gold modified with rabbit Fab′-SH, whereas Figure 2B is that for gold modified with whole molecule rabbit IgG. For comparison purposes, Figure 2C is an XPS spectrum for gold modified with 1,9-nonanedithiol and Figure 2D is that for gold modified with 1-decanethiol. All four spectra have been deconvoluted using the constraints described in the Experimental Section. The spectrum from the dithiol-modified surface (Figure 2C) contains two sets of doublets, indicating the presence of two chemically distinct forms of sulfur.[27,28] The higher binding energy doublet, centered at 164.9 and 163.7 eV, can be assigned respectively to the $2p_{1/2}$ and $2p_{3/2}$ of sulfur as a thiol, whereas the lower binding energy doublet, centered at 163.0 and 162.0 eV, can be respectively assigned to the $2p_{1/2}$ and $2p_{3/2}$ of sulfur as gold-bound thiolate. The latter assignment is confirmed by the single doublet present in the 1-decanethiol-modified surface (Figure 2D). In Figure 2D, the doublet, centered at 163.1 and 162.0 eV, arises from the $2p_{1/2}$ and $2p_{3/2}$ of sulfur as thiolate, the only detectable form of sulfur present in this adlayer.

Although difficult to dissect quantitatively, the envelope for gold modified with Fab′-SH (Figure 2A) is much broader than that for the gold modified with whole molecule IgG (Figure 2B). Importantly, the broadening for the Fab′-SH-modified sample occurs on the low-energy side of the envelope for the whole molecule-modified sample. Deconvolution of the Fab′-SH surface reveals two sets of doublets; the lower energy doublet is centered at 162.9 and 161.9 eV and is in good agreement with the thiolate doublets in Figure 2C,D. The envelope in the spectrum for Figure 2B, which includes the $S(2p_{1/2})$ and $S(2p_{3/2})$ bands at 165.5 and 164.2 eV, respectively, is consistent with the presence of the sulfur that forms the intact disulfides of whole molecule IgG. Similarly, Figure 2A is diagnostic of the presence of adsorbed Fab′-SH that contains disulfides (165.2 ($2p_{1/2}$) and 163.9 eV ($2p_{3/2}$)) and gold-bound thiolates (162.9 ($2p_{1/2}$) and 161.9 eV ($2p_{3/2}$)). These results, while not quantifiable because of the low signal-to-noise ratio and considerations of XPS depth sensitivity, clearly indicate that a portion of the sulfur in Fab′-SH is present in the adlayer as gold-bound thiolate.

The spectrum (Figure 2E) taken of gold modified with Fab′-SH fragments in which the sulfhydryl group was converted to a thioether by treatment with iodoacetamide supports the interpretation of Figure 2A,B. Thioethers adsorb on gold with both C−S
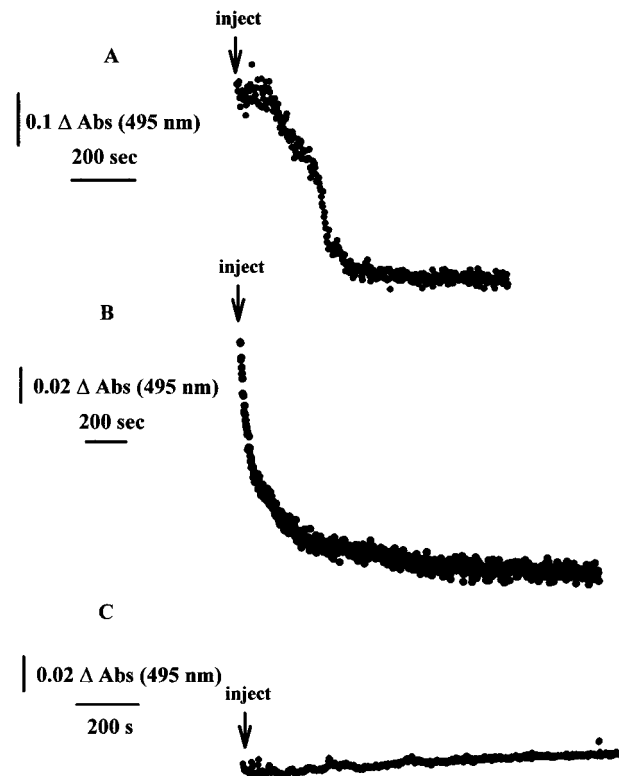

