

Label-Free Reading of Microarray-Based Immunoassays with Surface Plasmon Resonance Imaging

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A simple method is presented for patterning of protein antigens at a gold surface for use in surface plasmon resonance (SPR) imaging experiments. Microfluidic devices fabricated from poly(dimethylsiloxane) were used to flow various fluids over a gold substrate in spatially defined channels. This technique was used to pattern the surface chemistry of the gold as well as to adsorb antigens from solution to the modified substrates. The resulting antigen arrays were probed with complementary antibodies in order to demonstrate the effectiveness of the patterning for antibody capture experiments. SPR imaging was used to aid in the optimization of array fabrication and to observe the interactions of unlabeled antibodies with these microarrays. This work presents a means of fabricating microarrays with controlled surface density of antigens. SPR imaging provides both quantitative and qualitative evaluation of antibody binding in a label free format.

Immunoassays are a common and useful means of performing biochemical analysis. The strong, specific binding of an antibody to its antigen has been widely exploited in clinical applications as well as in sensor design. The most common approach to immunoassays is to immobilize either the antibody or antigen to a solid support and to detect binding with a labeled partner. Labels for immunoassays have had a range of composition and detection mechanisms and include enzymes,¹ fluorescent molecules,^{2–4} nanoparticles,^{5,6} and radiolabels.⁷ Recently, the widespread application of DNA microarrays (gene chips) for massively parallel analysis has prompted researchers to develop multisensing immunoassays in microarray format.^{8–10} As well, the high-

throughput demands of proteomics have driven development in the fabrication and reading of microarrays of a variety of proteins.^{2,4,11}

Microarrays of immunoreagents and other proteins continue to be developed largely by extending technologies used for gene chips² but remain much more problematic. For example, the fluorescence detection methods that are so successful for gene chips are much less convenient with protein chips due to synthetic challenges, multiple label issues, and the potential for interfering with the binding site. Also, signal-producing reactions catalyzed by commonly used enzyme-linked antibodies are difficult to implement in immunoassay microarray format. Strategies for the immobilization of proteins in the most efficient orientation and conformation for interactions with binding partners are much more complex.¹² Nonspecific adsorption is also a much greater problem for proteins than for DNA. Finally, it is not likely that a standard set of proteins will be made readily available in the future that has a large enough scope to meet all needs, so that custom-made protein chips that are easy to fabricate will be needed. In this paper, a facile method for creating arrays of antigen proteins on gold is demonstrated. Interactions of antibodies at these microarrays are detected by surface plasmon resonance (SPR) imaging. SPR detects changes in refractive index within a short distance from the surface of a thin metal film as variations in light intensity reflected from the back of the film and, thus, does not require labeling.

Microarrays are generally prepared by the robotic pin printing of biomolecules on glass slides.^{2,13} A number of laboratories have shown that microfluidic networks formed in poly(dimethylsiloxane) (PDMS) can be employed to pattern and array different types of biomolecules.^{8,14} Corn and co-workers have applied these techniques to create arrays of peptides,¹⁵ carbohydrates,¹⁶ and proteins¹⁷ on thin gold films and have measured interactions at

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these arrays in a label-free format with SPR imaging. Along with SPR imaging^{18–20} other label free methods such as mass spectrometry,²¹ ellipsometry,¹⁰ and Raman spectroscopy²² are also being developed for microarray reading. It is our belief that the strategies for both the attachment and patterning of proteins on surfaces that are compatible with label-free reading schemes will be important for widespread application of protein microarrays.

The method described here employs PDMS microfluidic networks to create patterns of amine-terminated alkanethiols and arrays of protein antigens on gold substrates. Label-free antibody binding to the array is monitored with SPR imaging. Given that SPR detects nonspecifically, it was of paramount importance to utilize surface treatments that would allow for unambiguous interpretation of signal changes. Ethylene glycol-terminated alkanethiolate monolayers were used to create a protein-resistant background between antigen spots. We demonstrate that SPR imaging provides a useful detection scheme for immunoassay microarrays, can be employed in array fabrication and optimization, and can yield information on the antibody–antigen binding characteristics.

