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Fabrication of Histidine-Tagged Fusion Protein Arrays for Surface Plasmon Resonance Imaging Studies of Protein−Protein and Protein−DNA Interactions

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The creation and characterization of histidine-tagged fusion protein arrays using nitrilotriacetic acid (NTA) capture probes on gold thin films for the study of protein−protein and protein−DNA interactions is described. Self-assembled monolayers of 11-mercaptoundecylamine were reacted with the heterobifunctional linker *N*-succinimidyl *S*-acetylthiopropionate (SATP) to create reactive sulfhydryl-terminated surfaces. NTA capture agents were immobilized by reacting maleimide-NTA molecules with the sulfhydryl surface. The SATP and NTA attachment chemistry was confirmed with Fourier transform infrared reflection absorption spectroscopy. Oriented protein arrays were fabricated using a two-step process: (i) patterned NTA monolayers were first formed through a single serpentine poly(dimethylsiloxane) microchannel; (ii) a second set of parallel microchannels was then used to immobilize multiple His-tagged proteins onto this pattern at discrete locations. SPR imaging measurements were employed to characterize the immobilization and specificity of His-tagged fusion proteins to the NTA surface. SPR imaging measurements were also used with the His-tagged fusion protein arrays to study multiple antibody−antigen binding interactions and to monitor the sequence-specific interaction of double-stranded DNA with TATA box-binding protein. In addition, His-tagged fusion protein arrays created on gold surfaces were also used to monitor antibody binding with fluorescence microscopy in a sandwich assay format.

Protein arrays can be used to identify and quantify bioaffinity interactions of proteins, DNA, RNA, and peptides in a multiplexed format. To date, protein arrays have been used to detect clinically interesting proteins in human serum at concentrations relevant to the analysis of blood samples.[1,2] Protein arrays have also been used as a proteomics screening tool to characterize biochemical processes by identifying novel protein−protein and protein−DNA binding interactions.[3,4] These results suggest that protein arrays will become essential tools in both clinical and research settings to identify the presence or absence of multiple proteins in a sample, to characterize protein function, and to design pharmaceutical agents that disrupt or stimulate specific bioaffinity interactions.

The successful implementation of these screening assays will depend on the development of optimized methods for the fabrication of high-fidelity protein arrays. Many protein arrays rely on the randomly oriented immobilization of proteins through amine groups present on the protein surface.[2,3,5] Randomly oriented immobilization can negatively affect the biological activity of a protein and has the additional disadvantage that the amount of protein adsorption may vary depending on the number of surface amines presented. To avoid these problems, surface capture agents can be used to immobilize fusion proteins, which are proteins that have been modified with a tag for specific interaction with the surface. This strategy is based on the established capture agent−fusion protein pairs that have been developed for purification via column chromatography. Many biologically active fusion proteins are available with popular fusion tags such as glutathione *S*-transferase,[6−8] maltose binding protein,[9,10] the FLAG peptide,[11,12] and hexahistidine.[13−15] Fusion

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(1) Pavlickova, P.; Jensen, N. M.; Paul, H.; Schaeferling, M.; C., G.; Kruschina, M.; Du, W. D.; Theisen, M.; Ibba, M.; Ortigao, F.; Kambhampati, D. *J. Protein Res.* **2002**, 227−231.
(2) Haab, B. B.; Dunham, M. J.; Brown, P. O. *Genome Biol.* **2001**, *2*, 1−13.
(3) Zhu, H.; Klemic, J. F.; Chang, S.; Bertone, P.; Casamayor, A.; Klemic, K. G.; Smith, D.; Gerstein, M.; Reed, M. A.; Snyder, M. *Nat. Genet.* **2000**, *26*, 283−289.
(4) Zhu, H.; Bilgin, M.; Bangham, R.; Hall, D.; Casamayor, A.; Bertone, P.; Lan, N.; Jansen, R.; Bidlingmaier, S.; Houfek, T.; Mitchell, T.; Miller, P.; Dean, R. A.; Gerstein, M.; Snyder, M. *Science* **2001**, *293*, 2101−2105.
(5) Schreiber, S. L.; MacBeath, G. *Science* **2000**, *289*, 1760−1763.
(6) Quetglas, S.; Leveque, C.; Miquelis, R.; Sato, K.; Seagar, M. *Proc. Nat. Acad. Sci. U.S.A.* **2000**, *97*, 9695−9700.
(7) Gokce, I.; Raggett, E. M.; Hong, Q.; Virden, R.; Cooper, A.; Lakey, J. H. *J. Mol. Biol.* **2000**, *304*, 621−632.
(8) Martzen, M. R.; McCraith, S. M.; Spinelli, S. L.; Torres, F. M.; Fields, S.; Grayhack, E. J.; Phizicky, E. M. *Science* **1999**, *286*, 1153−1155.
(9) Bach, H.; Mazor, Y.; Shaky, S.; Shoham-Lev, A.; Berdichevsky, Y.; Gutnick, D. L.; Benhar, I. *J. Mol. Biol.* **2001**, *312*, 79−93.
(10) Riggs, P. *Mol. Biotechnol.* **2000**, *15*, 51−63.
(11) Bouchard, B. A.; Furie, B.; Furie, B. C. *Biochemistry* **1999**, *38*, 9547−9523.
(12) Pearson, D. L.; Reimonenq, R. D.; Pollard, K. M. *Protein Expression Purif.* **1999**, *17*, 49−56.
(13) Schmitt, J.; Hess, J.; Stunnenberg, H. G. *Mol. Biol. Rep.* **1993**, *18*, 223−230.
(14) Bornhorst, J. A.; Falke, J. J. *Methods Enzymol.* **2000**, *326*, 245−254.