Figure 3. Absorbance versus time traces at 495 nm using a thin-layer cell for (A) rabbit Fab′-SH-modified gold exposed to FITC-labeled goat anti-rabbit IgG (extinction coefficient, 0.15 cm^{-1} (mg/mL)$^{-1}$) and (B, C) whole molecule goat IgG-modified gold exposed to (B) FITC-labeled rabbit anti-goat (extinction coefficient, 0.2 cm^{-1} (mg/mL)$^{-1}$) and (C) FITC-labeled goat anti-rabbit IgG (extinction coefficient, 0.15 cm^{-1} (mg/mL)$^{-1}$).

linkages intact.[21] Moreover, the S(2p) couplet for an adsorbed thioether is indistinguishable from that of its precursor (164.4 ($2p_{1/2}$) and 163.2 eV ($2p_{3/2}$))[21] as well as that of unbound disulfide. The conversion of the sulfhydryl group to a thioether moiety can then be used to assess whether the presence of the gold-bound thiolate in the Fab′-SH adlayer is a consequence of a reaction of the sulfhydryl group or of the chemisorbed-induced cleavage of any of the four disulfides in the Fab′-SH fragment. These results reveal features strongly diagnostic of sulfur present as disulfides and thioethers and at most a hint of a feature suggestive of a small amount of sulfur as a thiolate. The low signal strength of the features in Figure 2E with respect to those in Figure 2A further indicates that the presence of the sulfhydryl group results in a larger coverage for Fab′-SH than for the thiolether-modified Fab′-SH. We therefore believe that the sulfhydryl species in Fab′-SH binds to gold as a chemisorbed thiolate and plays an important role in the adsoprtion process. The next several sections critically assess the viability of these potentially intriguing immunosurfaces.

B. Thin-Layer Cell Spectroscopy. To determine whether Fab′-SH forms an immunosurface with a higher epitope density than a coating of whole molecule IgG, experiments using thin-layer cell (TLC) spectroscopy[22,29−31] were performed. Using this technique, we monitored the change in the absorbance of FITC-

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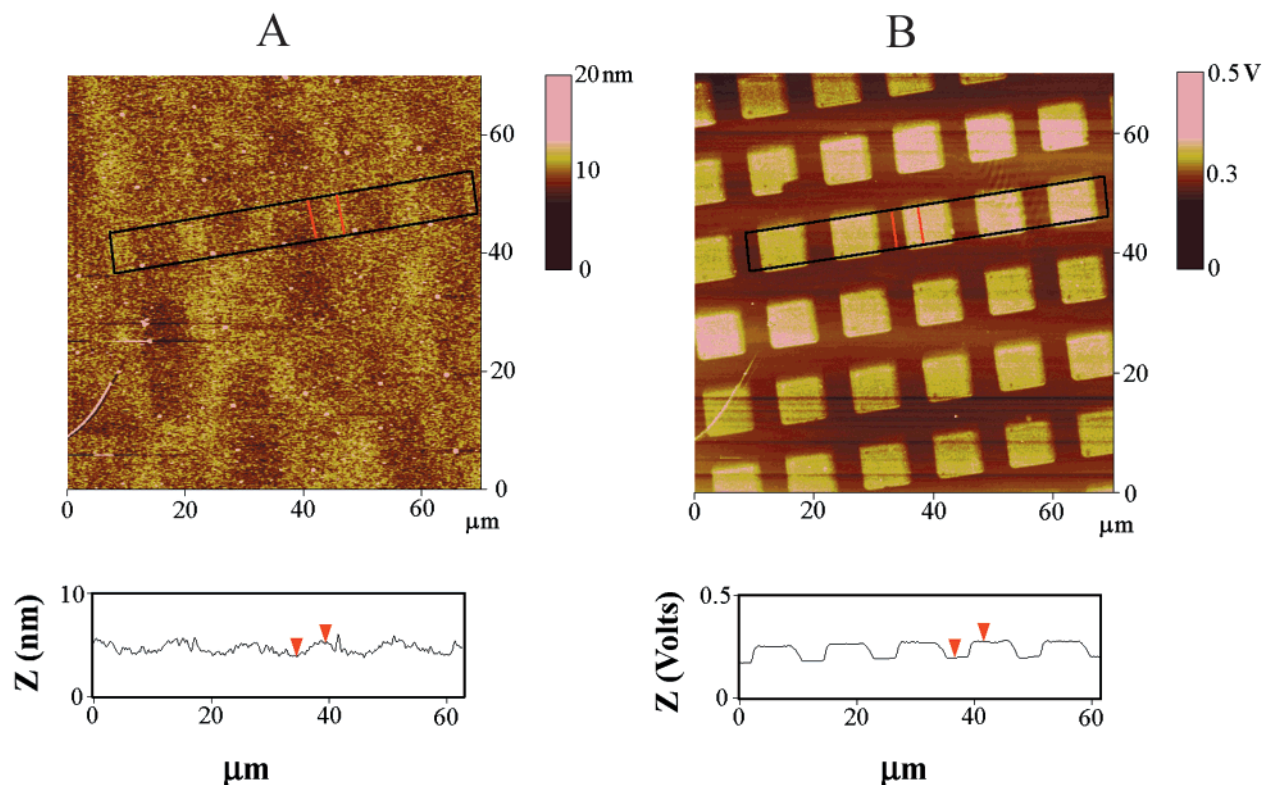
Figure 4. AFM contact mode height (A) and friction (B) images (70 μm $\times$ 70 μm) obtained in binding buffer of the patterned array (i.e., step 2). The cross-sectional analysis was performed by averaging the individual line scans contained in the boxed area of the array contained in the images.

