A Multistep Chemical Modification Procedure To Create DNA Arrays on Gold Surfaces for the Study of Protein−DNA Interactions with Surface Plasmon Resonance Imaging

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Abstract: A multistep surface modification procedure for the creation of DNA arrays on chemically modified gold surfaces that can be used in surface plasmon resonance (SPR) imaging studies of protein−DNA interactions is demonstrated. The multistep procedure is required to create an array of spots that are surrounded first by a hydrophobic background which allows for the pinning of aqueous DNA solutions onto individual array elements and then to replace that hydrophobic background with one that resists the nonspecific adsorption of proteins during in situ SPR imaging measurements. An amine-terminated alkanethiol monolayer is employed as the base layer, and Fmoc and PEG modifiers are used to create the sequentially hydrophobic and protein adsorption-resistant surfaces, respectively. Specifically, the chemical modification steps are the following: (1) the adsorption and self-assembly of an 11-mercaptoundecylamine (MUAM) monolayer on an evaporated gold thin film, (2) the reaction of the MUAM monolayer with an Fmoc protecting group to create a hydrophobic surface, (3) the photopatterned removal of the alkanethiol followed by (4) the readsorption of MUAM to create an array of MUAM squares (750 × 750 μm) surrounded by a hydrophobic MUAM-Fmoc background that can pin drops of aqueous solution, (5) the attachment of oligonucleotide sequences onto the MUAM squares by the reaction of the amine-terminated surface with the heterobifunctional cross linker SSMCC followed by a coupling reaction to a small volume (0.1 μL) of thiol-modified DNA, and (6) the removal of the Fmoc protecting group followed by (7) a pegylation reaction of the MUAM with PEG-NHS to create a protein adsorption-resistant background. A combination of polarization-modulation FTIR spectroscopy, contact angle, and scanning angle SPR measurements is used to characterize the surface modification procedure. An SPR imaging measurement of the adsorption of single-stranded DNA binding protein (SSB) onto an oligonucleotide array created by this procedure is used to demonstrate the utility of these surfaces.

I. Introduction

The binding of proteins to DNA plays a pivotal role in the regulation and control of gene expression, replication, and recombination. In addition, enzymes that recognize and modify specific oligonucleotide sequences are critical components of biological DNA manipulation and repair systems. An enhanced understanding of how these proteins recognize certain oligonucleotide sequences would aid in the design of biomedical systems which could, for example, be used to regulate the expression of therapeutic proteins. For this reason, the study of protein−DNA interactions is a rapidly growing area of molecular biology, aided in part by recent advances in NMR and X-ray structural determination methods. At the same time, the explosive increase in the amount of available genomic sequence information obtained from large-scale DNA sequencing efforts creates the need to survey this vast amount of new DNA sequence data for protein binding sites. In support of this effort, our goal is to use surface plasmon resonance (SPR) imaging techniques as a rapid and efficient method for screening the sequence or structure-specific binding of proteins to large arrays of DNA molecules immobilized on chemically modified gold surfaces.

The technique of surface plasmon resonance is a surface-sensitive, optical detection method well suited to the monitoring of reversible, protein−DNA interactions. The commercially successful BIACORE SPR instrument[1,2] has been used previously, for example, to study the interaction of DNA molecules with the MutS,[3,4] lac repressor,[5] and single-stranded DNA binding (SSB)[6] proteins of *E. coli*. Although powerful, the BIACORE instrument has no imaging capabilities; this severely limits the number of DNA sequences that can be screened in a single experiment. However, SPR imaging and microscopy have been used previously in our laboratory[7−10] and in others[11,12] to study biopolymer adsorption onto patterned surfaces, and is an

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ideal way to monitor the binding of proteins to arrays of surface-bound oligonucleotide sequences. In this technique, a light source (typically a HeNe laser) is used to illuminate a prism/thin gold film sample assembly at an incident angle that is near the SPR angle, and the reflected light is detected at a fixed angle with a CCD camera to produce an SPR image. The SPR image arises from variations in the reflected light intensity from different parts of the sample; these variations are created by any changes in organic film thickness or index of refraction that occur upon adsorption onto the modified gold surface. Since SPR imaging is sensitive only to molecules in close proximity to the surface (within ~200 nm), unbound protein molecules remaining in solution do not interfere with in situ measurements, making possible the monitoring of weak or reversible protein−DNA binding interactions. Such interactions cannot be measured with standard fluorescence imaging techniques due to overwhelming background fluorescence caused by excess tagged protein molecules present in solution.

The formation of robust, reproducible arrays of oligonucleotides tethered to gold-coated surfaces is an essential requirement for SPR imaging investigations of protein−DNA binding interactions. To use SPR imaging techniques, it is essential that the DNA array be constructed on a noble metal surface, and for this reason DNA arrays on glass supports from commercially available sources such as Affymetrix[13] are not a viable option. Using self-assembled monolayers of ω-substituted alkanethiols as a starting point, we have previously developed schemes to attach single-stranded DNA molecules to chemically modified gold surfaces.[8,9] At the same time, UV photopatterning and microcontact printing techniques allow alkanethiols to be assembled in a site-directed manner on the surface, enabling the creation of multicomponent arrays. A combination of these processing techniques along with the addition of novel surface chemical reactions serve as the basis for the DNA array fabrication scheme outlined here.