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protein arrays have the advantage that a single immobilization strategy can be used to create an array of multiple fusion proteins, and each protein will be immobilized in the same orientation through the fusion tag.

One of the most common fusion proteins used by molecular biologists is the addition of a hexahistidine tail through cloning means to a known protein forming a "His-tagged" fusion protein. The addition of this histidine tag is a relatively simple procedure, and there are currently a wide variety of commercially available His-tagged proteins. These His-tagged fusion proteins bind to the capture agent nitrilotriacetic acid (NTA) in the presence of Ni^{2+} ions and are used for the purification of proteins by column chromatography.[13,15,16] NTA capture agents have been used by several other researchers previously to make protein surface arrays.[17−21] The NTA modification chemistry used in column chromatography is not directly adaptable to the fabrication of protein arrays on planar surfaces, so other attachment strategies must be employed. This paper describes a simple surface attachment chemistry and methodology for fabricating oriented His-tagged fusion protein arrays on chemically modified gold surfaces.

One advantage of fabricating the protein arrays on gold surfaces is that the bioaffinity interactions can be measured using surface plasmon resonance (SPR) imaging. Most protein arrays currently developed rely on detection technologies that apply enzymatic or fluorescent tags. In contrast, SPR imaging is a label-free, surface-sensitive spectroscopic technique used to study bioaffinity interactions at gold thin films by measuring changes in the local index of refraction upon adsorption. SPR imaging has been successfully applied to the screening of bioaffinity interactions using DNA,[22−24] peptide,[25] and carbohydrate arrays.[26]

In this paper, we report the fabrication of oriented His-tagged protein arrays using NTA monolayers for SPR imaging measurements of protein−protein and protein−DNA interactions. Amine-terminated self-assembled monolayers of 11-mercaptoundecylamine (MUAM) on gold thin films were reacted with the heterobifunctional linker *N*-succinimidyl *S*-acetylthiopropionate (SATP) to create sulfhydryl surfaces. These surfaces were then modified with maleimide-NTA, creating a capture monolayer for His-tagged biomolecules. The robust NTA attachment chemistry was characterized with polarization−modulation Fourier transform infrared reflection absorption spectroscopy (PM-FT-IRRAS) measurements. Protein arrays were fabricated by a two-step process: first, patterned NTA monolayers were created using a single serpentine PDMS microchannel on a gold thin film and then a second set of microchannels with parallel channels was used to immobilize proteins to discrete regions of the array. SPR imaging measurements of these protein arrays were employed to characterize the immobilization of protein in the presence of nickel ions onto the NTA monolayers. SPR imaging measurements were also used to study antibody−antigen interactions with His-tagged fusion protein arrays and DNA-protein binding interactions with His-tagged arrays of TATA box-binding protein (TBP). In addition, fluorescence sandwich assays were performed on His-tagged protein arrays fabricated from NTA monolayers on gold surfaces.

Table 1. Oligonucleotide Sequences for Interaction with TBP Protein Arrays

symbol	sequence
dsDNA1	5′CTG CTA TAA AAG GCT G3′ 3′GAC GAT ATT TT C CGA C5′
dsDNA2	5′ATG AGT CTG ACG CTG A 3′ 3′TAC TCA GAC TGC GAC T 5′

EXPERIMENTAL CONSIDERATIONS

Materials. *N*-Succinimidyl *S*-acetylthiopropionate (Pierce), sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (Pierce), 11-mercaptoundecylamine (Dojindo Laboratories), *N*-[5-(3′-maleimidopropylamido)-1-carboxypentyl]iminodiacetic acid, disodium salt (maleimide-NTA, Dojindo Laboratories), and *N*-hydroxysuccinimidyl ester of methoxypoly(ethylene glycol) propionic acid MW 2000 (PEG-NHS, Shearwater Polymers Inc.) were used as received. Monoclonal anti-ubiquitin (Santa Cruz Biotechnology Inc.), His-tagged ubiquitin (His-TBP, EMD Biosciences, Inc.), *N*-terminal FLAG-tagged ubiquitin (Sigma), polyhistidine (MW 39 200, Sigma), His-tagged TATA binding protein (His-TBP, EMD Biosciences, Inc.), anti-FLAG M2 (Sigma), and Alexa Fluor 488 goat anti-rabbit (Molecular Probes) were used without further purification. His-tagged red fluorescent protein (RFP) and anti-RFP were a gift from the Marriott laboratory. DNA probes (see Table 1) and a peptide (HHHHHHSGDYKDDDDK) were synthesized at the University of Wisconsin Biotechnology center. Other standard chemicals were purchased from commercial vendors and used as received.

NTA Attachment Chemistry. Gold slides were immersed in 1 mM ethanolic solutions of MUAM for at least 4 h. SATP (14 mM, 10% DMF/90% PBS pH 7.4) was reacted with the MUAM monolayers for 1.5 h. The protecting group of the sulfhydryl was removed by exposing the monolayer to a solution of 0.5 M hydroxylamine, 0.05 M phosphate buffer, 0.025 M EDTA, and 0.05 M DTT for 30 min. The sulfhydryl surface was then reacted with maleimide-NTA (15 mM in TEA pH 8) overnight.

PM-FT-IRRAS Measurements. All FT-IR experiments employed samples that were prepared on commercial gold slides (5 nm of Cr, 100 nm of Au) obtained from Evaporated Metal Films. FT-IR spectra were collected using a Mattson RS-1 spectrometer and an HgCdTe detector. The optical layout has been previously described elsewhere.[27,28] Spectra were the result of 1000 scans collected at 4-cm^{-1} resolution.