labeled antibodies upon injection into the small solution cavity of the TLC. The decrease in absorbance that is observed when the solution-based antibody binds to the antigenic surface is used to estimate the number of available epitopes on the coated surface.

Figure 3A is a representative absorbance versus time trace for a solution of FITC-conjugated goat anti-rabbit IgG that was injected into a TLC cell containing an antigenic rabbit Fab'-SH surface. As evident, the absorbance observed upon injection of the solution into the TLC gradually decreases to a lower constant level. This decrease corresponds to the average loss of $2.8 \times 10^{13} \pm 4.1 \times 10^{12}$ ($n = 4$) labeled IgG molecules from the solution cavity. Figure 3B is a representative trace for the same type of experiment except that the gold surface is modified with whole molecule goat IgG and the solution contains FITC-labeled rabbit anti-goat IgG. The decrease in absorbance for the whole molecule antigenic surface reflects the average loss of $8.1 \times 10^{12} \pm 1.5 \times 10^{12}$ ($n = 3$) labeled IgG molecules. The absorbance decreases in both cases are attributed to the specific binding of the labeled IgG to each of the antigenic surfaces. We note that the shapes of both curves, which are indicative of multiple-step adsorption processes,[32,33] are representative of repeated trials performed in each system and are similar to those previous reported.[32-35]

To confirm that the absorbance decreases result from the specific binding of the labeled IgG to the modified gold surfaces and not from nonspecific adsorption onto any other portion of the

sample or the TLC, several control experiments were conducted. Figure 3C presents the results of one such an experiment in which a solution of FITC-conjugated goat anti-rabbit was injected into the TLC containing a surface modified with whole molecule goat IgG. No detectable change in absorbance is observed over the course of the experiment. This result confirms that the absorbance decreases in Figure 3A,B arise from the loss of labeled IgG because of its specific binding on the coated substrates.

The binding data indicate that the surface modified with a monolayer of Fab'-SH fragments contains nearly 4 times as many viable epitopes per square centimeter as the surface modified with whole molecule antibodies. In other words, a Fab'-SH modified surface has an average of 1.1×10^{13} epitopes/cm^2 whereas a whole molecule-modified surface has an average of 3.2×10^{12} epitopes/cm^2. We suspect that the lower number of epitopes on the whole molecule surface may be due, in part, to surface-induced denaturation. Gold is a high free energy surface, and recent investigations[36-39] argue that globular proteins undergo conformational changes upon adsorption, some of which may degrade epitope structures. It is intriguing to consider that thiolate bond

