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Surface Plasmon Resonance Imaging Measurements of DNA Hybridization Adsorption and Streptavidin/DNA Multilayer Formation at Chemically Modified Gold Surfaces

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A combination of scanning and imaging surface plasmon resonance (SPR) experiments is used to characterize DNA hybridization adsorption at gold surfaces and the subsequent immobilization of streptavidin. Single-stranded oligonucleotides are immobilized at gold surfaces, and the hybridization of biotinylated complements from solution is monitored with SPR. The subsequent attachment of streptavidin to the biotinylated complements provides a method of enhancing the SPR imaging signal produced as a result of the hybridization and leads to a 4-fold improvement in the hybridization detection limit of the SPR imaging apparatus. In situ scanning SPR experiments are used to measure a 60 ± 20% hybridization efficiency between immobilized single-stranded DNA and biotinylated complements. From the information provided by both the in situ imaging and scanning SPR experiments, an absolute surface coverage of immobilized single-stranded DNA is estimated to be ~3 × 10^{12} molecules/cm^2. The SPR signal resulting from hybridization onto immobilized probes is further amplified by the formation of streptavidin/DNA multilayers which grow by a combination of DNA hybridization and biotin−streptavidin binding. DNA/DNA multilayers without streptavidin are used as an additional method of amplifying the SPR signal.

The detection of a specific DNA sequence from solution by hybridization adsorption onto an array of immobilized DNA probes has been studied extensively in recent years as a method of rapidly assaying mixtures of DNA sequences. Detection of hybridization onto a combinatorial mixture of oligonucleotides immobilized at surfaces has many potential applications including DNA sequencing, diagnosis of genetic diseases, and DNA computing.[1−6] All of these applications benefit from rapid methods of detecting and characterizing DNA hybridization adsorption with high specificity and signal-to-noise ratio. Surface plasmon resonance (SPR) is a promising technique for measuring hybridization of both labeled and unlabeled oligonucleotides in an in situ environment as the presence of DNA in solution does not interfere with the detection of hybridization at the surface. The commercially available Biacore instrument takes advantage of this characteristic of SPR and has been used by many researchers to investigate DNA hybridization in real time.[7−10] SPR can also be performed in an imaging geometry which allows it to simultaneously measure hybridization across an array of immobilized oligonucleotides.[10−12]

Many applications would benefit from the ability to detect small amounts of hybridization at surfaces, for example: polymerase

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chain reaction (PCR) amplification typically provides nanomolar concentrations for detection, and some DNA computing applications have proposed hybridization to a single DNA sequence from a large combinatorial mixture immobilized on a surface.[6,13] Fluorescence is commonly used as a very sensitive technique for measuring small amounts of hybridized fluorescently labeled DNA; if evanescent wave fluorescence or scanning confocal fluorescent microscopy is used, it is even possible to detect hybridization adsorption in the presence of low concentrations of fluorescently tagged molecules in solution.[14−16] However, the amount of hybridization can be difficult to quantitate with fluorescence, particularly at gold surfaces where fluorescence quenching can occur.[17] In contrast, SPR is a technique that can be used to quantitatively measure the amount of hybridization that occurs in an in situ environment in real time.

SPR imaging is sensitive to small changes in the thickness or index of refraction of material at the interface between a thin gold film and a bulk solution.[18−21] SPR imaging has been shown to be a surface-selective technique that is sensitive *only* to molecules adsorbed to the interface.[22,23] This makes it possible to detect DNA hybridization adsorption in an in situ environment without the use of labels on the hybridized DNA, and SPR experiments of this type have been demonstrated elsewhere.[24] Although DNA hybridization can be measured by SPR without labeling, this method is sensitive only for surface coverages of immobilized single-stranded DNA (or probe DNA) of $\sim 10^{11}$ molecules/cm^2 or more. In order to improve the sensitivity of the SPR technique, a method of amplifying the SPR signal produced by DNA hybridization is developed here which involves binding streptavidin to biotin-labeled oligonucleotides hybridized to DNA immobilized at gold surfaces. This adsorption scheme is the reverse of the chemistry used in research reported previously which employs the binding between streptavidin and biotinylated oligonucleotides to immobilize DNA on streptavidin-coated surfaces with applications such as the purification of synthetic DNA, hybridization biosensors, and fabrication of MALDI mass spectroscopy samples.[25−30] Various methods of forming multilayers containing DNA have been reported previously,[31−34] and here we investigate two processes of DNA multilayer formation to further enhance the SPR sensitivity above that seen by the adsorption of a single layer of streptavidin.