(15) Hochuli, E. *J. Chromatogr.* **1988**, *444*, 293−302.
(16) Hoffman, A.; Roeder, R. G. *Nucleic Acids Res.* **1991**, *19*, 6337−6338.
(17) Sigal, G. B.; Bamdad, C.; Barberis, A.; Strominger, J.; Whitesides, G. M. *Anal. Chem.* **1996**, *68*, 490−497.
(18) Kubalek, E. W.; Le Grice, S. I.; Brown, P. O. *J. Struct. Biol.* **1994**, *113*, 117−123.
(19) Dietrich, C.; Boscheinen, O.; Scharf, K. D.; Schmitt, L.; Tampe, R. *Biochemistry* **1996**, *35*, 1100−1105.
(20) Gershon, P. D.; Khilko, S. *J. Immunol. Methods* **1995**, *183*, 65−76.
(21) Luk, Y. Y.; Tingey, M. L.; Hall, D. J.; Israel, B. A.; Murphy, C. J.; Bertics, P. J.; Abbot, N. L. *Langmuir* **2003**, *19*, 1671−1680.
(22) Smith, E. A.; Erickson, M. G.; Ulijasz, A. T.; Weisblum, B.; Corn, R. M. *Langmuir* **2003**, *19*, 1486-1492.
(23) Smith, E. A.; Kyo, M.; Kumasawa, H.; Nakatani, K.; Corn, R. M. *J. Am. Chem. Soc.* **2002**, *124*, 6810−6811.
(24) Lee, H. J.; Goodrich, T. T.; Corn, R. M. *Anal. Chem.* **2001**, *73*, 5525−5531.
(25) Wegner, G. J.; Lee, H. J.; Corn, R. M. *Anal. Chem.* **2002**, *74*, 5161−5168.
(26) Smith, E. A.; Thomas, W. D.; Kiessling, L. L.; Corn, R. M. *J. Am. Chem. Soc.* **2003**, *125*, 6140−6148.
(27) Green, M. J.; Barner, B. J.; Corn, R. M. *Rev. Sci. Instrum.* **1991**, *62*, 1426−1430.

Array Fabrication. A Denton DV-502A evaporator was used to vapor deposit thin gold films (45 nm) with an underlayer of chromium (1 nm) onto SF10 glass slides (Schott Glass); these films were used for all SPR imaging experiments. Microchannels were fabricated by curing poly(dimethylsiloxane) (PDMS) polymer on 3-D silicon master wafers at 70 °C as described previously.[24,29] A PDMS microchannel was constructed from a silicon master displaying a serpentine design (500-μm width, 14.2-cm total length, 35-μm depth) with 500-μm spacing between the folds, and entrance and exit holes were made for sample introduction. This channel was used to chemically pattern the gold surface with NTA capture agents using a total solution volume of 5 μL. Once NTA was immobilized, the PDMS channel was removed and replaced with a second set of PDMS microchannels featuring multiple parallel channels (300-μm width, 11-mm length, 35-μm depth) placed perpendicular to the pattern of immobilized NTA capture agents. These channels were used to immobilize His-tagged proteins in Tris buffer containing nickel ions (pH 7.4, 100 mM Tris-HCL, 100 mM NaCl, 40 mM $NiSO_4$). Additional biomolecules in Tris buffer were delivered to the protein chip through the same channels used to immobilize the proteins. A simple aspiration pumping system was used to introduce solutions through the microchannels. Further details of the array fabrication process can be found within the text and in Figure 3. These arrays could be regenerated by rinsing the microchannels with a Tris buffer that did not contain nickel ions. However, NTA arrays are best suited as disposable biosensors due to the residual nonspecific adsorption of proteins onto the surface.

SPR Imaging Measurements. All SPR imaging experiments were performed on an SPR imager apparatus using near-infrared excitation from an incoherent source.[30] The imager was designed with an optical configuration that allowed the microchannels attached to the protein array to remain in a horizontal position to reduce solution leakage. A polarized white light source was directed at the prism/sample assembly and through a narrow band-pass filter using mirrors. Images of the protein array were collected at a fixed angle using a CCD camera and V++ Precision Digital Imaging Systems, version 4.0 software. The software package NIH Image V.1.6.1 was used to further analyze the images.

RESULTS AND DISCUSSION

A. Preparation and Characterization of NTA Monolayers. NTA-modified self-assembled monolayers on gold thin films were developed in order to immobilize histidine-tagged proteins in an array format for SPR imaging measurements. The carboxylic acids of the NTA ligand have been shown to bind in the presence of nickel ions to hexahistidine peptide sequences that have been added to proteins by molecular biological methods. The chemical modification scheme used to prepare NTA monolayers is depicted in Figure 1. A packed self-assembled monolayer of MUAM is reacted with the heterobifunctional linker SATP. The NHS ester moiety of SATP reacts with the terminal amines of the packed