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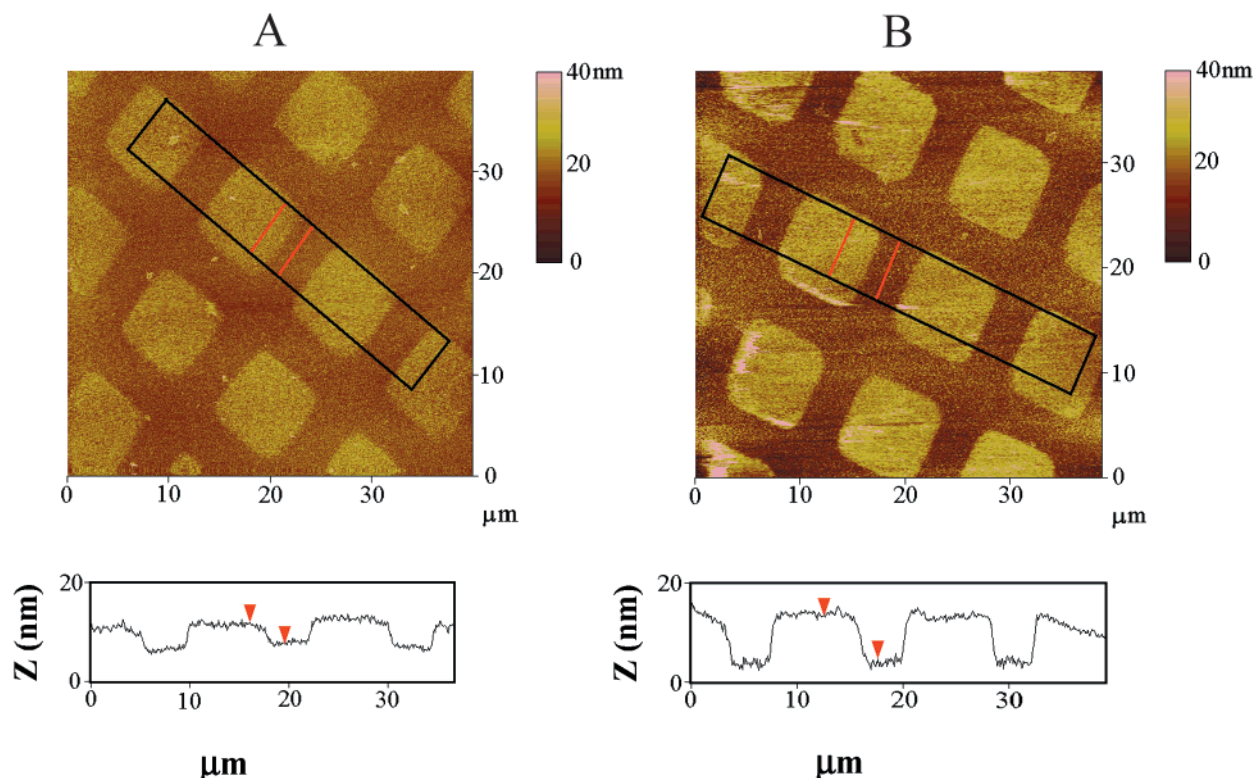
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Figure 5. (A) AFM tapping mode height image and height cross section of a rabbit Fab′-SH array (40 μm × 40 μm) obtained in binding buffer ~30 min after injection (5 μL of 5 μg/μL) of Fab′-SH fragments (final concentration in cell, 0.5 μg/μL). The cross-sectional analysis was performed by averaging the height of the individual line scans contained in the boxed area of the array. (B) AFM tapping mode height image and height cross section of a rabbit Fab′-SH array (40 μm × 40 μm) after the addition of goat anti-rabbit FITC-tagged secondary antibodies (5 μL injection of 0.1 mg/mL; final concentration in cell, 0.01 μg/μL). The cross-sectional analysis was performed by averaging the height of the individual line scans contained in the boxed area of the array.

formation to gold may minimize the surface-induced denaturation of a population of adsorbed Fab′-SH fragments. It is, however, unclear as to how the presence of the sulfhydryl species results in this minimization. With such systems, adsorption is a complex mixing of enthalpic and entropic considerations,[40] and we have not yet identified the energetic factors of importance for Fab′-SH adsorption. We are also uncertain whether a thiolate-directed orientation of the adsorbate may play a role. Interestingly, Caruso and co-workers found that antibodies modified with Traut's reagent, a reagent that converts free amines to sulfhydryl groups, and adsorbed on gold could bind twice the number of solution antigens as unmodified antibodies directly adsorbed onto gold.[41] Because the amines and therefore the sulfhydryl groups are distributed throughout the antibody structure, we suspect that thiolate bond formation is most likely not conferring any orientational advantage but, rather, may be affecting the structural viability of the immobilized antibodies. Our result with antigenic surfaces is in accord with their observations.

