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Accelerated Articles

Characterization and Optimization of Peptide Arrays for the Study of Epitope−Antibody Interactions Using Surface Plasmon Resonance Imaging

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The characterization of peptide arrays on gold surfaces designed for the study of peptide−antibody interactions using surface plasmon resonance (SPR) imaging is described. A two-step process was used to prepare the peptide arrays: (i) a set of parallel microchannels was used to deliver chemical reagents to covalently attach peptide probes to the surface by a thiol−disulfide exchange reaction; (ii) a second microchannel with a wrap-around design was used as a small-volume flow cell (5 μL) to introduce antibody solutions to the peptide surface. As a demonstration, the interactions of the FLAG epitope tag and monoclonal anti-FLAG M2 were monitored by SPR imaging using a peptide array. This peptide−antibody pair was studied because of its importance as a means to purify fusion proteins. The surface coverage of the FLAG peptide was precisely controlled by creating the peptide arrays on mixed monolayers of alkanethiols containing an amine-terminated surface and an inert alkanethiol. The mole fraction of peptide epitopes was also controlled by reacting solutions containing FLAG peptide and the non-interacting peptide HA or cysteine. By studying variants based on the FLAG binding motif, it was possible to distinguish peptides differing by a single amino acid substitution using SPR imaging. In addition, quantitative analysis of the signal was accomplished using the peptide array to simultaneously determine the binding constants of the antibody−peptide interactions for four peptides. The binding constant, K_{ads}, for the FLAG peptide was measured and found to be 1.5×10^8 M^{-1} while variants made by the substitution of alanine for residues based on the binding motif had binding constants of 2.8×10^7, 5.0×10^6, and 2.0×10^6 M^{-1}.

Peptide arrays are emerging as an effective tool for the study of binding recognition events between biologically active peptides and other biomolecules, such as protein, DNA, and RNA. Immobilized arrays of peptides have been used for several applications including the identification of important residues in protein−protein recognition,[1,2] the study of peptide−DNA interactions,[3] the enzymatic modification of peptides,[4,5] and the characterization of peptide motifs important to cell adhesion.[6,7] Many of these advances have been accomplished using multiplexed peptide arrays fabricated by SPOT synthesis.[8] In this method, the parallel synthesis of many peptides is performed at discrete locations on a cellulose membrane. Protein interactions with the immobilized peptides are then detected by labeling either the immobilized peptide or the target biomolecule with a fluorescent or radioactive tag. To enhance protein recognition and screening capabilities to

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the level required for large-scale proteomic measurements, the further development of functional peptide chips is necessary.

In support of this endeavor, surface plasmon resonance (SPR) imaging can be used to quantitatively study protein−peptide interactions with peptide arrays made on chemically modified gold surfaces. SPR imaging is an ideal surface-sensitive optical technique to detect the interactions of antibodies with peptides because fluorescent or radioactive labeling, which may adversely affect antibody structure, is not required. The utility of SPR imaging to the label-free study of reversible protein adsorption interactions was previously demonstrated in a series of protein−DNA measurements.[9−11] For example, the binding interactions of the mismatch binding protein, Mut S, and the single-stranded binding protein to DNA arrays have been studied,[9,10] and the sequence-specific transcription regulation proteins, OmpR and VanR, have been investigated using DNA arrays.[11]

The fabrication of robust arrays of peptides immobilized on gold films is a crucial element for the measurement of peptide−antibody interactions by SPR imaging. Only noble metals can be used as array substrates for SPR imaging measurements; therefore, the commercially available arrays made by SPOT synthesis techniques on membranes or by parallel synthesis on plastic pins cannot be used.[1,12] Instead, in this paper, we employ a self-assembled monolayer (SAM) of an ω-functionalized alkanethiol as the foundation of the peptide array, followed by chemical modification of the SAM to tether peptides to the surface. The chemical modification of SAMs has been used previously in our group in reaction schemes developed for the covalent attachment of DNA molecules.[13,14] Two methods were used to control the peptide surface coverage. The first approach involved the reaction of two-component solutions of alkanethiols with the gold surface to form mixed monolayers of the reactive amine-terminated alkanethiol and an inert carboxylic- or hydroxyl-terminated alkanethiol before chemically attaching the peptide. In the second methodology, mixtures of FLAG peptide and the noninteracting peptide HA or cysteine were reacted at the final step of the immobilization process.

In conjunction with the surface attachment chemistry, microfluidics were used both to immobilize peptides in an array format and to deliver small volumes of antibodies to the surfaces using poly(dimethylsiloxane) (PDMS) microchannels. These efforts follow our recent application of PDMS microchannels for the fabrication of DNA arrays for SPR imaging.[15] The peptide arrays were fabricated by a two-step process: (i) parallel microfluidic channels constructed from PDMS were used to immobilize multiple peptides onto a chemically modified gold chip in a series of lines; (ii) subsequently, target antibody solutions were delivered to the peptide "line array" through a second PDMS microchannel used as a small-volume flow cell (5 μl). Performing peptide attachment chemistry reactions within the microchannels facilitates the creation of multicomponent arrays and eliminates the need to use harsh chemicals to protect and deprotect the background during the peptide immobilization process.