In this paper we describe a multistep chemical modification procedure to create DNA arrays on gold surfaces specifically tailored for the study of protein−DNA interactions with surface plasmon resonance imaging. Arrays fabricated by this procedure meet three specific requirements, namely (i) the DNA sequences are covalently attached to the surface and remain active and accessible to hybridization and protein binding, (ii) the array background is, at first, sufficiently hydrophobic so as to allow for the "pinning" of aqueous solutions of DNA at specific array locations, and (iii) the final array background acts to inhibit the nonspecific binding of protein molecules to the surface. The key components of this fabrication scheme are the novel utilization of a reversible amine protecting group, 9-fluorenylmethoxycarbonyl (Fmoc), to control the surface hydrophobicity of a tethered alkanethiol monolayer and the attachment of a poly(ethylene glycol) (PEG) group to render the surface protein resistant. Polarization-modulation FTIR reflection absorption (PM-FTIRRAS) spectroscopy, contact angle, and SPR measurements are used to characterize each step in the surface modification procedure and to confirm that the array background inhibits the nonspecific binding of proteins. As a final test, an SPR imaging experiment which measures the adsorption of single-stranded DNA binding protein (SSB) to a dual component, oligonucleotide array demonstrates the utility of these surfaces for the monitoring of protein−DNA interactions.

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II. Experimental Considerations

Materials. The chemicals 9-fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide (Fmoc-NHS) (Novabiochem), *N*-hydroxysuccinimidyl ester of methoxypoly(ethylene glycol) propionic acid MW 2000 (PEG-NHS) (Shearwater Polymers, Inc.), ethanolamine (Aldrich), piperidine (Aldrich), tris(2-aminoethyl)amine (TAEA) (Aldrich), sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SSMCC) (Pierce), triethanolamine hydrochloride (TEA) (Sigma), bovine serum albumin (BSA)(Sigma), and single-stranded DNA binding protein (SSB) (Pharmacia Biotech) were all used as received. Solvents were of standard laboratory grade, and distilled water that was filtered through a Millipore purification system was used for all aqueous solutions and rinsing. The 11-mercaptoundecylamine (MUAM) and poly(ethylene glycol)-modified alkanethiol, $HS(CH_2)_{11}(OCH_2CH_2)_3OH$, were generous gifts from the laboratory of Professor George M. Whitesides, Harvard University. Gold substrates used in the PM-FTIR and contact angle measurements were purchased commercially (Evaporated Metal Films) and those used in scanning or imaging SPR measurements were prepared by vapor deposition onto microscope slide covers that had been silanized with (3-mercaptopropyl)trimethoxysilane (Aldrich) in a manner similar to that reported by Goss et al.[14] All oligonucleotides were synthesized on an ABI DNA synthesizer at the University of Wisconsin Biotechnology Center. Glen Research 5′-Thiol-Modifier C6 and ABI 6-FAM were used for 5′-thiol-modified and 5′-fluorescein-modified oligonucleotides, respectively, and Spacer Phosphoramidite 18 (Glen Research) was used for the addition of an ethylene glycol spacer region. Thiol-modified oligonucleotides were deprotected as outlined by Glen Research Corp.[15] Before use, each oligonucleotide was purified by reverse-phase binary gradient elution HPLC (Shimadzu SCL-10AVP) and DNA concentrations were verified with an HP8452A UV−vis spectrophotometer. The sequences of the DNA molecules used in the SSB experiment are as follows: D1 = 5′ $HS(CH_2)_6(T)_{16}$AAC GAT GCA GGA GCA A, D2 = 5′ $HS(CH_2)_6(CH_2CH_2O)_{24}$GCT TAT CGA GCT TTC G, and D2 complement = 5′ FAM-CGA AAG CTC GAT AAG C. The buffer used in the BSA and SSB SPR imaging experiments contained 20 mM phosphate, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, and 5 mM $MgCl_2$ and was buffered at pH 7.4.

Multistep Array Fabrication. A clean gold substrate was immersed in a 1 mM ethanolic solution of MUAM for at least 1 h to allow for the adsorption and self-assembly of the alkanethiol monolayer. The substrate was rinsed with ethanol and water, dried under a stream of N_2, and then reacted with a solution of Fmoc-NHS (3 mM in 1:1 DMSO:100 mM TEA buffer, pH 7) for 30 min. The sample was rinsed with water and then soaked briefly in DMSO to remove unreacted Fmoc-NHS from the surface. It was then photopatterned by irradiating the sample with UV light from a mercury−xenon arc lamp (Oriel) through a quartz mask (Photo Sciences Inc.) for 1 h at 400 W power. Subsequent rinsing of the sample with ethanol and water removed alkanethiol from the exposed areas. The sample was reexposed to the ethanolic MUAM solution resulting in an array of MUAM elements surrounded by a hydrophobic MUAM+Fmoc background. Single-stranded, 5′-thiol-modified DNA was then immobilized onto the array locations using an attachment scheme modified slightly from that used previously.[8,16] Briefly, the amine-terminated MUAM array elements were spotted with 0.1 μL of a 1 mM solution (in 100 mM TEA, pH 7) of the heterobifunctional linker SSMCC, creating a thiol-reactive, maleimide-terminated surface. 5′-Thiol-modified DNA sequences were then covalently attached to these maleimide-terminated array elements by spotting the sample with 0.1 μL drops of solutions containing 1 mM DNA onto the specific array locations and reacting for at least 2 h in a humid environment to prevent solvent evaporation. After exposure to the DNA solution, the surface was rinsed with water and soaked in buffer to remove unbound DNA sequences. The Fmoc was then removed from the background by immersing the array in a 1 M solution

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of TAEA in DMF for 10 min. The deprotected surface was rinsed with water and subsequently reacted with 4 mM PEG-NHS (in 100 mM TEA, pH 8) for 30 min to pegylate the array background, rendering it resistant to protein nonspecific binding.