C. AFM and Confocal Fluorescence Imaging. Characterizations using AFM and confocal fluorescence microscopy were carried out to evaluate the effectiveness of each step in the array fabrication at a microscopic level. Figure 4 presents in situ AFM images of the photopatterned surfaces prepared in steps 1 and 2, i.e., a gold substrate modified with a fluorinated alkanethiolate monolayer only in the grid regions. The topographic image at most hints at the presence of the patterned surface created in steps 1 and 2. As proposed in earlier investigations,[4,42] the inability to detect the 1.5−2.0-nm[12] difference in the height of the uncoated squares and the fluorinated monolayer-coated grids likely arises from the simultaneous contact of the tip with both parts of the array at the boundary between the squares and grids.

In contrast to the topographic image, the concurrent friction image in Figure 4B reveals an array composed of squares and grids. The shapes and the dimensions of the two elements of the image (grids, ~5 μm wide; squares, ~7.5 μm wide) are consistent with the expected photopattern created using a 2000-mesh TEM grid. Moreover, the friction image confirms a compositional difference between the grids and the squares, with the squares having a higher friction than the grids. Several laboratories,[43−45] including our own,[46] have recently shown that the friction observed at the microcontact formed between a high surface free energy Si_3N_4 tip (i.e., an uncoated tip) and a sample with high surface

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free energy is larger than the friction observed for a sample with low surface free energy. Since the uncoated gold squares have a higher surface free energy than the fluorinated grids,[12] it follows that the exposed gold squares of the photopatterned surfaces should exhibit a higher friction than the fluorinated grids. This result confirms the validity of steps 1 and 2 in the patterning process.

We next examined the effectiveness of step 3, i.e., the reliability of immobilizing viable rabbit Fab′-SH in the square regions of the pattern. This assessment was conducted using tapping mode AFM (TM-AFM) in order to reduce any contact-induced distortion of the topographic image.[47-49] This concern results from our earlier assessments of this immunoassay readout strategy[4] whereby the detected height changes were consistently larger when determined in tapping mode.

Figure 5A is an in situ TM-AFM height image for a patterned gold surface ~30 min after injection of a Fab′-SH solution into the cell. The height of the Fab′-SH layer is 4.4 nm larger than the fluorinated alkanethiolate monolayer in the grids (~1.5−2 nm[12]). This value is consistent with the molecular dimensions of Fab′-SH determined by others[50] after accounting for image distortions that arise from the finite size and shape of the probe tip.[51] This magnitude in the change in topography was observed in images of several samples and for different locations on each sample.

To demonstrate the viability of the Fab′-SH array, an antibody specific to the Fab′-SH surface was introduced into the imaging cell. Figure 5B is an in situ tapping mode height image of a different location of the same sample used in Figure 5A that was obtained ~15 min after injection of a dilute binding buffer solution of FITC-labeled goat anti-rabbit IgG into the cell. Cross section analysis of Figure 5B reveals a height difference of 8.6 nm, approximately double that obtained with only Fab′-SH fragments on the surface. This increase is consistent with the specific binding of goat anti-rabbit to the Fab′-SH-based array, thereby documenting that the Fab′-SH array is viable and contains intact epitopes.

Another confirmation of the viability and specificity of the immobilized Fab′-SH array is provided by the confocal fluorescence microscopic images presented in Figure 6. Figure 6A is an in situ fluorescence image (75 μm × 75 μm) obtained after the exposure of an antigenic Fab′-SH array to FITC-labeled specific antibody. As evident, the image is composed of a defined fluorescent pattern of 7.5-μm squares that are surrounded by nonfluorescent 5-μm-wide grids. This result indicates that the Fab′-SH array is viable as an immunosensing surface, with the regions of strong fluorescence corresponding to those displaying the height changes in Figure 5B that are diagnostic of the specific binding of the antibody to the antigenic array.