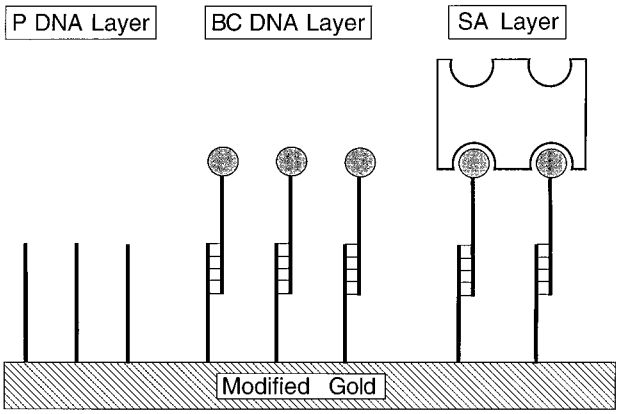

Figure 1. Schematic diagram showing DNA immobilization and hybridization, and streptavidin adsorption onto a modified gold surface: a layer of single-stranded probe DNA (P DNA), the probe DNA layer after hybridization of biotinylated complements (BC layer), and the biotinylated complement layer after adsorption of streptavidin (SA layer).

This paper describes the hybridization adsorption of biotinylated oligonucleotides to immobilized single-stranded DNA and the subsequent binding of streptavidin to these biotinylated complements. A thiol coupling surface chemistry that utilizes the self-assembly of alkanethiol monolayers[35−37] and has been presented in a series of previous papers was employed to immobilize single-stranded DNA at gold surfaces.[23,38−40] This resulted in a surface terminated with single-stranded DNA probes which are denoted as the "P DNA" layer in Figure 1. The DNA immobilization process has been characterized by a combination of polarization modulation-Fourier transform infrared reflection adsorption spectroscopy (PM-FTIRRAS) and scanning in situ SPR measurements. The probe DNA monolayer will hybridize to biotinylated complements (BC) from solution to form DNA duplexes terminated with biotin moieties as pictured in the "BC DNA" layer in Figure 1. Streptavidin can then be bound to the surface via the biotin moieties on the hybridized DNA, as depicted by the streptavidin (SA) layer in the figure. To aid in understanding how these layers form, in situ scanning SPR experiments have been used to characterize all three adsorbed biopolymers. SPR imaging experiments have also been used to investigate DNA hybridization and streptavidin adsorption onto probes with both perfectly

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matched and mismatched sequences. These SPR experiments indicate that the detection limit for DNA hybridization is significantly improved by the subsequent adsorption of streptavidin onto the biotinylated complements, and they have been used to estimate absolute surface coverages of the probe, biotinylated complements, and streptavidin. An additional set of SPR imaging experiments is used to demonstrate that the SPR signal resulting from the initial hybridization can be further increased by the formation of streptavidin/DNA multilayers. This multilayer deposition depends on a combination of both DNA hybridization and the streptavidin−biotin binding process. We also show in this paper that the SPR imaging signal can be enhanced by the formation of DNA/DNA multilayers that do not contain streptavidin.