SPR imaging measurements were then used to monitor the adsorption of antibodies onto the peptide arrays. As an example, we demonstrate the sequence-specific interaction of anti-FLAG M2 in solution with four surface-bound peptides. Langmuir isotherms of anti-FLAG binding to the four different peptides based on the biding motif of the FLAG fusion tag were also measured to determine the adsorption coefficients of four epitopes on one chip.

Table 1. Peptide Sequences and Antibodies Derived from Epitope Tags[a] and Measured Binding Constants

peptide	sequence (N- to C-terminus)	associated antibody	K_{ads} (M^{-1})	$C_{0.5\theta}$ (nM)
F1	CSGDYKDDDDK	M2	$1.5 \pm 0.5 \times 10^8$	6.5
F2	CSGDAKDDDDK	M2	$2.0 \pm 0.5 \times 10^6$	500
F3	CSGDYADDDDK	M2	$5.0 \pm 2.4 \times 10^6$	200
F4	CSGDYKDADDK	M2	$2.8 \pm 0.8 \times 10^7$	35
HA	CSGYPYDVPDYA	12CA5		

[a] Peptide sequences are modified at the N-terminus with the sequence CSG. The thiol functionality on the terminal cysteine is used to chemically attach the peptide to the surface. The serine and glycine act as a spacer between the active sequence and the cysteine. The underlined amino acids indicate alanine substitutions for residues in the original sequence, F1.

EXPERIMENTAL CONSIDERATIONS

Materials. *N*-Succinimidyl *S*-acetylthiopropionate (SATP, Pierce), 2,2′-dipyridyl disulfide (DPDS, Aldrich), 11-mercaptoundecylamine (MUAM, Dojindo Laboratories), 11-mercaptoundecanoic acid (MUA, Aldrich), 11-mercapto-1-undecanol (MUD, Aldrich), *N*-hydroxysuccinimidyl ester of methoxypoly(ethylene glycol) propionic acid MW 2000 (PEG-NHS, Shearwater Polymers Inc.), dithiothreitol (DTT, Aldrich), sodium chloride (Aldrich), potassium chloride, sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate dihydrate (Fluka), potassium dihydrogen phosphate (J.T. Baker Chem. Co.), and anti-FLAG M2 (Sigma) were used as received without any further purification. All rinsing steps were performed with Millipore-filtered water and absolute ethanol. Peptides were synthesized at the University of Wisconsin Biotechnology Center using an Applied Biosystems Synergy 432A. Purity was determined with MALDI-TOF mass spectrometry and HPLC, and the peptides used in these experiments had a minimum purity of 90%. The peptide sequences along with the associated monoclonal antibodies are listed in Table 1.

Array Fabrication. Thin gold films (45 nm) with an underlayer of chromium (1 nm) were vapor-deposited on SF10 glass slides (Schott glass) using a Denton DV-502A evaporator and were used for all SPR imaging experiments. PDMS polymer was used to fabricate microchannels by curing the polymer on 3-D silicon master wafers at 70 °C as described previously.[15−17] Peptide

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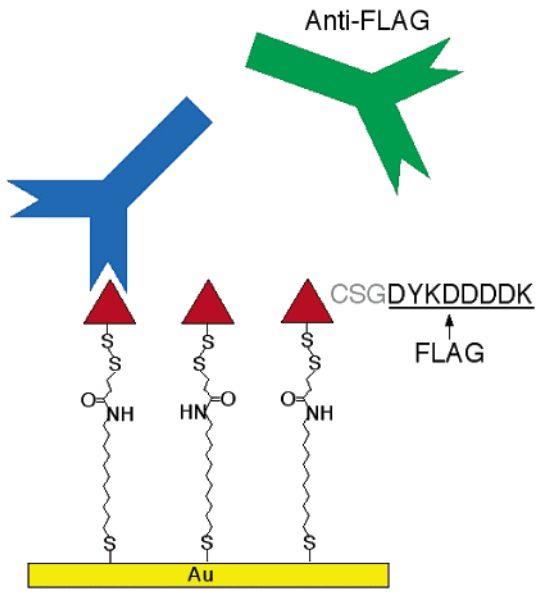

Figure 1. Schematic diagram illustrating the chemical attachment of peptides on gold surfaces. FLAG peptides were modified with a terminal cysteine for direct attachment onto a self-assembled monolayer on gold via a sulfhydryl−disulfide exchange reaction. The FLAG sequences contained an SG spacer between the cysteine and the FLAG sequence to maximize antibody accessiblity to the immobilized peptide.