EXPERIMENTAL SECTION

Mercaptoundecylamine hydrochloride (MUAM) was obtained from Dojindo Laboratories; $\text{HS}(\text{CH}_2)_{11}(\text{OCH}_2\text{CH}_2)_3\text{OCH}_3$ (abbreviated as EG3-OMe) was synthesized in general agreement with published methods.²³ Proteins used in this work were obtained in the highest available purity and used as received. Bovine serum albumin (BSA) was obtained from Sigma (St. Louis, MO), Bovine and goat immunoglobulins G (IgG), and polyclonal rabbit antisera to all proteins were obtained from ICN Biomedicals (Aurora, OH). All protein solutions were prepared in phosphate-buffered saline (PBS; 8.1 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , 137 mM NaCl, 2.7 mM KCl in 18 MΩ water). Antigen solution concentrations were determined from the measured weight and accurate molecular mass of the protein and are accurate to $\pm 5\%$. Antibody concentrations were determined by dilution of the commercial antisera.

PDMS microfluidic channels were fabricated according to established methods.^{8,14} Briefly, a relief pattern of photoresist on a silicon wafer was created photolithographically. By curing PDMS prepolymer and cross-linker (Sylgard 184, Dow Corning; Midland, MI) 10:1 by weight against this relief structure, a negative of the relief was formed in the PDMS. The microchannels measured 200 μm wide by 10–15 μm deep. The stamp was also through-bored at the ends of the channels to allow fluids access to the channels when the PDMS device was applied to a surface. Fluid flow was driven by vacuum applied through a pipet tip to one access point on the microchannel while the other end was connected to a reservoir of solution.

The protein arrays were constructed as described in Figure 1 on a 45-nm gold film deposited on SF10 glass (Schott; Toronto, ON, Canada) with a 1-nm adhesive layer of chromium. The arrays

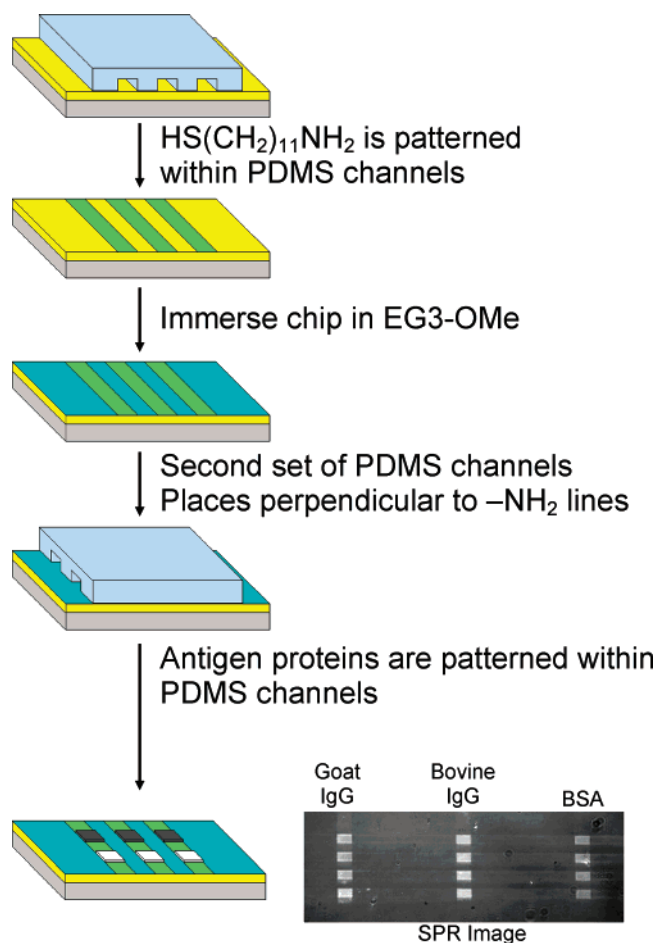


Figure 1. Schematic representation of the method for surface patterning of proteins.

were imaged using the GWC Instruments SPRImager (GWC Instruments; Madison, WI) and has been described in detail elsewhere.²⁴ Antibody solutions and buffer solutions are introduced to the entire array surface via peristaltic flow through a fluid cell. Images were generated by averaging 100 individual scans.