PM-FTIRRAS Measurements. PM-FTIRRAS spectra were collected on a Mattson RS-1 spectrometer equipped with either a narrow band HgCdTe detector (for spectra in the mid-IR region, 2000−1000 cm^{-1}) or an InSb detector (for spectra in the CH stretching region, 3400−2600 cm^{-1}). The optical layout and previously developed real-time interferogram sampling methods have been described elsewhere.[17,18] The PM-FTIRRAS differential reflectance values (% $\Delta R/R$) were converted to absorbance units for comparison with conventional IRRAS data. Spectra are an average of 1000 scans collected at 2 cm^{-1} resolution.

Contact Angle Measurements. Water contact angles were determined at ambient laboratory temperatures with a Model 100-00 Contact Angle Goniometer (Ráme-Hart, Inc.). Ten microliter droplets were dispensed from a Gilson pipet onto the surface and the angle measurement was recorded immediately. Reported contact angle values for both the Fmoc and PEG functionalized surfaces are the average of 12 different measurements taken on 4 individually prepared samples and the value for MUAM is the average of 30 measurements taken on 10 different samples.

Scanning Angle SPR Measurements. The optical technique of ex situ scanning SPR was used to determine the thickness of MUAM, MUAM+Fmoc, and MUAM+PEG assembled on BK7 coverslips (18 × 18 mm, Fisher) onto which 475 Å of Au was vapor deposited. Details of the SPR experiment and thickness calculations have been reported elsewhere.[19,20] Briefly, the reflectivity (R) of a p-polarized HeNe laser beam ($\lambda = 632.8$ nm) from a sample assembly (BK7 prism/Au/thin film/air) is monitored as a function of incident angle, to generate a SPR curve (% R vs angle). A steep drop in the reflectivity occurs at angles just past the critical angle. The exact position of the minimum is determined by the thickness and index of refraction of material adsorbed at the gold surface. A 4-phase complex Fresnel calculation was used to determine the film thickness and a refractive index of 1.45 was assumed for all the thin films measured here. This index of refraction is a typical value used previously by us and other authors to interpret SPR and ellipsometry data from various ω-functionalized alkanethiol monolayers.[19]

SPR Imaging Apparatus. The in situ SPR imaging instrument is a modified version of that described previously[7−10] in which the HeNe laser and beam expander have been replaced by a collimated white light source/band-pass filter combination. A more thorough discussion of this modification in the context of near-IR (NIR) SPR imaging is reported elsewhere.[21] In short, a collimated, polychromatic beam of light was used to illuminate an SF10 prism/Au/thin film/buffer assembly at a fixed incident angle near the SPR angle. The reflected light was passed through a 10 nm band-pass filter ($\lambda = 830$ nm) and was collected with an inexpensive CCD camera (iSC2050, i Sight, Inc.). Differences in the reflected light intensity measured at various locations on the sample create the image and are a direct result of differences in the thickness and/or refractive index of the material bound at the gold surface. The images shown here were collected in situ for samples constructed on SF10 substrates (18 × 18 mm, Schott Glass) onto which 450 Å of Au had been deposited. Data workup was done using NIH Image v.1.61 software.

III. Results and Discussion

A. Chemical Modification of Alkanethiol-Coated Gold Surfaces To Create DNA Arrays. The fabrication of the multicomponent DNA arrays for SPR imaging experiments requires a series of seven surface chemical reactions that are outlined in Figure 1. These steps are as follows: (1) Adsorption of a self-assembled monolayer of 11-mercaptoundecylamine (MUAM). (2) Reaction of the MUAM surface with the hydrophobic protecting group Fmoc. (3) Photopatterning of the surface to create an array of bare gold areas. (4) Adsorption of MUAM to fill in the bare gold array elements. (5) Covalent attachment of DNA onto array elements. (6) Removal of Fmoc from the array background. (7) Reaction of the background with poly(ethylene glycol) to make it protein resistant.

The progression of the multistep chemical reactions involved in the array fabrication was monitored using PM-FTIRRAS, contact angle measurements, and scanning-angle SPR. IR spectral characterization is based on the band assignments of terminally substituted alkanethiols[22] and poly(ethylene glycol)[23] and on band assignments found in general texts on the subject.[24,25]

Step 1. In step one, a monolayer of the amine-terminated alkanethiol, 11-mercaptoundecylamine (MUAM), is self-assembled from an ethanolic solution onto a gold-coated glass substrate. Self-assembled monolayers of amine-terminated alkanethiols on gold have been used previously,[26−28] and are believed by most to form well-ordered, monomolecular films. However, Tien et al.[29] report that preliminary ellipsometry and contact angle measurements indicate that $HS(CH_2)_{11}NH_3^+$ and $HS(CH_2)_{10}C(NH_2)_2^+$ can form hydrophobic bilayer structures on gold. IR, contact angle, and SPR measurements collected in our laboratory (and discussed below) give no evidence that such bilayer formation occurs under our experimental conditions. It has also been suggested that the terminal-amine groups of the monolayer might react with CO_2 to form carbamate salts on the surface[30] rendering them inert to chemical modification; we have verified with IR that if left exposed for extended periods of time carbamates can form on the MUAM monolayer surface so care must be taken when handling these samples.