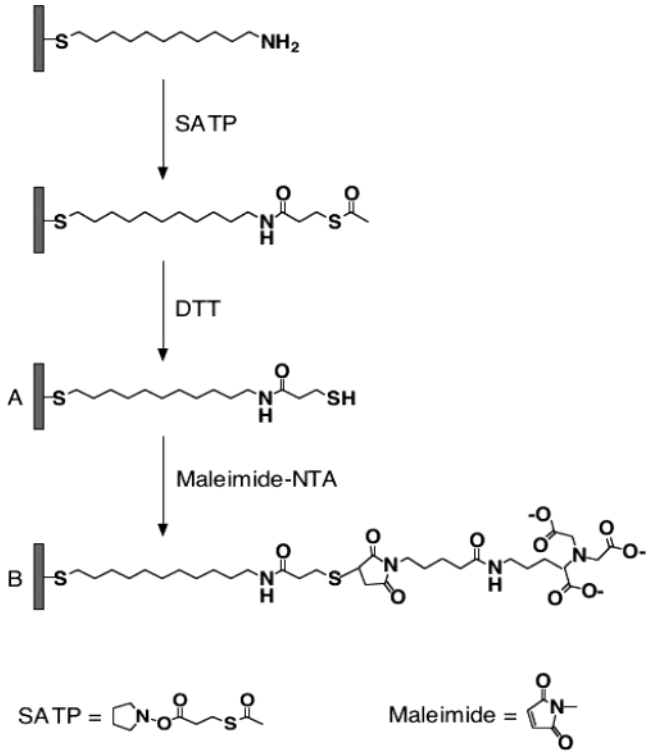

Figure 1. Surface reaction scheme showing the steps involved in the immobilization of NTA capture agents onto a gold thin film. First, the heterobifunctional linker SATP is reacted with a MUAM monolayer forming an amide bond. (A) The protecting group of the sulfhydryl is removed to reveal an active sulfhydryl surface. (B) The reaction of maleimide-NTA with the sulfhydryl surface results in the formation of a thioether bond linking the NTA capture agents to the surface.

monolayer forming a stable amide linkage. A hydroxylamine solution containing DTT is used to remove the acetyl protecting group from the sulfhydryl, revealing an active sulfhydryl surface (Figure 1A).[31] The sulfhydryl groups are then exposed to a 15 mM solution of maleimide-NTA, forming a stable thioether linkage through the alkylation of the double bond of the maleimide (Figure 1B).

PM-FT-IRRAS was used to characterize each modification step in the creation of an NTA monolayer on a gold thin film, and the spectra are shown in Figure 2. Table 2 summarizes the frequencies and assignments of the vibrational bands observed for each step. Several characteristic bands were observed in the spectrum taken of the surface after the reaction of SATP with MUAM and the removal of the acetyl protecting group (spectrum A, Figure 2). The amide I and amide II bands were observed at 1660 and 1550 cm^{-1} respectively, and a small carbonyl band was observed at 1730 cm^{-1} from the protecting group of residual unreacted SATP.[31] Spectrum B in Figure 2 shows the PM-FT-IRRAS spectrum after the reaction of the sulfhydryl surface with NTA. Additional bands appear in this spectrum; these bands are readily apparent in the difference spectrum which is shown in Figure 2 and labeled B − A. An increase in the amide I band was observed due to the presence of an additional amide linkage in the maleimide-NTA linker. From the intensity of these amide bands, the NTA surface coverage was estimated to be $\sim 10^{13}$ molecules/cm^2. This number

(28) Barner, B. J.; Green, M. J.;.I., S. E.; Corn, R. M. *Anal. Chem.* **1991**, *63*, 55−60.
(29) Duffy, D. C.; McDonald, J. C.; Schueller, O. J. A.; Whitesides, G. M. *Anal. Chem.* **1998**, *70*, 4974−4984.
(30) Nelson, B. P.; Frutos, A. G.; Brockman, J. M.; Corn, R. M. *Anal. Chem.* **1999**, *71*, 3928−3934.
(31) Smith, E.; Wanat, M. J.; Cheng, Y.; Barreira, S. V. P.; Frutos, A. G.; Corn, R. M. *Langmuir* **2001**, *17*, 2502−2507.

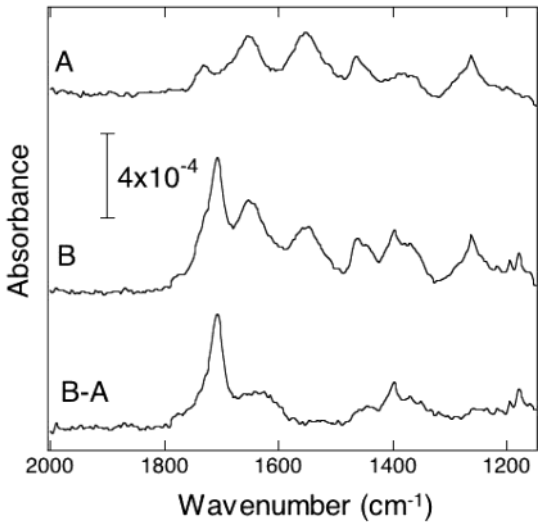

Figure 2. PM-FT-IRRAS spectra of the mid-IR region for the surfaces involved in the immobilization of NTA capture agents to the protein array. (A) Spectrum taken of the sulfhydryl surface created from the reaction of MUAM with SATP bands attributed to the formation of amide bonds. (B) Spectrum taken after the reaction of maleimide-NTA with the sulfhydryl surface. (B − A) The difference spectrum of the SATP spectrum from the NTA spectrum clearly shows the increase in the amide I band and the formation of additional carboxylic acid stretching bands indicating the covalent attachment of NTA capture probes.