immobilization steps were performed within PDMS microchannels constructed from a silicon master displaying parallel channels (300-μm width, 1.4-cm length, and 700-μm spacing). Further details can be found within the text and in Figures 1 and 2. Peptides were immobilized for 3 h in phosphate buffer (pH 7.4, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 14 mM NaCl, 2.7 mM KCl). A flow cell for sample delivery to the line array was constructed from a single PDMS microchannel (500-μm width, 14.2-cm total length, 35-μm depth). The channel was constructed to wrap around the chip surface with 500-μm spacing between folds, which are aligned perpendicular to the line array. Solutions were delivered to the surface through the microchannels by a simple aspiration-based differential pressure pumping system. The peptide array was regenerated by exposing the surface to 100 mM triethylamine, pH 11.2, for 5 min to remove adsorbed antibody from the surface. The peptide could be regenerated and exposed to antibody solutions for five cycles before nonspecific protein adsorption resulted in degradation of the peptide array.

SPR Imaging Measurements. The SPR imaging experiments were performed on an SPR imager apparatus (GWC Instruments), which used near-infrared excitation from an incoherent source.[18] Immobilized peptides were exposed to antibody solutions in phosphate buffer with a total sample volume of 5 μL delivered through the wraparound PDMS flow cell. All SPR images were taken under equilibrium conditions, 10 min after antibody introduction into the microfluidic channel. Images were collected using V++ Precision Digital Imaging Systems, version 4.0 software and further analyzed using the software package NIH Image V.1.6.1.

Fluorescence Wash-Off Measurements. The surface density of peptide monolayers prepared by surface thiol−disulfide exchange reactions with fluorescently labeled peptides was estimated by measuring the fluorescence of a DTT solution used to cleave the disulfide bonds immobilizing the peptides onto the surface.[14] A peptide monolayer was formed by reacting a 1 mM solution of fluorescein-labeled F1 peptide (see Table 1 for sequence) on a chemically modified gold-coated slide. The slide was then soaked in a 1 M DTT solution for 1 h. The fluorescence emission at 517 nm was measured with a Hitachi F-4500 fluorescence spectrophotometer for each cleaved peptide solution. The peptide surface coverage estimated by this method should be regarded as an upper limit, because the gold-coated substrates used for these fluorescence measurements were assumed to be morphologically smooth.

RESULTS AND DISCUSSION

I. Creation of FLAG Epitope Peptide Arrays. Peptide sequences based on the FLAG peptide tag were studied as a model system for peptide−antibody interactions. This hydrophilic eight-amino acid peptide is frequently fused to proteins for two purposes: (1) for the detection of proteins, where the tag provides a means to identify proteins in cell-based systems allowing the study of protein functions and cellular locations,[19−22] and (2) for protein purification, where the peptide tag is used to selectively bind to an associated antibody allowing purification from cell extracts.[23,24] The interaction of FLAG peptide and anti-FLAG M2 has been well-characterized, and the amino acids essential to binding have been previously identified[25,26] making this antigen−antibody pair ideal for showing the feasibility of SPR imaging for label-free quantitative analysis of adsorption interactions on multipeptide arrays. We studied variants of the FLAG peptide based on the binding motif of the antibody−peptide interaction. Four FLAG variants were immobilized on one array and were used to demonstrate the utility of SPR imaging to distinguish peptides differing by a single amino acid and to identify the essential residues in an antibody−peptide binding motif.

A. Surface Attachment Chemistry. Peptides were covalently immobilized on gold surfaces using reaction schemes previously developed for the attachment of thiol-modified DNA.[14] Briefly, the NHS ester of SATP was reacted with an amine-terminated alkanethiol monolayer of MUAM, resulting in the formation of a protected thiol surface. A free sulfhydryl surface was formed, upon removal of the protection group, with a hydroxylamine solution containing DTT. The sulfhydryl surface was then reacted with dipyridyl disulfide to create a pyridyl surface. Next, peptides modified with a terminal cysteine were attached to this surface via a thiol−disulfide reaction (see Figure 1).

B. Peptide Array Fabrication. A two-step fabrication method was used to create the peptide arrays. As shown in Figure 2a,

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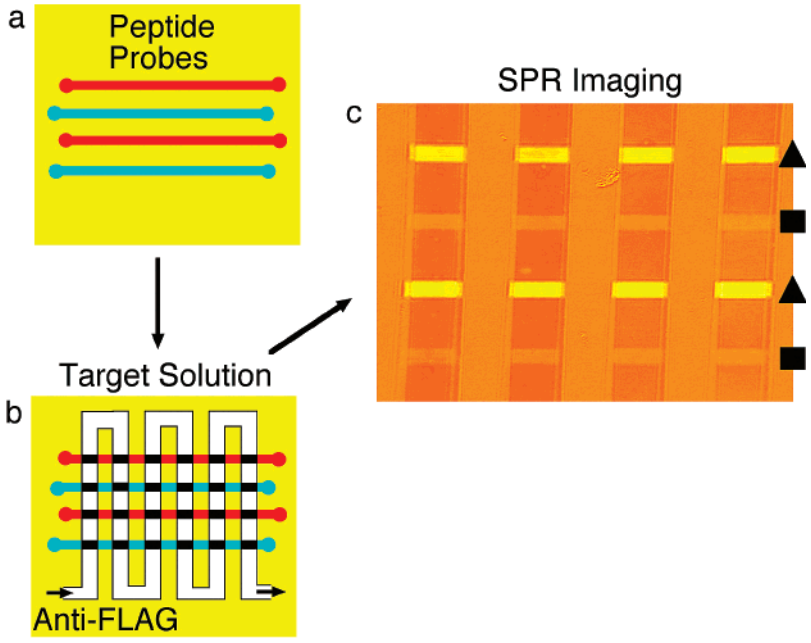