The PM-FTIRRAS spectrum of MUAM in the mid-IR region is shown in Figure 4A. The small peaks centered at 1608 and 1545 cm^{-1} have been assigned as the asymmetric and symmetric NH_3^+ deformations, respectively. The presence of these peaks suggests that after a rinsing with ethanol and water (pH ∼6), a significant portion of the terminal amine groups exist in the protonated form. Variation in the intensity of the 1545 cm^{-1} peak can be effected by rinsing the surface in solutions of differing pH. Bands at 1465 and 1258 cm^{-1} have been assigned to the CH_2 scissoring and twist deformations of the alkane chains, respectively. The frequencies of the peaks due to the CH_2 asymmetric stretching mode at 2923 cm^{-1} and the CH_2 symmetric stretching mode at 2853 cm^{-1} (spectrum not shown) indicate that the monolayer exists in a relatively

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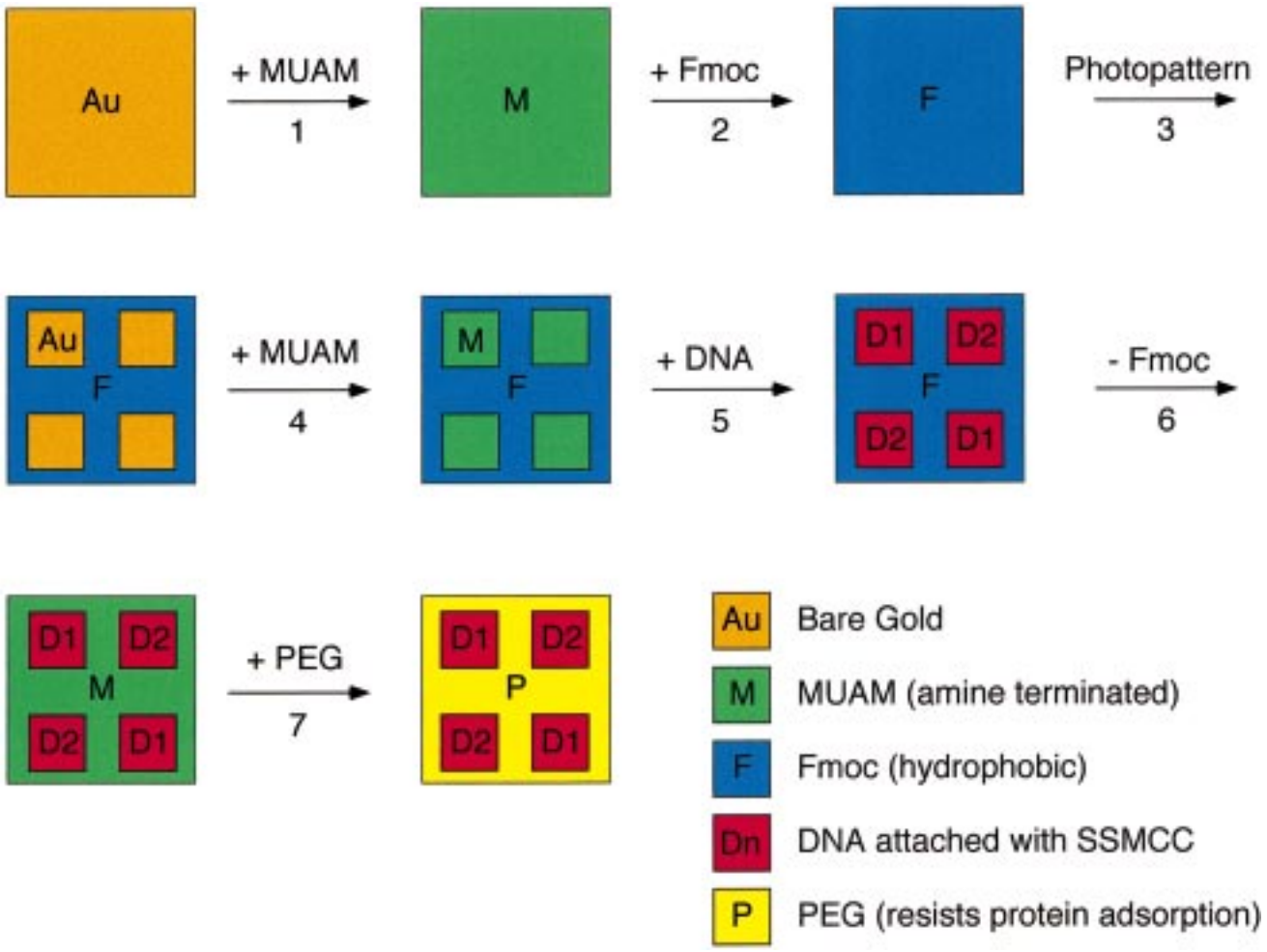

Figure 1. Fabrication scheme for the construction of multi-element DNA arrays. A clean gold surface is reacted with the amine-terminated alkanethiol MUAM, and subsequently reacted with Fmoc-NHS to create a hydrophobic surface. This surface is then exposed to UV radiation through a quartz mask and rinsed with solvent to remove the MUAM+Fmoc from specific areas of the surface, leaving bare gold pads. These bare gold areas on the sample surface are filled in with MUAM, resulting in an array of MUAM pads surrounded by a hydrophobic Fmoc background. Solutions of DNA are then delivered by pipet onto the specific array locations and are covalently bound to the surface via the bifunctional linker SSMCC. In the final two steps, the Fmoc-terminal groups on the array background are removed and replaced by PEG groups which prohibit the nonspecific binding of analyte proteins to the background.

ordered state. Absent from the spectrum in the CH stretching region is a band due to the N−H stretch (∼3200−3500 cm^{-1}) of the amine groups; it is assumed that this band is too weak to be detected. Due to its terminal amine groups, a MUAM monolayer surface is quite hydrophilic, which is verified by a contact angle measurement of 36.2 ± 2.5° (see Table 1) and is consistent with monolayer formation. Ex situ scanning SPR was used to measure a thickness of 17.5 ± 0.4 Å for a gold surface modified with MUAM (see Table 1); this thickness is consistent with that expected for a fully extended MUAM monolayer oriented nearly normal to the surface.