In Figures 3 and 4, the SPR signal is shown as raw intensity units. The signal in Figures 5 and 6 are given in percent reflectivity (%R). The value of %R, or $\Delta\%R$ in the case of difference images, is obtained, as specified by the manufacturer, by $\%R = (0.85I_p/I_s) \times 100\%$, where I_p and I_s refer to the reflected light intensity detected using p- and s-polarized light, respectively. The adsorption of bovine IgG to MUAM was studied on chips consisting of MUAM rows surrounded by EG3-OMe. Each bovine IgG solution was introduced to the array, in sequence from lowest to highest concentration, and allowed to adsorb for 1 h before being flushed from the cell with PBS. Signals due to adsorption generally reached a steady-state value after 10 min. Images were collected in PBS and integration of cross-sectional profiles provided the signal used to construct the adsorption isotherm. For the determination of antibody binding curves, the surface was blocked with 1% BSA in PBS for 1 h. prior to the introduction of antibodies. Each antibody solution was introduced to the array, in sequence

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from lowest to highest concentration, and allowed to adsorb for 10 min before being flushed from the cell with PBS. Signals due to antibody binding generally reached a steady-state value after a few seconds.

RESULTS AND DISCUSSION

To establish a platform for SPR imaging of antigen–antibody interactions, the surface chemistry of the sensor chip had to possess several characteristics. Given that monolayers of alkanethiolates containing oligoethylene glycol groups have been demonstrably effective in resisting protein adsorption at gold surfaces,^{25,26} we employed EG3-OME to give a strongly protein-resistant background region. In addition to an invariant reference plane, the experimental design took into consideration the need to adsorb proteins to the surface without a high degree of conformational perturbation. Drawing on the use of polyamines in assays that require proteins to be bound via physisorption,^{9,27} an amine-terminated monolayer was used to localize adsorbing antigens from solution.

Arrays of antigenic proteins were fabricated using microfluidic channels formed in PDMS using three steps as shown in Figure 1. First, a PDMS microchannel device was brought into conformal contact with the gold surface of the chip. A 3.5–7 mM aqueous solution of 1 mM MUAM was introduced into all channels. The thiol molecules were adsorbed for 10 min, the channels were rinsed with water, and the PDMS was removed from the gold. This resulted in a pattern of 200- μ m-wide lines of amine-terminated monolayer on the chip surface. We have found that using aqueous solutions within the channels is the best means of preserving the conformal seal between the PDMS and the gold substrate. The second step involved immersing the substrate in an ethanolic solution of EG3-OME for at least 8 h to modify the remaining gold surface with a layer that resists protein adsorption. Separate infrared spectroscopy studies show negligible displacement of the MUAM by the EG3-OME under these conditions (data not shown). Finally, after removing the chip from the EG3-OME solution, rinsing, and drying, a second PDMS microchannel device was placed with its channels perpendicular to the NH₂ lines. Separate solutions containing bovine IgG, goat IgG, and BSA, all at 670 nM in PBS, were introduced to each channel and physisorbed for 1 h. Due to the protein-resistant nature of the EG3-OME monolayer, adsorption of the antigen proteins occurred primarily to the NH₂ terminal MUAM regions. Thus, we are using the microfluidic channels to confine the adsorption of the antigens in one direction and the patterned surface chemistry to control adsorption in the other. This procedure results in a chip featuring spots of physisorbed protein supported on a NH₂-terminated monolayer.