Figure 2. Schematic drawing showing fabrication of a peptide array on a gold surface. (a) A set of parallel PDMS microchannels was used to immobilize peptides onto a gold film, (b) A second PDMS microchannel was used as a small-volume sample cell (5 μL) to deliver target molecules (e.g., antibody) to the peptide line array. (c) SPR difference image showing the adsorption of anti-FLAG M2 onto a two-component peptide array.

peptides were immobilized within a set of parallel microfluidic channels constructed from PDMS to create a "line array" of different peptide monolayers on a gold substrate. In a second step (see Figure 2b), a second PDMS channel with a wraparound design was used to deliver a small volume (5 μL) of antibody solution to the peptide line array. The surface chemistry described in section A was performed within the first set of parallel channels to create a pyridyl-terminated disulfide surface. Then different peptide solutions (1mM) were delivered through the channels and reacted with the pyridyl-terminated surface for 3 h to covalently attach the peptides to the gold surface. The microchannels used to create the peptide arrays were 300 μm wide with 700-μm spacing so that a maximum of 10 different species could be immobilized for simultaneous analysis by SPR imaging. By changing the spacing and width of the microchannels, it is possible to immobilize up to 100 different peptides on one 1.8 cm $\times$ 1.8 cm chip.

Once the peptide immobilization was complete, the parallel microchannels were removed and the amine moieties on the surface surrounding the immobilized peptide lines were reacted with PEG-NHS. The PEG surface provided a background with respect to the peptide array that is resistant to the nonspecific adsorption of proteins. Polarization modulation Fourier transform infrared spectroscopy (PM-FT-IR) measurements demonstrated that while the N-terminus and lysine residues of the peptides contain amine functionalities, PEG-NHS reacted with relatively few of them, in comparison with the alkanethiol background, and consequently did not negatively modify the peptide structure to a significant extent (supporting FT-IR data are not shown).

After the background of the peptide of the array was prepared, a second microchannel was used as a small-volume flow cell to introduce antibody solutions to the surface (Figure 2b). This microchannel was placed perpendicular to the peptide line array resulting in discrete intersections, where antibody solution contacts the peptide array, defined by the flow path. A maximum number of intersections are created by this approach while the total sample volume is 5 μL.

SPR imaging experiments were used to monitor the adsorption of the antibodies to the peptide array (Figure 2c). Images of a peptide array made by this fabrication process were taken before and after the introduction of 100 nM anti-FLAG M2 through the PDMS microchannel and subtracted to produce the SPR difference image shown in Figure 2c. The array was composed of two peptide epitopes immobilized in alternate channels onto the gold surface, and differential antibody binding to the two peptides was observed based on the sequence specificity of the antibody−peptide interaction.

C. Optimization of Peptide Arrays for Antibody Binding Measurements. Optimization of the peptide arrays for SPR imaging measurements of antibody binding has components in two essential areas: (i) optimization of the surface attachment chemistry for the peptide epitopes and (ii) optimization of the surface density of the peptides. Specifically, the surface attachment chemistry was optimized by studying the effect the addition of spacer residues to the F1 peptide sequence had on anti-FLAG M2 binding. The surface density of the peptides was modified by two different methods that targeted the initial and final steps of the peptide immobilization process, respectively.

Spacer Residues and Peptide Accessibility. The oriented attachment of peptides was accomplished through the addition of a terminal cysteine. The terminal cysteine was coupled to the peptide sequence of interest with spacer residues, to maximize the availability of the peptide to antibody. Short uncharged spacers have often been inserted between a peptide tag and a protein for the purpose of increasing accessibility of the target to the tag when fusion proteins were purified.[27−29] Peptides containing the F1 sequence listed in Table 1 were synthesized without a spacer, with a serine−glycine (SG) spacer, and with an SGSG spacer and

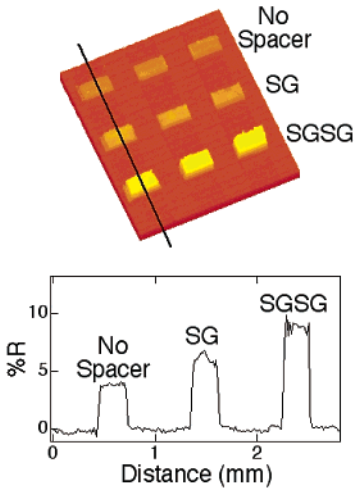

Figure 3. SPR difference image showing the binding of a 100 nM solution of anti-FLAG M2 to a peptide array composed of peptides containing the F1 sequence (refer to Table 1) modified with either an SGSG spacer, an SG spacer, or no spacer. The most antibody adsorption was observed at the peptide with the SGSG spacer.