Step 2. In step two of the array fabrication, the MUAM-covered surface is reacted with the reversible amine protecting group, Fmoc, to create a hydrophobic surface; Fmoc is a bulky, hydrophobic, base-labile, amine protecting group routinely used in the solid-phase synthesis of peptides.[31] The specific chemical reaction is shown in Figure 2. The *N*-hydroxysuccinimide ester of Fmoc (Fmoc-NHS) reacts with the terminal amine moiety of the MUAM molecule to form a stable carbamate (urethane) linkage, covalently attaching the Fmoc group to the surface. The IR spectrum shown in Figure 4B, collected for the MUAM surface after reaction with Fmoc-NHS, gives evidence that the surface reaction proceeded as expected. Prominent peaks at 1720, 1544, and 1267 cm^{-1} are due to the carbamate linkage that tethers the Fmoc group to the MUAM surface. The band at 1720 cm^{-1} has been assigned to the carbonyl stretching vibration (amide I), that at 1544 cm^{-1} to the CHN group vibration, and that at 1267 cm^{-1} to the coupled C−N and C−O stretches (amide IV). The peak at 1450 cm^{-1} is ascribed to the C═C ring stretch of the fluorenyl group and the band centered at 1147 cm^{-1} is attributed to the Fmoc C−O−C (ether) stretch. After reaction with Fmoc-NHS, the surface properties of the array are changed significantly; the surface is extremely hydrophobic as confirmed by the measured contact angle of 74.4 ± 2.5°. In addition, an increase in the film thickness to 22.8 ± 0.5 Å is measured with scanning angle SPR. Using bond lengths predicted for a fully extended Fmoc molecule, we estimate that this ∼5 Å thickness change corresponds to the addition of roughly 70% of an Fmoc monolayer.

Step 3. In step three, UV photopatterning is used to create a patterned surface. The surface is exposed, through a quartz mask with 750 × 750 μm square features, to UV radiation which photooxidizes the gold−sulfur bond that anchors the alkanethiol

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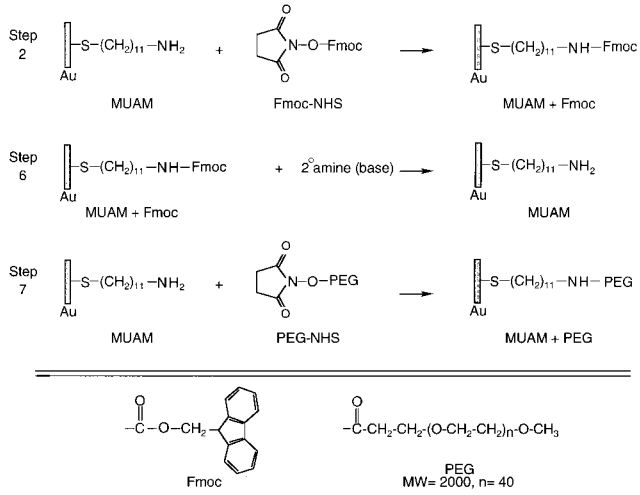

Figure 2. Surface reaction scheme showing the steps involved in the reversible modification of the array background. (Step 2) The starting amine-terminated alkanethiol surface (MUAM) is reacted with the Fmoc-NHS protecting group to form a carbamate linkage thus creating a hydrophobic Fmoc-terminated surface. (Step 6) After DNA immobilization (see Figure 3), the hydrophobic Fmoc group is removed from the surface with a basic secondary amine, resulting in the return of the original MUAM surface. (Step 7) In the final array fabrication step, the deprotected MUAM is reacted with PEG-NHS to form an amide bond that covalently attaches PEG to the array surface.

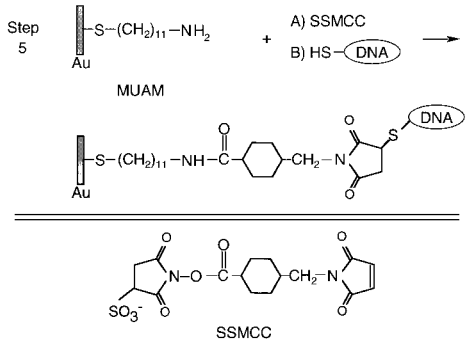

Figure 3. Surface reaction scheme showing the immobilization of thiol-terminated DNA to the array surface. In Step 5 of the DNA array fabrication, the heterobifunctional linker SSMCC is used to attach 5′-thiol modified oligonucleotide sequences to reactive pads of MUAM. This linker contains an NHSS ester functionality (reactive toward amines) and a maleimide functionality (reactive toward thiols). The surface is first exposed to a solution of the linker, whereby the NHSS ester end of the molecule reacts with the MUAM surface. Excess linker is rinsed away and the array surface is then spotted with 5′-thiol-modified DNA that reacts with the maleimide groups forming a covalent bond to the surface monolayer.

monolayers to the surface.[32,33] The surface is then rinsed, removing the photooxidized alkanethiol and leaving an array of bare gold pads surrounded by a hydrophobic MUAM+Fmoc background. With the photopatterning technique we have been able to create features with dimensions as small as 50 μm, and microcontact printing methods can be used to create patterned surfaces with features as small as ~100 nm.[34]

Step 4. In step four, the surface is again exposed to an ethanolic solution of MUAM, whereby the alkanethiol assembles into the bare gold regions producing a surface composed of hydrophilic MUAM pads surrounded by the hydrophobic Fmoc background. This difference in hydrophobicity between the reactive MUAM regions and the background is essential for the pinning of small volumes of aqueous DNA solutions onto individual array locations.