Table 2. PM-FT-IRRAS Band Assignments for Surface Modification with NTA Probes

surface	frequency (cm^{-1})	assignment	Figure
SATP	1732	C═O symmetric stretch (protecting group)	2A
	1653	amide I	
	1550	amide II	
	1466	CH_2 scissor deformation	
	1370	CH_2 wagging	
NTA	1709	C═O asymmetric stretch (maleimide)	2B
	1653	amide I	
	1550	amide II	
	1466	CH_2 scissor deformation	
	1380	CH_2 wagging	

is comparable to that observed for the reaction of SATP monolayer with peptides.[25]

B. Fabrication of His-Tagged Protein Arrays. Oriented His-tagged fusion protein arrays were fabricated using a two-step process in which His-tagged proteins were immobilized onto the NTA monolayers using PDMS microfluidics. In the first step, a patterned surface of NTA capture agents was attached to a chemically modified gold surface by delivering reaction solutions through a single microchannel with a serpentine design (Figure 3a). In the second step, a set of parallel microchannels was used to immobilize proteins onto discrete regions of the array (Figure 3b). This was accomplished by removing the first microchannel after the NTA capture agents were immobilized overnight and replacing it with a set of parallel microchannels with 300-μm widths, oriented perpendicular to the NTA pattern. Next, a succinimide ester derivative of PEG was introduced through each of the parallel microchannels to react with the amine groups in

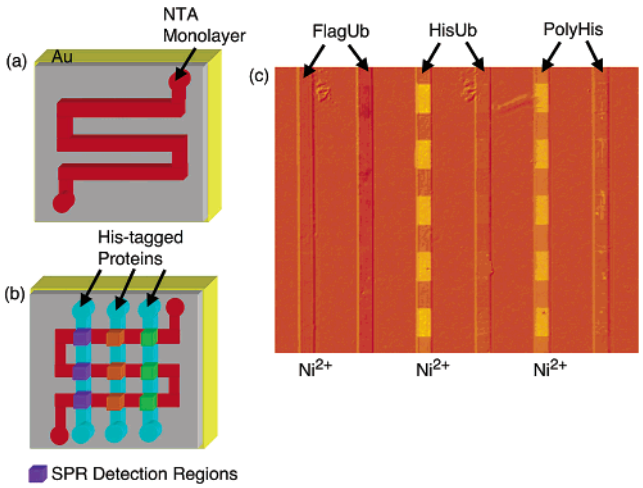

Figure 3. (a) Patterned NTA monolayer created using a serpentine microfluidic channel onto a MUAM surface. (b) After NTA immobilization, the microchannel is removed and a second set of parallel microchannels is placed perpendicular to the NTA pattern and used to deliver PEG molecules to react with the amine monolayer surrounding the NTA and prevent nonspecific protein adsorption. These channels are then used for the immobilization of His-tagged proteins. (c) The SPR difference image obtained by subtracting images taken before and after the introduction of proteins shows the specific adsorption of His-tagged ubiquitin and polyhistidine in the presence of nickel ions to the NTA-modified array. No adsorption is observed for the His-tagged ubiquitin or the polyhistidine when nickel ions are present. No interaction between the NTA-modified surface and FLAG-ubiquitin is observed either in the presence or in the absence of nickel ions.

the regions surrounding the pattern of immobilized NTA capture agents. Discrete pads of protein surrounded by a PEG background were created by delivering His-tagged proteins through each of the parallel microchannels. The same channels were then used to deliver target proteins or DNA to the protein array. Since PEG resists the nonspecific adsorption of protein, target molecules binding to the individual protein regions can be compared to the PEG background.

SPR imaging measurements were used to characterize the specific immobilization of the His-tagged proteins onto the NTA array. Figure 3c shows an SPR difference image obtained from images taken before and after the immobilization of three different proteins in the NTA array. These His-tagged proteins were introduced through the microchannels by a simple vacuum, and then the flow was stopped and the proteins were reacted with the NTA surface for 10 min. The channels were then rinsed with several aliquots of buffer in order to remove any nonspecifically adsorbed molecules, and an image was immediately taken. The binding of both 250 nM polyhistidine (PolyHis) and 2.0 μM His-tagged ubiquitin (HisUb) was observed when the proteins were solvated with Tris buffer containing 40 mM nickel sulfate. However, no adsorption of either HisUb or PolyHis was observed in the absence of nickel ions in solution. The specific chelation interaction between NTA and the His-tagged proteins involves the octahedral coordination of the nickel ion, with two valences occupied by two imidazole groups from the His tag and four ligands donated by the NTA molecule.[14,32] The specificity of Ni^{2+}-

(32) Hochuli, E.; Dobeli, H.; Schacher, A. *J. Chromatogr.* **1987**, *411*, 177−184.

NTA and Ni^{2+}-histidine binding was demonstrated by the necessity of Ni^{2+} ions to be present in the buffer for protein adsorption to be observed. As a control experiment, the patterned NTA monolayer was also exposed to solutions of 2.0 μM ubiquitin modified with a FLAG fusion tag (DYKDDDDK) in place of a hexahistidine tag (the two channels labeled FLAGUb in Figure 3c). The SPR image shows that there is virtually no interaction of these fusion proteins with the NTA surface. These control experiments confirm that a His tag must be present for the specific immobilization of fusion proteins onto the NTA surface.

Once adsorbed to the surface, the His-tagged fusion proteins could be removed by exposing the surface to a buffer solution that did not contain Ni^{2+} ions. A maximum amount of His-tagged fusion protein adsorption to the NTA surface was observed for buffers with nickel sulfate concentrations above 30 mM. Lower surface protein densities could be obtained with lower nickel sulfate concentrations. The ability to reduce the amount of protein immobilized on the surface in a controlled fashion is important for two reasons: (i) to eliminate any possible protein−protein interactions in the monolayer and (ii) in the case of high-affinity binding events, to reduce the signal in the SPR imaging measurements in order to maintain a linear response relationship with surface coverage.[25,33]