immobilized onto an array. Figure 3 shows an SPR difference image taken after the introduction of 100 nM anti-FLAG M2 solution to the surface. The line profile distinctly shows the increase in antibody adsorption onto peptides containing spacers. The addition of an SG spacer increased the SPR response by 179% from the original sequence, whereas the addition of an SGSG spacer resulted in an increase of 238%. Subsequent peptide immobilizations were performed using peptides incorporating an SG spacer to enhance the SPR signal observed while minimizing the addition of amino acids added to the short peptide sequence.

Control of Peptide Surface Density. The attachment of peptides to the gold surface is composed of two coupling reactions: (i) the formation of the amide linkage to the amine-terminated MUAM surface to create a free thiol and (ii) the conversion of this thiol into a disulfide linkage to the cysteine of a modified peptide. The surface coverage of a full peptide monolayer was measured by fluorescence wash-off experiments of fluorescently labeled peptides to be 1.5×10^{13} molecules/cm^2 (see Experimental Section), while the number of alkanethiol molecules in a packed monolayer was determined by others to be 4×10^{14} molecules/cm^2. [30,31] To account for the order of magnitude difference between the alkanethiol monolayer and the peptide surface coverage, two methods were explored to vary the peptide surface coverage based on the two steps of the peptide immobilization strategy. The first method involved controlling the surface coverage of the amine-terminated monolayer using mixed monolayers to change the initial step of the attachment chemistry. The second method explored the reaction of mixed peptide solutions to control the peptide surface coverage through the disulfide linkage.

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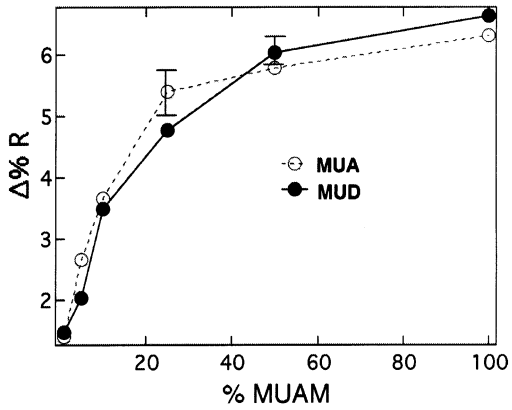

Figure 4. Comparison of the change in percent reflectivity upon anti-FLAG adsorption (100 nM) onto F1 peptide arrays fabricated on mixed monolayers containing various percentages of MUAM in MUA (○) and MUAM in MUD (●). Representative error bars have been included for these measurements. The dotted and solid lines are included as an aid to the eye.

Mixed alkanethiol monolayers were used to reduce the amine surface coverage and thereby change the surface density of immobilized peptides. Mixtures were made of MUAM and an alkanethiol terminated with either a carboxylic acid (MUA) or a hydroxyl group (MUD). These alkanethiols were selected as diluents since neither MUA nor MUD can be chemically modified to attach peptides to the surfaces using the reaction scheme described above. Arrays of mixed monolayers were fabricated on gold thin films using a set of parallel PDMS michrochannels. Two-component alkanethiol solutions with different ratios of MUAM/MUD or MUAM/MUA were introduced into the microchannels to create a line array of different SAMs. These sets of SAMs were then used to create peptide arrays by performing the rest of the chemical modification steps within the microchannels.

SPR imaging measurements were used to measure antibody adsorption onto the peptide arrays. Figure 4 shows the results from SPR imaging experiments where a peptide array created with mixed alkanethiol monolayers was exposed to 100 nM anti-FLAG. The changes in percent reflectivity were plotted as a function of the solution composition of alkanethiol exposed to the gold surface to create the peptide lanes. A maximum in the change in percent reflectivity ($\Delta\%\ R$) due to antibody adsorption was observed on peptide array elements composed of greater than 50% MUAM for both MUAM/MUD and MUAM/MUA solution mixtures.