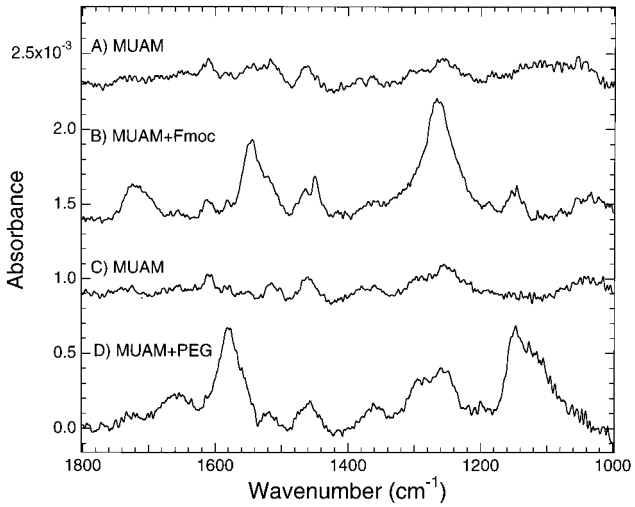

Figure 4. PM-FTIRRAS spectra in the mid-IR region for the surfaces involved in the array background modification. (A) The starting MUAM surface. (B) After reaction with Fmoc-NHS, bands indicative of the carbamate linkage and the Fmoc ring stretch (for specific assignment see text) appear in the spectrum. (C) The surface is deprotected and reverts back to the MUAM surface as evidenced by the similarities between spectra A and C. (D) After reaction with PEG-NHS, bands indicative of the amide linkage as well as those associated with the ethylene glycol groups are present.

Table 1. Contact Angle and SPR Thickness Measurements

surface	contact angle[a]	thickness[b]
MUAM	36.2 ± 2.5°	17.5 ± 0.4 Å
MUAM + Fmoc	74.4 ± 2.5°	22.8 ± 0.5 Å
MUAM + PEG	37.3 ± 2.6°	23.8 ± 0.8 Å

[a] The contact angle value is an average of 10 (MUAM) or 4 (Fmoc, PEG) different samples. [b] The thickness value is an average of 5 (MUAM, PEG) or 2 (Fmoc) different samples.

Step 5. At this point in the fabrication scheme, DNA is covalently attached to the surface as is shown in Figure 3. The MUAM reactive pads are first exposed to a solution of the bifunctional linker SSMCC, which contains both an *N*-hydroxysulfosuccinimide (NHSS) ester and a maleimide functionality. The NHSS ester end of the molecule reacts with the free amine groups on the MUAM surface creating pads terminated in maleimide groups which are reactive toward thiols. Small volumes (0.08−0.1 μL) of 1 mM solutions of 5′-thiol-modified DNA sequences are then spotted at discrete array locations and react to form a covalent attachment to the surface. A variation on this attachment scheme in which thiol−DNA is linked via SSMCC to a MUA/PL (11-mercaptoundecanoic acid/poly-L-lysine) bilayer has been used quite extensively in this laboratory.[8,16,35] Other researchers have used the direct self-assembly of thiol-terminated DNA molecules on gold to prepare functionalized surfaces,[36,37] and the fabrication scheme presented here could easily be modified to accommodate this method of

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oligonucleotide attachment. The attachment scheme implemented in our laboratory has two distinct advantages: (i) it avoids the nonspecific adsorption of DNA onto the bare gold surface, and (ii) the density of the surface-bound DNA probes can be manipulated to optimize conditions for hybridization adsorption and protein binding. The DNA squares in this paper are spotted by using a pipet; with robotics and inkjet printing systems for the delivery of DNA solutions, we estimate that DNA arrays with squares as small as 25 μm can be fabricated.[12]

Step 6. In step 6 the Fmoc protecting group is removed from the array surface by exposure to a 1 M solution of the secondary amine, tris(2-aminoethyl)amine (TAEA), in DMF. Many basic secondary amines can be used to remove Fmoc from the surface;[38] we have also had success using 1 M solutions of ethanolamine and piperidine. TAEA was chosen specifically as the deprotection agent since it effectively scavenges the dibenzofulvene byproduct[39] and is efficiently rinsed from the array surface. After this deprotection step, the array background reverts to the original MUAM surface. The spectrum of a deprotected MUAM surface is shown in Figure 4C; note the strong similarity between it and the original MUAM spectrum. The prominent bands due to the carbamate linkage no longer appear, indicating that the Fmoc protecting group has been completely removed from the surface. The deprotected surface was also measured with scanning SPR; the thickness measured was within ±1 Å of that measured for the starting MUAM surface and this gives additional proof that the Fmoc protecting group is removed completely from the surface.

Step 7. In the final step of the array fabrication, the MUAM background is reacted with an NHS ester derivative of poly(ethylene glycol) (PEG-NHS) to create a background that is resistant to the nonspecific binding of proteins. To effectively monitor the binding of proteins to arrays of surface-bound DNA molecules, the array background must prohibit the nonspecific adsorption of protein molecules. Significant nonspecific binding would undoubtedly obscure the measurement of small amounts of protein binding at specific array locations. Poly(ethylene glycol) is well-known for its ability to resist the nonspecific binding of proteins, and PEG-functionalized surfaces are currently being developed for medical and biochemical applications.[40] Alkanethiols have been successfully modified with terminal PEG groups and have been shown to self-assemble onto gold surfaces.[41] These PEG-thiols are quite effective at resisting nonspecific protein adsorption, and ellipsometric,[42] acoustic plate mode sensor,[43] and scanning angle SPR[44] measurements have shown the resistance of PEG-thiol SAMs to proteins such as fibrinogen, Rnase A, lysozyme, and pyruvate kinase. SPR imaging results from our laboratory (data not shown) indicate that we can successfully use the poly(ethylene glycol) modified alkanethiol, $HS(CH_2)_{11}(OCH_2CH_2)_3OH$, to resist the nonspecific binding of the protein BSA. However, this SAM presents a hydrophilic surface and thus cannot function as our initial array background because it cannot be used to pin drops of aqueous DNA solutions. Conversely, alkanethiol surfaces sufficiently hydrophobic to pin drops of DNA (e.g., C18-thiol)[45] are not able to resist the nonspecific binding of proteins. This fact necessitated the application of a method to reversibly protect the array background.