A SPR image, collected under PBS, of a chip containing spots of the three proteins is shown in the lower right of Figure 1. The localized adsorption of antigen proteins to regions of the MUAM monolayer generates a change in refractive index resulting in the observed pattern of contrast. The protein patterning procedure described here differs from that described previously by Delamarche et al.¹⁴ in that the use of unmodified PDMS allows for a

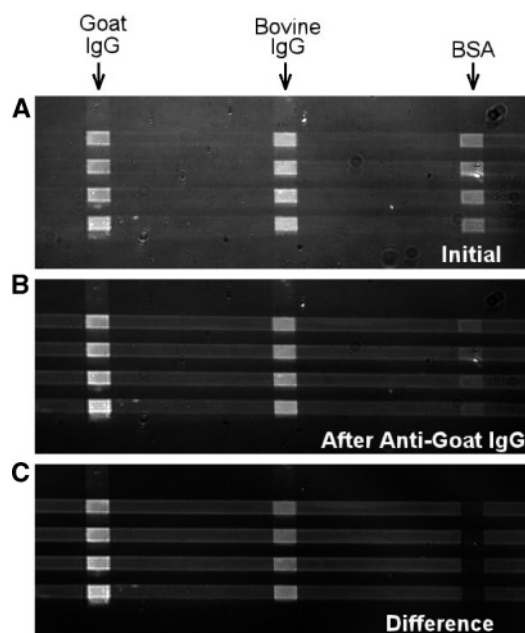


Figure 2. Difference images used to visualize the changes upon antibody binding. The observed increase in the reflected intensity from the bovine IgG regions after exposure to anti-goat IgG is attributable to interspecies cross-reactivity. The lack of change on the BSA regions indicates that nonspecific adsorption was minimized.

strongly sealed contact between the PDMS device and the substrate. In addition, by using vacuum pressure to drive fluid flow, we are able to incorporate rinsing steps directly within each channel, reducing the likelihood of cross-contamination or loss of spatial resolution.

The antigen array chips prepared as described above were exposed to solutions of polyclonal antibodies. Figure 2 contains the SPR image sequence in an antibody capture experiment. Figure 2A is an image of the initial array collected in PBS. A solution of 130 nM polyclonal anti-goat IgG was introduced into the flow cell for 10 min. After flushing the cell with PBS, the image in Figure 2B was collected. The net result of the anti-goat IgG binding to the chip is more apparent in the difference image of Figure 2C, which is produced from the subtraction of Figure 2A from Figure 2B. Importantly, the contrast generated in SPR difference image is due to the variation in refractive index resulting from the localized binding of the antibodies and requires no labeling of the antibodies. The results contained in Figure 2C are explored quantitatively in Figure 3.

SPR signal intensities from an image can be measured from cross-sectional profiles. Figure 3 contains the difference image following anti-goat IgG binding shown in Figure 2C as well as several cross sections collected along various lines through the image. Cross sections a–c correspond to the SPR signal increase observed on the goat IgG, bovine IgG, and the BSA spots, respectively. The largest signal increase for anti-goat IgG binding occurs at the immobilized goat IgG spots (38 ± 6 RIU over 4 spots). A significant signal (25 ± 2 RIU) is also observed at the bovine IgG spots due to cross reactivity with the anti-goat IgG. The polyclonal anti-goat IgG used here is directed against whole IgG and will bind light-chain sites common among immunoglobulins, leading to the observed cross reactivity. Since the goat IgG and the bovine IgG were adsorbed from solutions with