In a second set of experiments, we controlled the mole fraction of peptide epitopes on the array by the attachment of mixed solutions of peptides via the thiol−disulfide exchange reaction (see Figure 1). Peptide attachment was performed with solutions containing mixtures of either F1 and HA peptides (refer to Table 1 for sequences), or mixtures of F1 and cysteine. The HA sequence, a peptide tag derived from the influenza hemagglutinin protein,[32,33] was chosen as a noninteracting peptide, because anti-FLAG does not bind to the HA sequence and it has an associated antibody. F1 and HA were mixed in solutions with mole fractions

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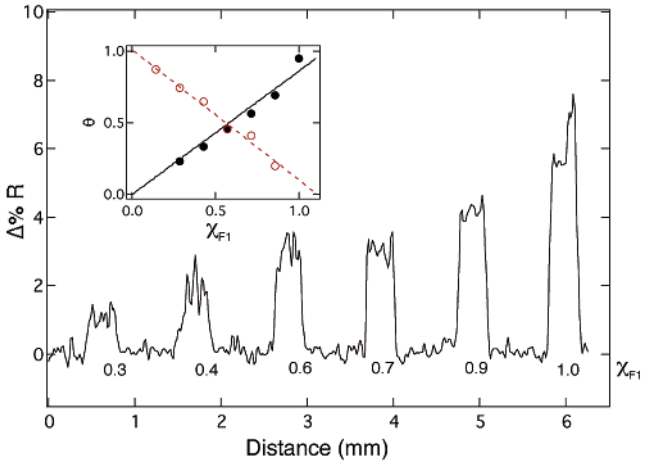

Figure 5. Line profile showing the adsorption anti-FLAG M2 (50 nM) onto a peptide array composed of F1 peptide diluted into the noninteracting HA peptide in mole fractions ranging from 0.29 to 1.00 F1. The graph inset shows the antibody surface coverage obtained when the array is exposed sequentially to anti-FLAG (●) and 10 nM anti-HA (○).

of F1 peptide ranging from 0.29 to 1.00 and immobilized onto the surface in a line array format.

Figure 5 shows line profiles obtained from SPR imaging experiments after the exposure of the peptide line array to antibody solutions. Exposure of the array to a 50 nM solution of anti-FLAG resulted in antibody binding that correlated to the composition of the array elements, with low coverages of immobilized F1 resulting in less antibody adsorption than higher F1 coverages. A similar relationship between anti-HA binding and the composition of the peptide array element was obtained in the converse experiment, where 10 nM anti-HA was introduced to the peptide array. An additional SPR imaging experiment was perfomed using peptide arrays fabricated by immobilizing solutions of FLAG and cysteine in varying ratios. The linear relationship between antibody adsorption and the mole fraction of FLAG was always observed for FLAG arrays made from mixtures of either HA or cysteine.

The inset in Figure 5 depicts the quantitative relationship between the surface coverage and the molar ratio of peptides reacted with the surface. These data were obtained by integrating the line profile to determine the change in percent reflectivity at each array element. This value was converted to surface coverage based on the signal measured for fully packed HA and FLAG peptide monolayers after maximum antibody binding, which was determined to be repeatable from array to array. If the HA and FLAG peptides were immobilized with equivalent reaction efficiencies to the surface, the surface coverage at 50% would correspond to 0.5 mole fraction of FLAG to HA. However, the HA and FLAG lines intersect at ~60% FLAG in HA, suggesting that immobilization efficiencies might differ slightly for the two peptides.

Using the results from these two surface dilution experiments, we can conclude that (i) the first surface attachment reaction limits the surface density of the peptides, and (ii) there are no steric hindrance effects due to antibody−antibody interactions. Figures 4 and 5 demonstrate that modifications to the first and second steps of the peptide immobilization process cause differing changes to the surface coverage of the peptide arrays, as can be seen in the patterns of anti-FLAG M2 adsorption onto the peptide arrays. The linear relationship between antibody adsorption and the percentage of FLAG immobilized in Figure 5 implies that there are few antibody−antibody interactions on these monolayers. More specifically, a system where steric hindrance limits the antibody surface coverage would be associated with a curve that reaches a maximum in antibody adsorption with increasing peptide surface coverages. Consequently, the maximum antibody coverage observed on peptide arrays created with mixed alkanethiol monolayers, shown in Figure 4, cannot be attributed to antibody−antibody interactions and is instead the result of a chemical peptide attachment effect. Two explanations can account for this behavior; either the MUA and MUD alkanethiols adsorb preferentially to the gold surface or one of the peptide attachment chemical modification steps reacts nonlinearly as a function of amine surface coverage.

To determine which of these explanations is correct, additional measurements of the mixed monolayers were performed using PM-FT-IR. PM-FT-IR measurements of surfaces exposed to either a 100% MUA solution or a 50% MUA in MUAM solution showed that there is an equivalent decrease in the intensity of the carboxylic acid stretch associated with the surface exposed to a decreased MUA concentration. This demonstrates that preferential alkanethiol adsorption onto the gold surface did not account for the leveling of peptide coverage in Figure 4. Additional PM-FT-IR experiments were performed to determine which of the surface modification steps affected the peptide coverage on the mixed monolayers. Spectra were taken of surfaces prepared with 50% MUAM in MUD, 50% MUAM in MUA, and 100% MUAM after reaction with SATP. The spectra of the SATP attachment chemistry showed that the intensity of the amide I and amide II bands due to the formation of an amide linkage were comparable for the full monolayer of MUAM and the mixed monolayers (data not shown). Since the intensity of the amide bands can be used to compare the relative amount of SATP immobilization on the three surfaces, this proves that the SATP modification step is not linearly related to the amine surface coverage and this nonlinearity results in the curvature of the SPR antibody binding signal in Figure 4.