To create a background that is resistant to the nonspecific binding of proteins, we react the MUAM surface with PEG-NHS as is shown in Figure 2. As was the case in the Fmoc-NHS + MUAM reaction, PEG-NHS reacts with the terminal amine groups of the MUAM to form an amide linkage, covalently attaching the PEG polymer chain to the surface. The specific PEG-NHS polymer used here has an average molecular weight of 2000 and contains one NHS ester moiety per molecule, allowing for a single point of attachment. The IR spectrum collected for a MUAM surface reacted with PEG-NHS is shown in Figure 4D. The peaks which appear at 1660 and 1576 cm^{-1} have been assigned as amide I and II bands, respectively. The bands at 1457 and 1250−1260 cm^{-1} are ascribed to the scissoring and twist deformations of the CH_2 groups contained in both the MUAM alkyl chains and the ethylene glycol (EG) groups. The band at 1352 cm^{-1} is due to an EG CH_2 wagging mode, and the band centered at 1148 cm^{-1} is due to the C−O−C (ether) stretch of the ethylene glycol units. The spectra collected for both the mid-IR and CH stretching regions of this pegylated-MUAM surface appear quite similar to spectra reported in the literature for oligo(ethylene glycol)-functionalized alkanethiol molecules self-assembled on gold surfaces.[46] After reaction of the deprotected surface with PEG-NHS, the surface remains hydrophilic and has a measured contact angle of 37.3 ± 2.6°. A total thickness of 23.8 ± 0.8 Å was measured for a MUAM monolayer film after reaction with PEG-NHS. This increase of only 6 Å of PEG suggests that only a small fraction of the amine groups of the MUAM are modified and that the oligo(ethylene glycol) chains are lying flat across the surface. SPR imaging experiments (results not shown) were used to measure the nonspecific adsorption of the protein bovine serum albumin (BSA) to a dual component surface (C18-thiol/MUAM+PEG) and showed that MUAM+PEG effectively resists the nonspecific adsorption of proteins.

B. SPR Imaging Measurements of the Binding of Single-Stranded DNA Binding Protein to Arrays of Single- and Double-Stranded DNA Sequences. As a demonstration showing that these DNA arrays can be used in conjunction with imaging SPR to monitor protein−DNA binding, we constructed a checkerboard surface containing both single- and double-stranded DNA and then monitored, in situ, the binding of single-stranded DNA binding protein to the array surface. As its name implies, SSB (a tetramer of four identical subunits with a total molecular weight of 75000) binds tightly, selectively, and cooperatively to single-stranded DNA and plays a central role in DNA replication, repair, and recombination. Previously, Fischer et al. investigated the binding of SSB to surface-immobilized DNA using the BIACORE SPR instrument and developed a mathematical model to describe the kinetics of binding of the protein to a 70-base sequence of poly(deoxythymidylic acid).[6] Figure 5 shows the difference between two images collected immediately before and after the exposure of the surface to SSB. The raised areas on the image are a measure of the change in % R upon adsorption of the protein to the surface. The array locations at which the protein bound correspond to those regions which were modified with single-stranded DNA sequences.

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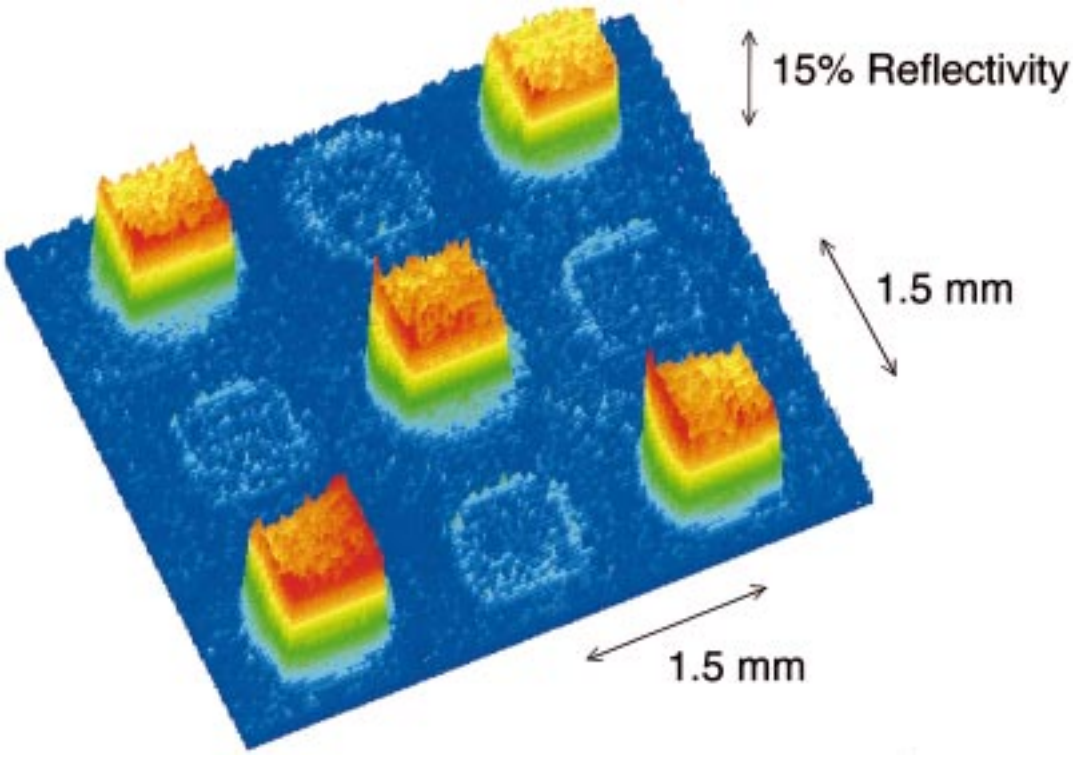