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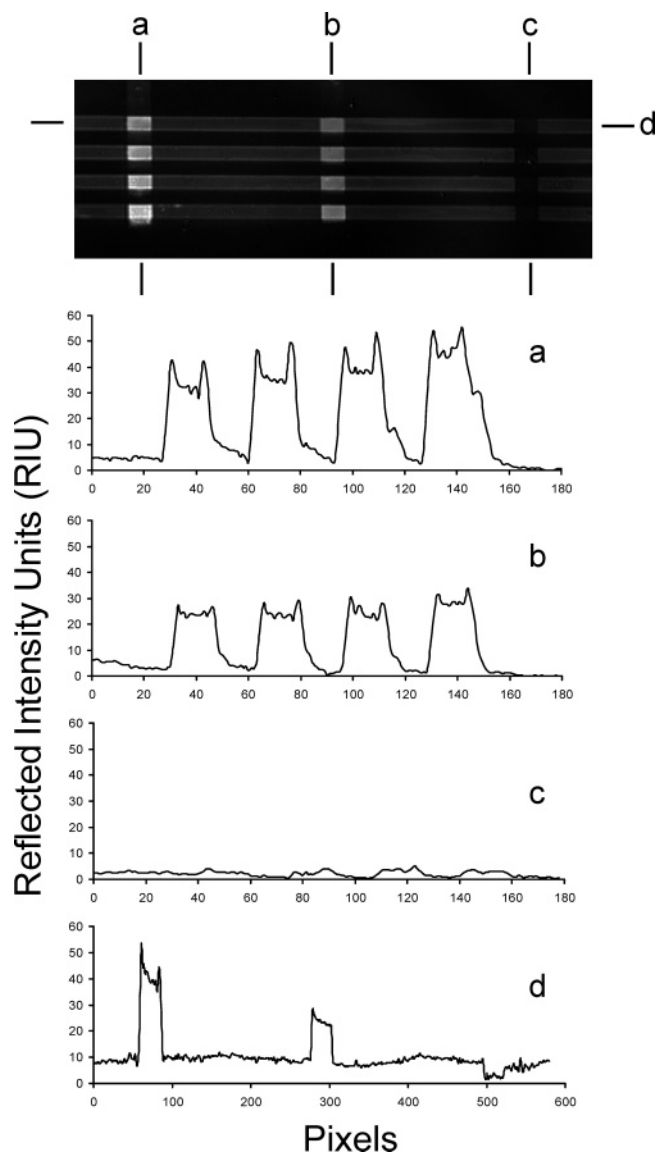


Figure 3. Line profiles of the anti-goat IgG difference image. The profiles show that the specific target of the antibody was indeed bound (a) and the cross-reactivity with bovine IgG was also significant (b). The change in background signal was negligible due to the protein resistance of the EG3-OMe groups (a–c), yet some background increase was apparent on the amine-terminated regions due to the physisorption of the antibody (d).

identical concentrations, we expect that their surface density is similar. Thus, the difference in anti-goat IgG binding observed reflects the difference in specificity of the antibody for the two IgGs. A negligible signal change is observed at the BSA spots in Figure 3, suggesting minimal nonspecific binding to the protein spots.

It is crucial that the background signal of the chip remains low following antibody exposure for sensitive and accurate quantitative binding analysis. In profiles a and b, the signal in the regions between and outside the spots is generally less than 2 RIU due to the resistance of the EG3-OMe groups to nonspecific protein adsorption. This is contrasted by profile d, which traces along a MUAM line. The regions between spots in profile d show significant signal (~ 10 RIU) due to the nonspecific adsorption of anti-goat IgG to the amine-terminated monolayer. Although this

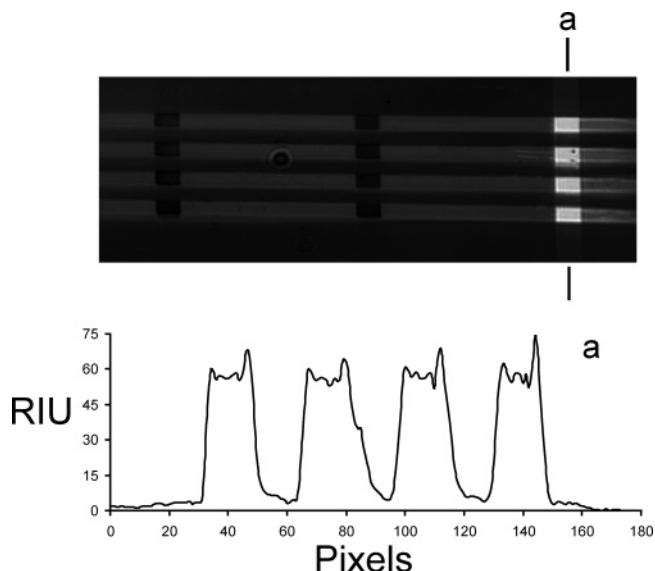


Figure 4. SPR image of the same array as in Figures 2 and 3 following the binding of anti-BSA. The array functions well even after successive introductions of at least three antibody probes.