C. Measuring Bioaffinity Interactions with His-Tagged Protein Arrays. Antibody−Antigen Interactions. A series of SPR imaging and fluorescence measurements were performed in order to demonstrate the utility of NTA protein arrays for the study of protein−protein and protein−DNA interactions. In the first set of experiments, SPR imaging measurements of His-tagged protein arrays were used to identify specific antibody−antigen interactions. A three-component array was made by immobilizing 2 μM His-tagged RFP, 2 μM ubiquitin, and 30 μM of a modified His-FLAG peptide in consecutive parallel channels. The modified His-FLAG peptide consisted of six histidines, a serine−glycine spacer, and the FLAG sequence (DYKDDDDK). The concentration of the His-tagged probes was selected to allow the formation of a complete monolayer as determined by Langmuir isotherm measurements of the binding of His-tagged ubiquitin to NTA. The K_{ads} for this interaction was determined to be 2×10^6 M^{-1} ($\theta_{1/2} = 5.0$ μM). After the immobilization of His-tagged probes, the surface was rinsed with buffer and an image was taken. The off rate of the His-tagged protein from the NTA array is relatively slow, suggesting that the protein array does not degrade in the 2 min required to flow antibody into the microchannels. In fact, no change in protein surface coverage was observed when the immobilized His-tagged proteins were exposed to a solution of buffer containing Ni^{2+} ions for 10 min. The array composed of RFP, ubiquitin, and FLAG peptide was then sequentially exposed to three different antibodies, and the SPR imaging results from these experiments are shown in Figure 4. All three components of the protein array (labeled RFP, Ub, and FLAG in the figure) were first exposed to a 50 nM solution of the antibody anti-FLAG M2 for 5 min to allow for equilibrium binding. This concentration was selected with respect to our previously reported K_{ads} of 1.5×10^8 M^{-1} ($\theta_{1/2} = 6.5$ nM) for anti-FLAG binding to FLAG peptide.[25] A difference image of the array was obtained by subtracting images taken directly before and after antibody binding with the antibody still present in solution and is shown at the top of Figure 4. The specific interaction of anti-FLAG with the immobilized His-tagged FLAG peptide is clearly observed in a line profile taken across the image, with little nonspecific adsorption observed to either RFP or ubiquitin fusion proteins.

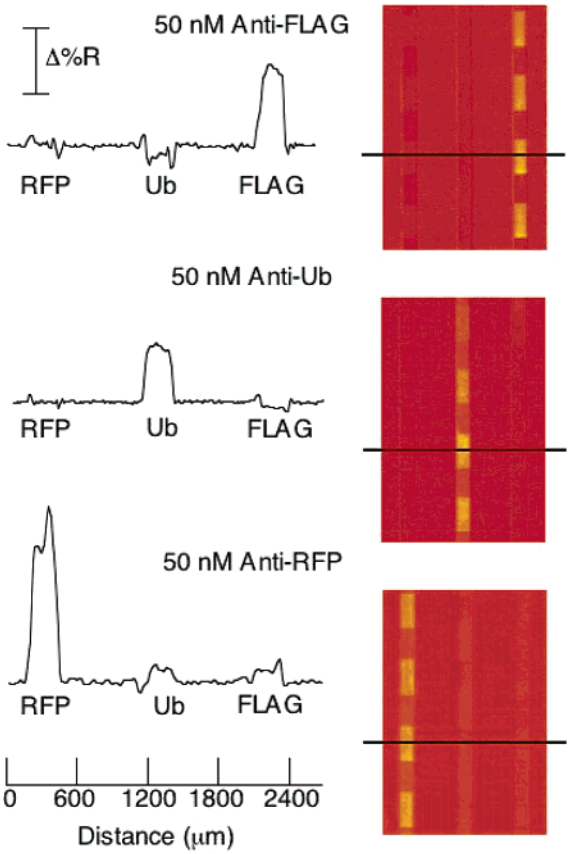

Figure 4. Three-component array created using His-tagged Ub, RFP, and FLAG peptide to study antibody−antigen interactions. An SPR difference image was obtained by subtracting images taken directly before and after 50 nM anti-FLAG was introduced to the protein array (top). The line profile taken across the image shows the increase in percent reflectivity as anti-FLAG adsorbed to the FLAG peptide with little nonspecific adsorption to either Ub or RFP. In another experiment, an SPR difference image was taken after 50 nM anti-Ub was exposed to the protein array showing the specificity of anti-ubiquitin to ubiquitin (middle). Similar specificity of 50 nM anti-RFP to RFP was observed in a third SPR imaging experiment (bottom).

In a second and third experiment, a three-component protein array containing His-tagged RFP, ubiquitin, and FLAG peptide was exposed to 50 nM solutions of the antibodies anti-Ub and anti-RFP. The results are shown in the two lower panels of Figure 4; antibody binding was observed to the His-tagged Ub or RFP for anti-Ub and anti-RFP, respectively. A small amount of nonspecific adsorption was observed for anti-RFP; no nonspecific adsorption was observed for anti-FLAG or anti-Ub. Moreover, the antibody adsorption onto the His-tagged protein arrays required 40 mM Ni^{2+} ion in solution. This adsorption was only partially reversible because of denaturation of the antibodies onto the surface; rinsing with a Tris buffer solution without Ni^{2+} led to the removal of 70% of the adsorbed antibody−antigen complex. As a consequence, three different arrays were used in Figure 4. The partial irrevers-

(33) Nelson, B. P.; Grimsrud, T. E.; Liles, M. R.; Goodman, R. M.; Corn, R. M. *Anal. Chem.* **2001**, *73*, 1−7.