Figure 6B is a fluorescence image of a Fab′-SH array after the addition of FITC-labeled nonspecific antibodies. The absence of a detectable fluorescent pattern demonstrates that the Fab′-SH array contains viable epitopes that are only recognizable by the antibody raised against the immobilized antigen. Therefore, the
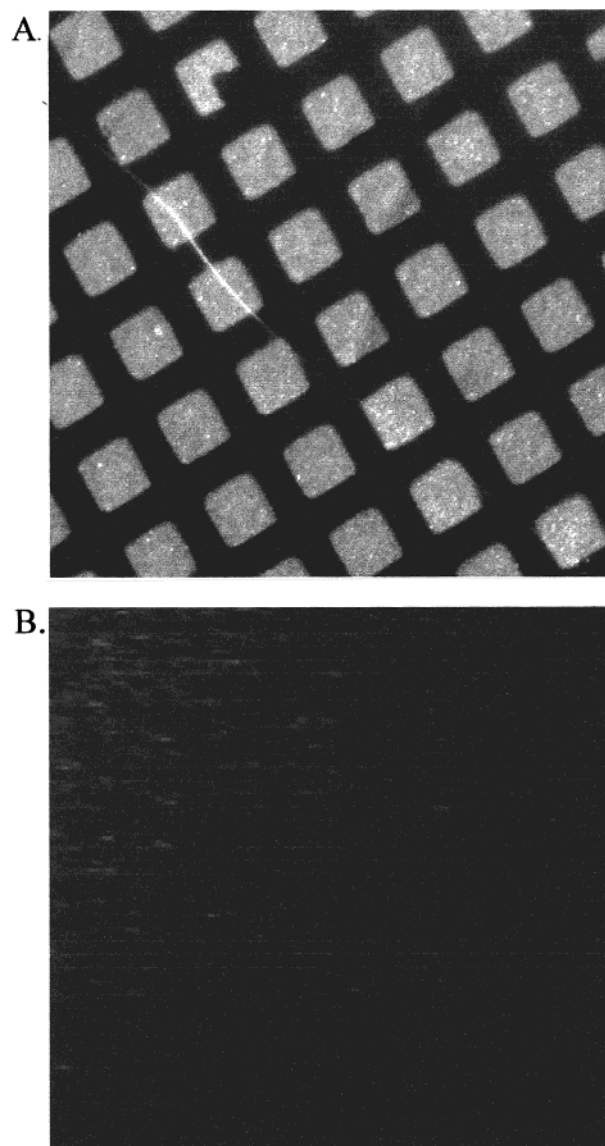
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Figure 6. Fluorescence images (75 μm × 75 μm) of a rabbit Fab′-SH array after the addition of goat anti-rabbit (A) or goat anti-mouse (B) FITC-tagged secondary antibodies. The images were obtained under deionized water.

cleavage process and subsequent immobilization of the fragments to the bare gold squares produces a viable immunosurface.

CONCLUSIONS AND FUTURE WORK

Rabbit Fab′-SH fragments have been used to construct viable antigenic immunosurfaces on gold. X-ray photoelectron spectroscopy argues that the immobilization of Fab′-SH fragments involves the free sulfhydryl group created in the fragment during the controlled reduction process of rabbit F(ab′)$_2$ and results in the formation of a gold-bound thiolate. TLC spectroscopic investigations reveal that an antigenic Fab′-SH monolayer on gold contains ~4 times more intact epitopes per square centimeter than an antigenic monolayer of whole molecule antibodies, a property that may prove useful in the detection of lower affinity antibodies and, therefore, earlier detection of disease. These studies suggest that thiolate bond formation regulates adsorption in a way that results in immunosurfaces of larger epitope density than those prepared

with adsorbed whole molecule antibodies. Furthermore, attachment through spontaneous chemisorption to gold eliminates the need for chemical linkers, simplifying the array fabrication process. Finally, both in situ AFM characterizations and fluorescence assays conducted on compositionally patterned surfaces of Fab′-SH fragments demonstrated that the coatings are viable immunosurfaces. Experiments to further assess the role of the sulfhydryl group in controlling orientation, along with evaluations of whether higher epitope densities enhance the detection of lower affinity antibodies, are planned.