EXPERIMENTAL SECTION

Materials. 11-Mercaptoundecanoic acid (MUA, Aldrich), poly(L-lysine)·HBr (PL, MW 14 000, Sigma), sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SSMCC, Pierce), streptavidin (Sigma), $NaHCO_3$ (Fluka), NaCl (Fluka), $NaHPO_4$ (Fluka), ethylenediaminetetraacetic acid (EDTA, Sigma), sodium dodecyl sulfate (SDS, Fluka), triethanolamine (TEA, Sigma), urea (Aldrich), and absolute ethanol (Pharmco) were all used as received. All oligonucleotides were synthesized by The University of Wisconsin Biotechnology Center and purified by reversed-phase binary gradient elution HPLC prior to use. Glen Research 5′-thiol-modifier C6 and 5′-biotin phosphoramidite were used in the synthesis of 5′-thiol- and 5′-biotin-modified oligonucleotides, respectively. Millipore-filtered water was used for all aqueous solutions and rinsing.

Surface Attachment Chemistry. The SPR experiments utilized thin (47 or 57 nm) gold films that had been vapor-deposited onto SF10 glass slides (18 × 18 mm^2, Schott Glass) as described previously.[41,42] The 47 and 57 nm gold films were used for in situ scanning and imaging experiments respectively. MUA monolayers were formed on gold films by immersing vapor-deposited gold surfaces into a 1 mM ethanolic solution for at least 18 h followed by thorough rinsing with ethanol and water. PL was adsorbed by immersing MUA-coated surfaces into a 0.7 mM PL solution in 5 mM $NaHCO_3$ for 30 min and then rinsing with water and ethanol. Thiol-terminated DNA was adsorbed to the PL surface by first exposing the entire surface to a 1 mM SSMCC solution in 0.1 M TEA, pH 7, for 15 min, then placing 0.6−1.8 μL drops of 0.5 mM thiol-terminated DNA in 0.1 M TEA, pH 7, on the surface, and allowing this to react overnight in a humid atmosphere. After reacting DNA onto the modified gold surfaces, slides were soaked in 300 mM NaCl, 20 mM $NaHPO_4$, 2 mM EDTA, pH adjusted to 7.8 (2×SSPE), and 0.2% SDS buffer for 1 h and thoroughly rinsed with water and ethanol before use for hybridization experiments. All oligonucleotide hybridization adsorption solutions contained 2 μM DNA in 2×SSPE, and streptavidin adsorption steps used 0.44 μM streptavidin in 2×SSPE unless otherwise noted. The modified gold films were thoroughly rinsed with either water or buffer after each adsorption step.

SPR Experiments. Both in situ scanning and imaging SPR experiments are described in this paper. An ex situ scanning SPR instrument has been described in detail previously,[19,40,43] and only small modifications to this instrument were required for in situ scanning SPR experiments. The ex situ scanning instrument was modified to perform in situ experiments by attaching a Kel-F flow cell with a 60 μL volume to the prism/sample assembly so that a 2 cm^2 area of the gold surface was in contact with solution. The other modification to the ex situ instrument involved replacing the BK7 prism with an SF10 prism ($n = 1.727$, Howard Johnson Optical Labs).[44] The in situ scanning SPR instrument generates plots (denoted as SPR curves) of the percent reflectivity (R) as a function of incident angle. The in situ imaging SPR experiments did not require any modifications to be made to the instrument described previously.[23,24] This instrument uses a CCD (iSight, iSC2050) camera to measure the R across a large incident beam and in this way generates an image of the surface. The percent reflectivity measured at a fixed incident angle can be related to the thickness of the material adsorbed to the surface so that different percent reflectivities will be measured from parts of the surface with different thicknesses.