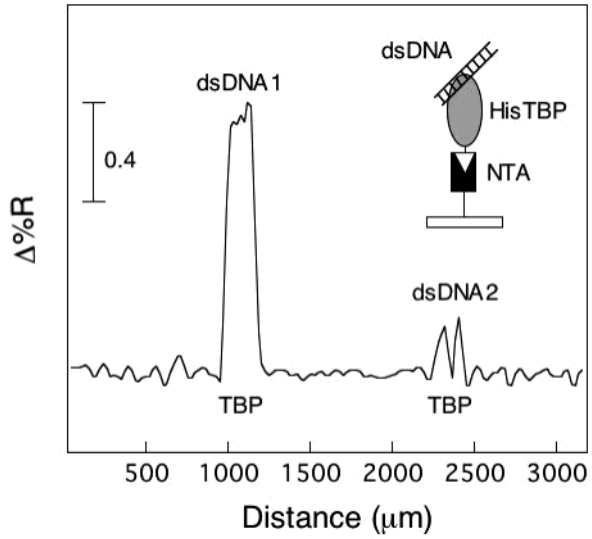

Figure 5. Line profile showing the sequence-specific interaction of dsDNA to His-tagged TATA box-binding protein (TBP) immobilized on an NTA array. Two dsDNA sequences were flowed through parallel channels of a TBP array. An increase in percent reflectivity shown in the line profile occurred when 2 μM dsDNA1 containing the TATA motif interacted with TBP. However, the control sequence dsDNA2, containing a randomized DNA sequence, shows little adsorption to the TBP elements.

ibility of the antibody adsorption onto these surfaces suggests that these protein arrays are more suitable to antibody detection and identification rather than quantification of the strength of the antibody−antigen interactions.

DNA-TATA Binding Protein Interactions. In a second set of SPR imaging experiments, we observed the interaction between double-stranded DNA (dsDNA) and a His-tagged fusion protein array of the TATA box-binding protein (TBP). TBP is a component of the multi-protein complex required to initiate transcription in the cell by binding to promoter DNA containing the TATA box motif.[34,35] First, an array of His-tagged TBP was immobilized onto the patterned NTA monolayer and thoroughly rinsed with Ni^{2+}/Tris buffer. Then, this His-tagged protein array was exposed to two different 16-mer dsDNA sequences, dsDNA1 and dsDNA2, for 5 min. The DNA sequences are given in Table 2; the dsDNA1 sequence contains the TATA element of the adenovirus major later promoter (TATAAAAG). This 16-mer was found previously to bind to TBP[36] and has been used to determine the crystal structure of the TBP−DNA complex.[37] The dsDNA2 sequence is a randomized control sequence with a stability comparable to dsDNA1 (T_m of 65.1 and 60.9 °C as calculated using the parameters of Breslauer et al.[38]). The individual strands of dsDNA1 and -2 were hybridized in solution prior to exposure to the His-TBP array.

SPR imaging measurements were used to demonstrate the sequence-specific binding of dsDNA to the His-TBP array. Figure 5 shows a line profile taken across the difference image of a two-channel His-TBP array which was exposed to 2 μM solutions of dsDNA1 and dsDNA2. It is clear from the figure that the TBP preferentially interacts with the dsDNA1 sequence. The amount of dsDNA binding was roughly constant for solutions from 0.25 to 10 μM. Above 10 μM, additional binding of dsDNA2 to the surface was observed. The sequence specificity of TBP−dsDNA binding has been noted previously to depend on stringency and dsDNA concentration.[35,39,40] Unlike the antibody−antigen binding, the DNA−protein interactions were more reversible and almost all of the dsDNA could be removed from the surface using a Tris buffer that did not contain Ni^{2+} ions. This suggests that these His-tagged fusion protein arrays will be useful in measurements of the strength of DNA−protein interactions.

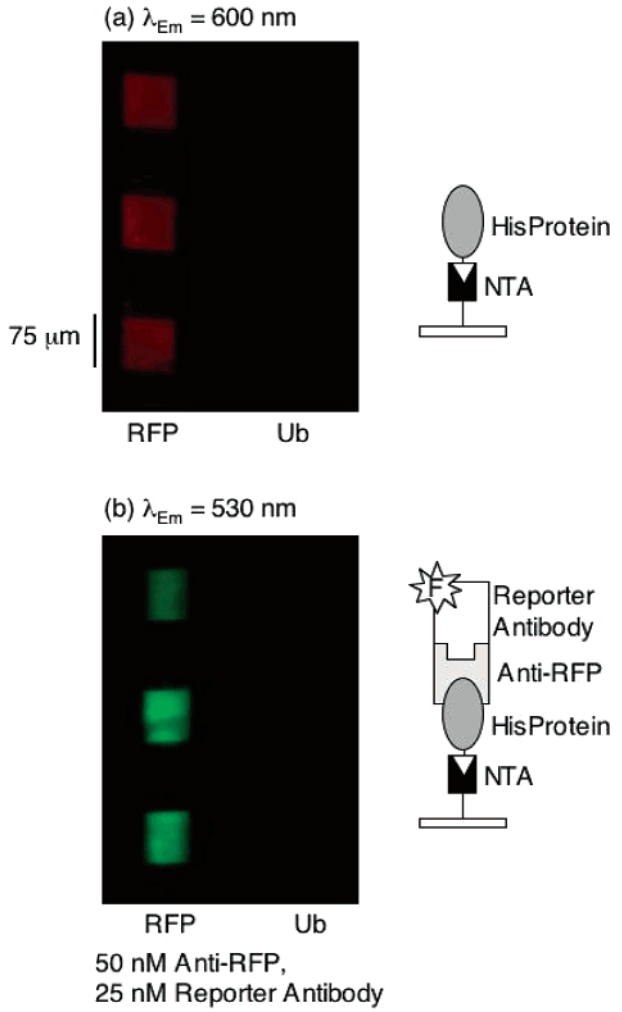

Figure 6. (a) Fluorescence image taken using a TRITC filter after the immobilization of His-tagged RFP and ubiquitin using NTA capture agents in a parallel set of microchannels (75-μm width, 12.8-mm length, 35-μm depth). RFP, which is naturally fluorescent, is visible in the image, while His-tagged ubiquitin is not. (b) A fluorescence image taken using a FITC filter after exposing the array to anti-RFP and a 488-nm fluorescently tagged reporter antibody. This sandwich assay results in specific adsorption to the RFP array elements with little nonspecific adsorption to the immobilized ubiquitin regions.