nonspecific adsorption does not affect quantitative measurements, the exposed NH_2 layer can be blocked with a secondary protein before the analysis is performed as described below.

Figure 4 demonstrates that our chip chemistry and detection method function well for sequential sample solution introductions. Following the incubation in unlabeled anti-goat IgG, the array in Figure 2 was exposed to $5.8 \mu\text{M}$ anti-BSA. The difference image in Figure 4 shows a large signal change at the BSA spots (57 ± 1 RIU) and negligible signal on the other spots, indicating exclusive binding to the immobilized BSA. The signal at the EG3-OMe regions remains low (< 2 RIU), reflecting minimal nonspecific adsorption to the background after multiple successive antibody solutions. The results in Figures 2–4 show that the three-step procedure described here produces robust antigen microarrays on gold that can be used in repeated SPR imaging experiments of antibody binding.

SPR imaging can also be employed in array design and fabrication. The surface density of immobilized binding partners in a heterogeneous assay must be optimized to best reflect biomolecular binding in a homogeneous environment. In addition, the ability to control signal levels by varying the surface densities of the array elements is especially useful for SPR imaging as the change in percent reflectivity ($\Delta\%R$) is linear with surface concentration when ΔR is 10–20% or less.^{19,28} A number of studies have shown that the surface concentration of adsorbed proteins such as IgG can be controlled by varying their concentration in solution.^{29,30} To examine this quantitatively for our system, we studied the adsorption of bovine IgG, the antigen, from various solution concentrations at array chips composed of NH_2 -terminated MUAM rows surrounded by EG3-OMe. The percent reflectivity change is plotted versus solution concentration of bovine IgG to construct the adsorption isotherm in Figure 5. For all concentra-

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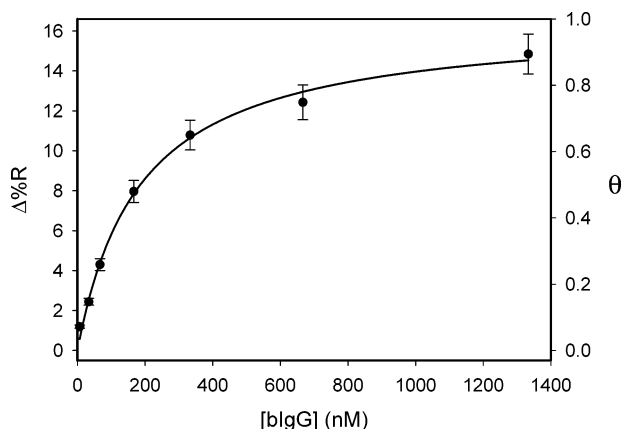


Figure 5. Plot of $\Delta\%R$ vs bulk solution concentration for bovine IgG adsorption to MUAM as determined with SPR imaging. The error bars represent the relative standard deviation (7%) of the mean for measurements over four spots. The solid line through the points is the Langmuir fit of the data. The fit yields $\Delta\%R_{\text{sat}} = 16.5 \pm 0.5$, $K_{\text{ads}} = 5.4 \pm 0.5 \times 10^6 \text{ M}^{-1}$, and $R^2 = 0.99$. The right axis plots the fractional surface coverage (θ) computed by $\Delta\%R/\Delta\%R_{\text{sat}}$.