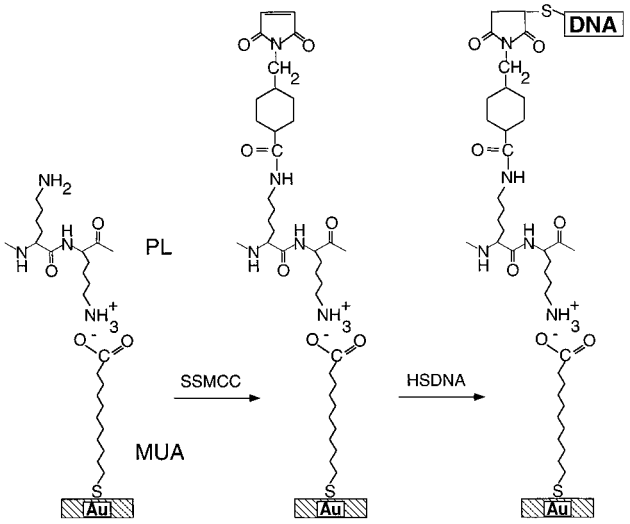

Figure 2. Surface modification scheme for attaching thiol-terminated DNA onto gold. Monolayers of 11-mercaptoundecanoic acid (MUA), and electrostatically bound poly(L-lysine) (PL) are deposited on a gold surface. The bifunctional linker SSMCC will react with some of the lysine residues on the PL to create a surface terminated with reactive maleimide groups, mal-PL surface. These maleimides will react with thiols, and in this way, it is possible to bind thiol-terminated DNA to the modified gold surface.

PM-FTIRRAS Measurements. PM-FTIRRAS spectra were obtained from a Mattson RS-1 spectrometer and a narrow-band HgCdTe detector using 3000 scans at 2 cm^{-1} resolution. The real-time interferogram sampling methods and optical layout have been described previously.[41,42,45] The PM-FTIRRAS differential reflectance values were converted to absorbance units for comparison with conventional IRRAS and FTIRRAS data.[43]

RESULTS AND DISCUSSION

A. Immobilization of Thiol-Terminated DNA onto Modified Gold Surfaces. Single-stranded DNA has been immobilized at vapor-deposited gold surfaces and the attachment chemistry was characterized with PM-FTIRRAS and SPR. The steps employed in the surface modification are shown in Figure 2. A gold

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surface was first coated with a self-assembled monolayer (SAM) of MUA and then exposed to a dilute PL solution, which resulted in the electrostatic adsorption of a monolayer of PL, as depicted in the leftmost structure in Figure 2.[40] This PL−MUA bilayer was used to minimize the nonspecific adsorption of DNA to the gold surface. The PL-modified surface was then exposed to a solution of the bifunctional linker SSMCC, which contains an *N*-hydroxysulfosuccinimide (NHSS) ester and a maleimide functionality. The NHSS ester end of the molecule reacted with some of the free lysine residues on the electrostatically adsorbed PL,[39] resulting in a PL surface containing reactive maleimide groups (mal-PL surface), depicted by the middle structure in Figure 2. PM-FTIRRAS experiments performed previously have estimated the percent of lysine residues on covalently bound PL that are modified by the SSMCC reaction. This estimate used the intensity of the 1708 cm^{-1} band, which is due to an in-phase stretch of the maleimide carbonyl groups.[39] Similar experiments (data not shown) performed on electrostatically adsorbed PL have shown that ∼15% of the lysine residues were modified with maleimide groups. A surface coverage of 4×10^{14} lysine residues/cm^2 in an electrostatically adsorbed PL monolayer has been measured previously,[38] and from this number and the percent of modified lysine residues, a surface coverage of $\sim 6 \times 10^{13}$ maleimide groups/cm^2, is estimated. Maleimide functional groups react with thiols (sulfhydryl groups), and by this method, one is able to attach thiol-modified DNA oligonucleotides to the mal-PL surface, as shown by the final reaction in Figure 2.