Figure 5. In situ SPR difference image showing the binding of single-stranded DNA binding protein (SSB) to a checkerboard array of single- and double-stranded oligonucleotide sequences. Images collected immediately before and after exposure of the surface to SSB were subtracted to produce the one shown above. Significant binding of the protein to array locations with covalently bound single-stranded DNA sequences occurred, whereas very little binding occurred at the array locations which contained double-stranded DNA sequences.

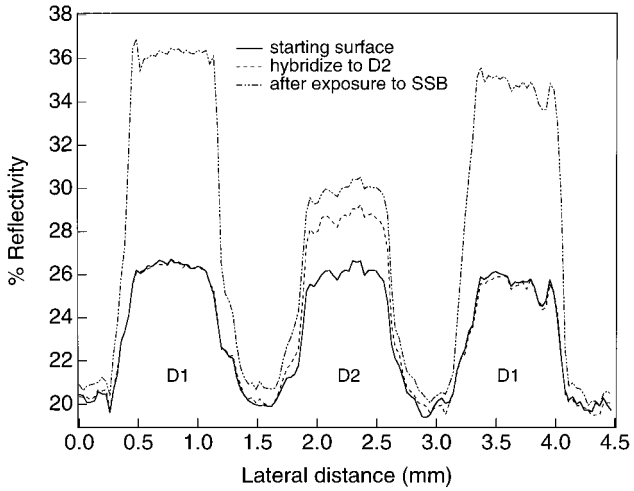

Figure 6. Line profiles showing in situ hybridization and the adsorption of SSB onto a dual component DNA array containing oligonucleotide sequences D1 and D2. The solid line is the percent reflectivity measured for the starting surface composed of alternating DNA probe spots D1 and D2. The dashed line is the % *R* measured after exposing the surface to a 2 μM solution containing the complement to D2. Apparent is an increase in % *R* at position D2 upon binding of the complementary DNA sequence. The dot−dashed line is the % *R* measured after exposing the surface to a 200 nM solution of SSB. While measurable binding did occur at array location D2 (which contained double-stranded DNA), the protein clearly bound more abundantly to the single-stranded sequence D1.

Figure 6 shows various line profiles taken from images collected during the course of the experiment. These "line profiles", which provide quantitative information, are constructed by averaging the % *R* values measured for each column of pixels in a selected rectangular region drawn across the image and plotting this average value against that column's lateral position. The solid line shows the starting surface in which two 5′-thiol-modified, single-stranded DNA sequences, D1 and D2, were immobilized in a checkerboard pattern onto the array surface. The sequences of these two DNA probe strands can be found in the Experimental Section. Each sequence contains a 5′-thiol modifier, a spacer region, and a 16 base long variable sequence. The variable regions were specifically chosen from a library developed for the purposes of DNA computing;[35] they and their complements exhibit no cross hybridization. To position the DNA sufficiently far from the surface so that steric hindrance does not interfere with the hybridization adsorption process, a spacer region is incorporated. A 15T spacer region was used for D1, but sequence D2 contained a similar length EG spacer instead. This was necessary given the fact that SSB is known to bind quite strongly to polyT sequences.[47] The dashed line shows the effects of exposing the surface, in situ, to a 2 μM solution containing the 16-mer complement to D2. A measurable change in % *R* occurred at location D2, indicating that hybridization adsorption of the complementary sequence took place; no increase in signal was seen at D1. The dot−dashed line shows the surface after exposure to a 200 nM solution of SSB. As expected, the protein bound strongly to locations on the array which were single stranded but also bound slightly to those locations that contained double-stranded sequences. Since SSB does not bind to double-stranded DNA,[48] we attribute the increased signal at location D2 to the binding of SSB to single-stranded DNA present at these locations as a result of incomplete hybridization. It is important to note that the array background successfully resisted the nonspecific binding of both comple-

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mentary DNA molecules and single-stranded binding protein; this allowed us to measure small changes in % R without interference from a high background signal.

IV. Conclusions

In this paper we describe a fabrication procedure used to construct multicomponent arrays suitable for use in SPR imaging studies of protein−DNA interactions. The success of the procedure hinges on the novel application of the reversible protecting group Fmoc, commonly used in peptide synthesis, to reversibly modify amine-functionalized alkanethiols self-assembled on gold substrates. The surface reactions involved in the array fabrication process were thoroughly characterized with PM-FTIRRAS, contact angle measurements, and scanning angle SPR spectroscopy. The utility of these surfaces was demonstrated with an SPR imaging measurement that monitored the selective binding of SSB to an array of single- and double-stranded DNA sequences. While the fabrication scheme presented here specifically used the Fmoc protection of MUAM, any number of reversible protecting groups could be used to modify ω-functionalized alkanethiol SAMs. Preliminary results from our laboratory indicate that chloride derivatives of both Fmoc and trityl (triphenylmethyl) to can be used to reversibly modify hydroxyl-terminated alkanethiols, and methods to reversibly modify carboxylic acid-terminated alkanethiols are currently being investigated.[49] Full control of these surface reactions will allow for the custom fabrication of arrays of biomolecules, with properties specifically tailored for each application.

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