(34) Juo, Z. S.; Chiu, T. K.; Leiberman, P. M.; Baikalov, I.; Berk, A. J.; Dickerson, R. E. *J. Mol. Biol.* **1996**, *261*, 239−254.
(35) Weideman, C. A.; Netter, R. C.; Benjamin, L. R.; McAllister, J. J.; Schiedekamp, L. A.; Coleman, R. A.; Pugh, B. F. *J. Mol. Biol.* **1997**, *271*, 61−75.
(36) Matsui, T.; Segall, J.; Weil, P.; Roeder, R. *J. Biol. Chem.* **1980**, *255*, 11922−11996.
(37) Nikolav, D. B.; Chen, H.; Halay, E. D.; Hoffman, A.; Roeder, R. G.; Burley, S. K. *Proc. Nat. Acad. Sci. U.S.A.* **1996**, *93*, 4862−4867.
(38) Breslauer, K. J.; Blocker, H.; Frank, R.; Marky, L. A. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 3746−3750.
(39) Coleman, R. A.; Pugh, B. F. *J. Biol. Chem.* **1995**, *270*, 13850−13859.
(40) Hanhn, S.; Buratowski, S.; Sharp, P. A.; Guarente, L. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 5718−5722.

Fluorescence Sandwich Assays. In a final set of demonstration experiments, His-tagged protein arrays were used to examine multicomponent sandwich assays using fluorescence measurements. A two-channel His-tagged protein array of His-RFP and His-ubiquitin was immobilized onto an NTA monolayer from 2 μM protein solutions. Parallel channels with 75-μm width were used in this array in order to be compatible with the field of view of the fluorescence microscope. The immobilization of RFP, which is naturally fluorescent,[41,42] is shown in the image in Figure 6a, which was taken with a TRITC filter (λ_{Em} = 600 nm). After the immobilization of the His-proteins, both channels were exposed to a 50 nM solution of anti-RFP. After 5 min, the array was rinsed to remove nonspecifically bound molecules, and then 25 nM solution of fluorescently labeled goat anti-rabbit reporter antibody (λ_{Ex} 488 nm, λ_{Em} = 530 nm) was introduced into the microchannels. After another 5 min, the array was rinsed again and the fluorescence image in Figure 6b was obtained using a FITC filter (λ_{Em} = 530 nm). This image clearly shows specific binding of the reporter antibody to the anti-RFP. In a separate experiment (not shown), where anti-RFP was replaced with anti-Ub, only the His-Ub array elements fluoresced. These results demonstrate that the His-tagged protein arrays are compatible with fluorescence measurements and can be used to study protein complexes containing multiple binding partners.

CONCLUSIONS

The fabrication of robust and reliable protein arrays is extremely important for the large-scale protein screening assays required in modern proteomics research. The chemical attachment of molecules onto alkanethiol monolayers at gold surfaces such as the NTA monolayers described in this paper is an attractive route for the creation of these protein arrays. Moreover, the ability to examine the chemical structure of the monolayers on gold surfaces with FT-IR vibrational spectroscopy, and the subsequent use of SPR imaging measurements to monitor biomolecular adsorption at these interfaces, make the use of chemically modified gold surfaces an appealing alternative to the more typical glass substrates and fluorescence assay techniques.

In this paper, we have demonstrated how to create His-tagged protein arrays from patterned NTA monolayers, and we have shown that these protein arrays can be used to monitor antibody−antigen binding and DNA−protein binding with both SPR imaging and fluorescence measurements. The microfluidic fabrication methods employed in this paper are a simple yet effective means for assaying a small number of proteins. We have used a maximum of 10 protein channels with this array format; however, by changing the width and spacing of the PDMS microchannels, 150 species could be immobilized on a 1.8 cm by 1.8 cm chip. As an alternative, a spotting methodology could be employed for the preparation of more dense protein arrays.

SPR is emerging as a useful tool for the study of both irreversible and reversible bioaffinity interactions. In this paper, SPR imaging measurements were applied to the study of dsDNA binding to TBP. In future studies, we will examine the formation of multicomponent complexes of dsDNA, TBP, and other TATA binding-associated factors.[43] These His-tagged protein arrays can also be used to study the binding of transcription factors to dsDNA for the control of gene expression. One limitation in the use of His-tagged protein arrays is the required presence of nickel ions in solution. For this reason, we are also pursuing the development of gold surfaces that will work with other fusion proteins (e.g., glutathione-*S*-transferase, FLAG).

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(41) Mizuno, H.; Sawano, A.; Eli, P.; Hama, H.; Miyawaki, A. *Biochemistry* **2001**, *40*, 2502−2510.
(42) Jakobs, S.; Subramaniam, V. S., A.; Jovin, T. M.; Hell, S. W. *FEBS Lett.* **2000**, *479*, 131−135.
(43) Burley, S. K.; Roeder, R. G. *Annu. Rev. Biochem.* **1996**, *65*, 769−799.