The adsorption of thiol-modified DNA onto maleimide-functionalized surfaces has been followed by PM-FTIRRAS and in situ SPR. Figure 3 shows PM-FTIRRAS spectra for DNA immobilized on a gold surface and for bulk DNA. Figure 3a shows the difference between two PM-FTIRRAS spectra, one of a mal-PL surface and one of the same surface after the specific adsorption of thiol-terminated DNA. Figure 3b is a bulk spectrum of DNA obtained from a sample made by placing a DNA solution on a gold film and allowing the water to evaporate. The agreement between the positions and relative intensities of the bands in the two spectra indicate that thiol-terminated DNA can be immobilized at a maleimide-modified gold surface. The intensity of four IR absorption bands increases upon the immobilization of thiol-terminated DNA to a mal-PL surface. The assignments of the bands in Figure 3a are based on work reported in the literature:[46,47] the absorption at 1704 cm^{-1} is due to double-bond stretching vibrations of the DNA bases, the 1278 cm^{-1} band results from an NH bending vibration on the base thymine, and the bands at 1223 and 1073 cm^{-1} are respectively the antisymmetric and symmetric stretching vibrations of the phosphates. The small decrease at 1567 cm^{-1} is due to a shift in the amide II band of the mal-PL layer after the adsorption of DNA.

The sequences and symbols of oligonucleotides used in these experiments are shown in Table 1. The thiol-terminated probes that were immobilized at the gold surface are denoted as P1, P2, and P4. The 15 thymines adjacent to the thiol on the probes help to promote hybridization to complementary oligonucleotides by distancing the duplex-forming region from the surface.[2] The complements to these oligonucleotides are BC1, BC2, and C4a, respectively. The right-hand column in Table 1 contains cartoons

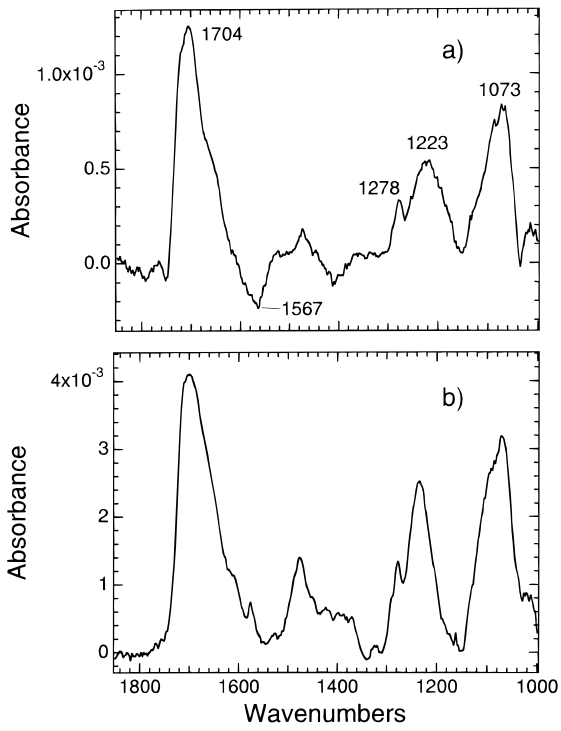

Figure 3. PM-FTIRRAS spectra of thiol-terminated DNA immobilized on a mal-PL surface and bulk DNA. (a) Spectrum showing the difference between two PM-FTIRRAS spectra, one of a mal-PL surface and one of the same surface after the specific adsorption of thiol-terminated DNA. (b) Bulk spectrum of DNA obtained from a sample made by placing a DNA solution on a gold film and allowing the water to evaporate. The similarity between the two spectra indicates that thiol-terminated DNA can be attached to a gold surface modified with maleimides.

indicating how hybridization occurs among these oligonucleotides. The modified gold surface is shown as a vertical line on the left of the cartoons, the horizontal lines represent DNA oligonucleotides, the shorter vertical lines indicate hybridization between perfectly matched complements, and the gray circles represent biotin moieties. Experiments using each of these oligonucleotides will be discussed in the following sections.