tions studied, the SPR signal due to bovine IgG adsorption reached a steady-state value after ~ 10 min. Each point in Figure 5 was collected after an adsorption time of 1 h. The general shape of the isotherm is consistent with those measured for IgG adsorption to other surfaces,²⁹ and the results fit a Langmuir isotherm, which is commonly used to describe protein adsorption.^{31,32} The curve through the data points in Figure 5 is the Langmuir fit and yields a saturation $\Delta\%R$ ($\Delta\%R_{\text{sat}}$) of 16.5. Based on the assumption that this signal represents saturation monolayer coverage of bovine IgG on MUAM, the relationship between solution concentration and fractional surface coverage (θ) can be established by normalizing $\Delta\%R$ to 16.4 (i.e., $\theta = \Delta\%R/\Delta\%R_{\text{sat}}$, right axis in Figure 5). This relationship was then used to quantitatively explore the effect of antigen surface density on antibody binding.

An array was prepared consisting of bovine IgG immobilized at various θ by adsorbing from solutions of different concentrations. For these experiments, the chip surface was blocked with 1% BSA in PBS for 1 h. Figure 6 shows an SPR image of such an array after exposure to 670 nM anti-bovine IgG and rinsing with PBS. As shown in the cross-sectional profile, the amount of antibody binding that is detected scales with the surface density of antigen.

Traditional, nonimaging SPR is widely employed to measure binding strength of biological interactions.³³ Wegner et al. demonstrated that SPR imaging could be used to measure binding isotherms and determine adsorption coefficients (K_{ads}), which are related to binding constants.¹⁷ Isotherms were constructed for bovine IgG/anti-bovine IgG binding from antigen arrays such as that shown in Figure 6A. The antibody binding data for each surface density of antigen was fitted with a Langmuir isotherm, and K_{ads} and $\Delta\%R_{\text{sat}}$ for antibody binding were determined and are listed in Table 1. Two observations are noteworthy from the results in Table 1. First is that the maximum amount of antibody