In addition, dilution of the peptide monolayer can be used to quantitatively reduce the measured change in percent reflectivity due to antibody adsorption when necessary. Changes in percent reflectivity less than 10% have been previously found by SPR imaging to be linearly proportional to changes in the index of refraction, correlating to the number of molecules adsorbed onto the surface.[34] Since antibodies have large molecular weights, antibody adsorption onto the peptide surface may cause changes in the index of refraction greater than 10%. In these cases, the peptide surface coverages can be precisely reduced (refer to Figures 4 and 5) to maintain levels of antibody binding within the linear range of SPR.

II. SPR Imaging Measurements of FLAG Peptide Arrays. As a demonstration of the utility of these peptide arrays, we examined the binding of anti-FLAG M2 to an array composed of four different peptide epitopes based on the binding motif of the

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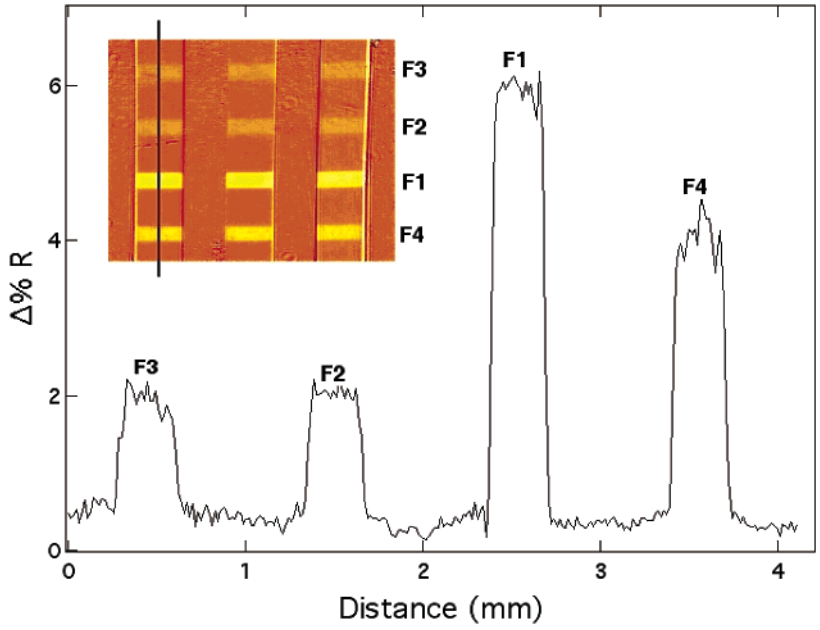

Figure 6. SPR difference image showing the binding of 100 nM solution of anti-FLAG M2 to a peptide array. The line profile shows the differential binding of antibody to the original sequence (F1) and peptide variants produced by replacing one of the original residues with alanine (F2, F3, F4). The peptide sequences are listed in Table 1.

antibody−peptide interaction. The critical residues from the FLAG binding motif are given by Asp-Tyr-Lys-*X*-*X*-Asp-*X*-Lys, where *X* denotes nonessential residues.[25,26] The original FLAG sequence is identified as F1. Variants were made by single alanine substitutions for amino acids derived from the original sequence. F2 and F3 contained alanine substitutions for the residue 2 tyrosine and residue 3 lysine, respectively. F4 was made by substituting alanine for the residue 5 aspartic acid, which was not identified as part of the binding motif.

Differential binding of anti-FLAG M2 to an array of FLAG peptides is shown in the SPR image in Figure 6. The line profile taken across one of the columns in the image clearly shows the difference in binding to each of the peptide lanes. The most SPR signal was observed for F1, the original peptide. The F4 sequence had the next highest signal. Since this peptide contained an alanine substitution for a nonessential amino acid, higher binding would be expected for F4 than for F2 or F3. The latter peptides, which contained alanine substitutions for amino acids in the binding motif, resulted in the least amount of antibody adsorption. At low concentrations, very little binding was observed at the F2 and F3 peptides while the F1 and F4 peptides were comparable and had a higher response. These data confirm that SPR imaging can be used to distinguish sequences with a single amino acid substitution and qualitatively discriminate between amino acids either important or superfluous to the antibody−peptide interaction.