B. Scanning SPR Experiments on DNA Hybridization and Streptavidin Adsorption. Hybridization adsorption of biotinylated DNA onto oligonucleotides immobilized at gold surfaces and the subsequent binding of streptavidin have been investigated using in situ scanning SPR. Scanning SPR experiments performed in water were employed to characterize the formation of a mal-PL surface used to covalently attach thiol-terminated DNA. The shifts in the angle at which a minimum reflectivity is measured (SPR angle) as compared to that of bare gold are shown in Table 2. Also shown in the table are the total effective thickness and the additional increase in effective thickness after each adsorbed layer, along with indices of refraction for the adsorbed layers, bulk solutions, and the number of phases used in the Fresnel calculations (e.g., for $n = 5$ the phases were prism, gold, two phases of adsorbed film with different indices of refraction, and solution). Thicknesses calculated from SPR data are reported as effective thicknesses at a particular index of refraction since they measure an average thickness over the size of the incident beam and small errors in estimated indices of refraction can result in relatively large errors in absolute calculated thicknesses.[43,44] The effective thicknesses measured by in situ scanning SPR experi-

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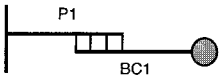
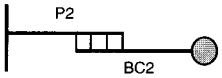
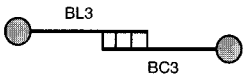
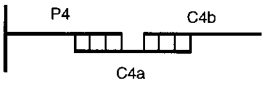
Table 1. Oligonucleotide Sequences and Hybridization Schemes

symbol	sequence	hybridization cartoon[a]
P1	5' thiol $(T)_{15}$ GCT TTC CTG AAG TTC G 3'	
BC1	5' biotin CGA ACT TCA GGA AAG C 3'	
P2	5' thiol $(T)_{15}$ GCT TCA ACA CCA TTC G 3'	
BC2	5' biotin CGA ATG GTG TTG AAG C 3'	
BL3	5' biotin $(T)_5$ CGA ACG AAG TTG GTT GAA GC 3'	
BC3	5' biotin $(T)_5$ GCT TCA ACC AAC TTC GTT CG 3'	
P4	5' thiol $(T)_{15}$ CGA ACG AAG TTG GTT GAA GC 3'	
C4a	5' GCT TGC TTG AAG GAA GTT CGG CTT CAA CCA ACT TCG TTC G 3'	
C4b	5' CGA ACT TCC TTC AAG CAA GCC GAA CGA AGT TGG TTG AAG C 3'	

[a] Oligonucleotides on top of each cartoon are shown left to right 5′ to 3′ and those on the bottom are shown 3′ to 5′.

Table 2. SPR Angle Shifts and Calculated Film Thicknesses

layer	total $\Delta\theta$ (deg)[a]	solution n[b]	layer n	no. of phases[c]	effective total thickness (Å)	effective additional thickness (Å)[d]
MUA	0.147 ± 0.018[e]	1.333	1.40[f]	4	17.0 ± 1.4[e]	17.0
PL	0.279	1.333	1.52[f]	5	27.0	10.0
SSMCC	0.294	1.333	1.52[f]	5	28.3	1.3
P2	0.457	1.336	1.46[g]	6	45.8	17.5
BC2	0.503	1.336	1.46[g]	6	51.0	5.2
SA1	0.715	1.336	1.45[f]	7	75.2	24.2

[a] Total shift in SPR angle from that of bare Au. [b] Measured by refractometry. [c] Number of phases used in Fresnell calculation when effective thicknesses are determined. [d] Increase in the effective thickness from the previous layer. [e] Total $\Delta\theta$ and thickness values are the average of three samples with the errors resulting from sample-to-sample variation; they do not include errors from estimating n. [f] Index of refraction estimated from bulk values. [g] From ref 11.

ments for MUA and PL layers are 17.0 and 10.0 Å, respectively; these values compare reasonably well with previous ex situ measurements on similar surfaces.[40] Upon the adsorption of SSMCC to the PL surface, a change in thickness of only 1.3 Å was observed. This small increase is reasonable for a surface coverage of 6×10^{13} maleimide groups/cm^2 as estimated from the PM-FTIRRAS data.