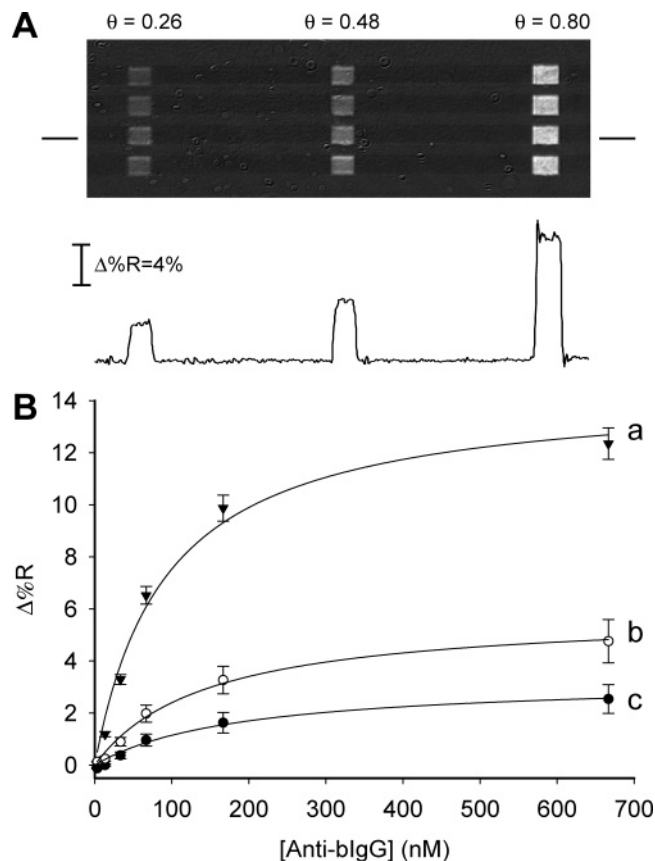


Figure 6. Effect of antigen surface concentration on antibody capture. (A) SPR image of a bovine IgG array following exposure to a 667 nM solution of anti-bovine IgG. The array consists of bovine IgG (antigen) adsorbed at different fractional coverage (θ) as described in Figure 5. To obtain a fractional coverage, $\theta = 0.26$, the antigen was adsorbed from 67 nM bovine IgG. To achieve $\theta = 0.48$, the concentration was 167 nM, and for $\theta = 0.80$, the concentration was 667 nM. The line profile shows the magnitude of the signal from antibody binding through a row of array elements. (B) Binding curves for anti-bovine IgG adsorption to bovine IgG immobilized at different surface densities. The error bars represent the standard deviation of the mean for measurements over four spots. The solid lines through the points are Langmuir fits (see Table 1). (a) Binding curve for antigen spots, where $\theta = 0.80$. (b) Binding curve for antigen spots, where $\theta = 0.48$. (c) Binding curve for antigen spots, where $\theta = 0.26$.

Table 1. Curve-Fitting Parameters for Anti-Bovine IgG Binding Curves Shown in Figure 6B^a

soln concn of antigen (nM)	θ	$K_{\text{ads}} (\text{M}^{-1})$	$\Delta\%R_{\text{max}}$	R^2
67	0.26	$5.3 (1.3) \times 10^6$	3.3 (0.3)	0.91
167	0.48	$6.9 (1.2) \times 10^6$	5.9 (0.4)	0.94
667	0.80	$1.1 (0.1) \times 10^7$	14.4 (0.5)	0.98

^a Values in parentheses are standard deviations of the indicated values.

binding ($\Delta\%R_{\text{sat}}$) is governed by the surface density of antigen. This implies that the SPR signals are mainly due to antibody–antigen interactions with little contribution from nonspecific adsorption. A number of covalent schemes,^{2,34,35} specific interac-

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tions requiring fusion proteins,^{13,17} and nonspecific physisorption^{9,36,37} have been used to immobilized proteins in microarrays. It is notable that the method applied here, simple physisorption to an amine-terminated monolayer, can be used to predictably control the surface density of protein arrayed and thus optimize the observed readout signal.

Second, K_{ads} values determined from the Langmuir fit increase with increasing surface density of antigen. Smith et al. have noted that K_{ads} values obtained from Langmuir fits are not equivalent to binding association constants, but K_{ads} can be used to rank binding strengths in similar systems.¹⁶ Thus, the trend in K_{ads} from Figure 6B implies stronger antibody affinity to spots with a higher surface density of antigen. A recent report has shown that the affinity between immobilized antibodies and their antigens increases at high surface density.³⁸ This observation has been ascribed to a lowering of the dissociation rate constant at higher surface densities of antibodies. Although our format is antibody capture, similar arguments can be made. At lower antigen surface densities, a dissociating antibody must diffuse larger distances to interact with another immobilized antigen. As the surface density of antigens increases, the distance across the surface between binding sites decreases, increasing the likelihood of antibody diffusion into the bulk solution rather than toward another adsorption site. The overall effect is a decrease in the dissociation rate constant and an apparent increase in binding constant.³⁸

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CONCLUSIONS

We have demonstrated a simple method for the fabrication and reading of antigen microarrays. The use of self-assembled monolayers and microfluidic networks enables the patterning of surface chemistry to support proteins in array elements and creates a background plane on which there is a very low level of nonspecific adsorption. Microarrays with controlled surface density of antigens can be fabricated using this method. While we have demonstrated the fabrication of low-density arrays here, the technique has the potential to produce arrays of 64 or more spots.¹⁹ This work provides further demonstration of the use of SPR imaging for label-free reading of microarrays. The differential imaging technique employed is able to study antibody binding and cross-reactivity while confirming the absence of nonspecific adsorption for numerous solution exposures to the chip. SPR imaging provides both quantitative and qualitative evaluations of antibody binding in a label-free format. We have studied the effect of bovine IgG surface density on SPR imaging signal changes upon polyclonal anti-bovine IgG binding. The affinity between the immobilized bovine IgG and its antibody increases with antigen surface density.

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