A quantitative evaluation of the signal was obtained by simultaneously measuring the adsorption constants of antibodies onto peptides immobilized in an array format. This was accomplished by integrating the line profile of the peptide elements at increasing antibody concentrations. Data obtained in this way are shown in Figure 7 where the SPR signal resulting from antibody adsorption is plotted as a function of anti-FLAG M2 concentrations ranging from 1 to 150 nM. Anti-FLAG M2 solutions were introduced to the surface, in 5-μL aliquots, in order of increasing concentration. Measurements were obtained without the removal of antibody between concentrations, since the basic solutions used to denature the antibodies and remove them from the surface resulted in eventual degradation of the sample. Multiple aliquots were used for low-concentration samples to prevent stoichiometric limitations, since the surface coverage of peptides was larger than the quantity of antibodies present in a 5-μL sample. The detection limit for the FLAG and anti-FLAG M2 binding pair was found to be 0.5 nM. Data from the F1 peptide were fit using a Langmuir adsorption isotherm (solid line) with an adsorption coefficient, K_{ads}, of 1.5×10^8 M^{-1}. The adsorption coefficient can be used to determine the concentration at 50% surface coverage ($C_{0.5\theta}$), which can be directly compared to solution measurements of equilibrium dissociation constants. From our measurements, the F1 peptide was determined to have a $C_{0.5\theta}$ of 6.5 nM, which corresponds well to a literature report of an equilibrium dissociation constant (K_d), of 3.0 nM for the solution interaction of anti-FLAG M2 with a FLAG fusion protein displayed on a rat oocyte.[35]

Adsorption isotherms were also measured for the F4, F2, and F3 peptides on the same chip. The dashed line (Figure 7) shows the calculated Langmuir fit for the F4 peptide resulting in an adsorption coefficient of 2.8×10^7 M^{-1}. This value is less than the F1 adsorption coefficient, suggesting that some contribution to the peptide−antibody interaction was made by the residue 5

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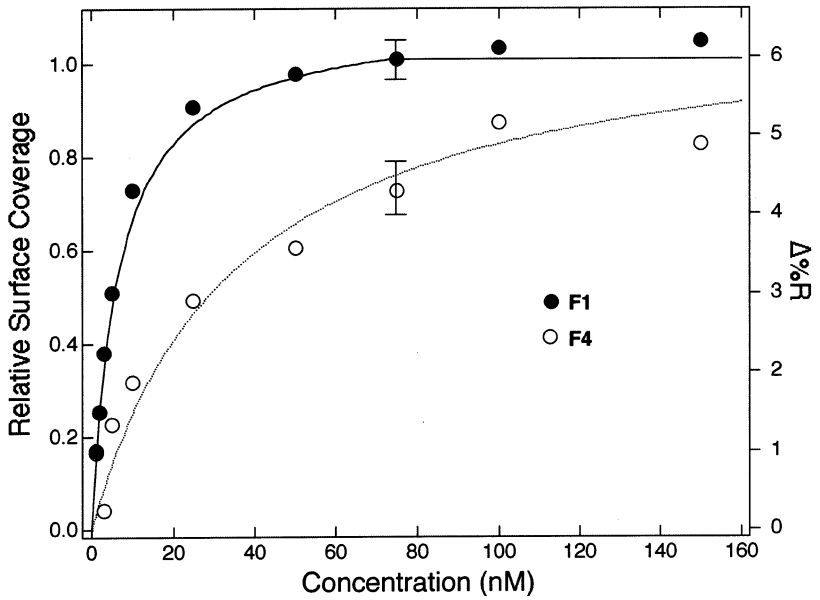

Figure 7. Langmuir isotherm fits for the adsorption of anti-FLAG M2 onto an array containing F1 peptide (●) and F4 peptide (○) using concentrations ranging from 1 to 150 nM. The Δ% *R* upon antibody adsorption to the peptide array is calculated by integration of the line profiles for the F1 and F4 peptides. The calculated Langmuir adsorption isotherms for F1 and F4 peptides are shown by a solid and a dashed line, respectively. The calculated adsorption coefficients (K_{ads}) were found to be 1.5×10^8 M^{-1} for F1 and 2.8×10^7 M^{-1} for F4.

aspartic acid that was not made by the substituted alanine. Adsorption constants were estimated by Langmuir fits for the F2 and F3 peptides to be 2.0×10^6 and 5.0×10^6 M^{-1}, respectively. The significant decrease in the adsorption coefficients is due to the adverse impact that an alanine substitution of an essential amino acid has on the antibody binding interaction. This experiment demonstrates that quantitative comparisons can be made between peptides containing single amino acid substitutions.

CONCLUSIONS

In this paper, we have developed a methodology for creating peptide arrays on chemically modified gold films using microfluidics for the study of peptide−antibody interactions with SPR imaging. The peptide arrays made by this process are robust and can be used for up to 30 solution cycles over several days. These arrays can be easily adapted to simultaneously study hundreds of peptide−protein interactions. These experiments also demonstrate that we can use SPR imaging of peptide arrays in the future to study linear epitope mapping of antibody−antigen interactions and enzymatic modifications of peptides. For example, these peptide arrays can be employed to characterize antigen−antibody pairs for the development of peptide tags[4,36] and for the identification of active peptide mimotopes against the original antigen that serve as a basis for novel drug development strategies.[37,38] Furthermore, the ability to monitor single amino acid substitutions with SPR measurements of antibody binding makes these peptide arrays amenable to the study of sequence-specific kinases and phosphatases using the immobilized peptides as model substrates.[39]

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