Figure 4 shows the experimental SPR curve for a mal-PL surface on which the thiol-terminated oligonucleotide P2 has been adsorbed (open circles) and the solid line is a six-phase Fresnel fit to the data. This and all subsequent in situ experiments were performed in 2×SSPE, which promoted DNA hybridization. The inset in Figure 4 is an expanded view of the SPR minima for the P2 surface and the same surface after the hybridization adsorption of perfectly matched biotinylated complements (BC2, open squares). The calculated effective thicknesses of these two layers are shown in Table 2. P2 has 31 nucleotide bases (31-mer), and the thickness of the P2 layer measured in 2×SSPE is 17.5 Å. As the surface coverage of maleimides is only $\sim 6 \times 10^{13}$ molecules/cm^2, it is expected that the surface attachment chemistry would form only a partial monolayer of probe DNA oligonucleotides. The measured thickness of the P2 layer is in agreement with a partial monolayer as a fully packed layer of an extended 31-mer oligonucleotide is estimated to be ~100 Å thick by assuming the single-stranded DNA will have the same length per base as double-stranded DNA. The hybridization of a layer of biotinylated complements, BC2, shows an additional increase in the effective thickness of 5.2 Å. This is ~30% of the effective thickness measured for P2 in 2×SSPE, and since BC2 is about half the size of P2, a hybridization efficiency of 60 ± 20% is determined. This hybridization efficiency was not found to be highly sequence dependent as similar shifts in SPR angles were observed for the adsorption of P1 and BC1. A hybridization efficiency of ~60% is in good agreement with those measured by other researchers on similar systems, who have found hybridization efficiencies between 40 and 80% for DNA oligonucleotides immobilized to gold, silicon, and carboxy-methylated dextran.[9,10,48,49]

In situ scanning SPR experiments have also shown that it is possible to specifically adsorb the protein streptavidin onto biotinylated DNA hybridized onto immobilized oligonucleotide probes, as depicted by the rightmost cartoon in Figure 1. The open triangles in Figure 4 are the SPR curve taken after streptavidin has been adsorbed onto BC2. The expanded view of the SPR minima shown in the inset to Figure 4 clearly indicates that the SPR angle has shifted significantly upon the adsorption of streptavidin as compared to the smaller shift seen for just the biotinylated complements. The calculated effective thickness increase for the streptavidin adsorption is 24.2 Å, and this is a 5-fold increase in the effective thickness of streptavidin and BC2 as compared to just BC2. A 24.2 Å thick streptavidin film

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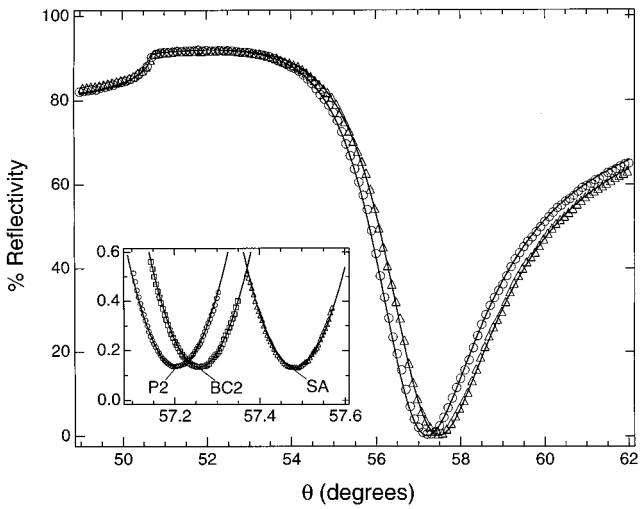

Figure 4. In situ scanning SPR curves of DNA immobilization and hybridization and streptavidin adsorption, taken in 2×SSPE buffer. The full SPR curves are shown after the deposition of the single-stranded probes and streptavidin. The inset is an expanded view of the SPR minima for the probe (P2), hybridized biotinylated complement (BC2), and streptavidin (SA) layers. The open circles, squares, and triangles show respectively the experimental percent reflectivities for P2, BC2, and SA as a function of incident angle, θ. The solid lines are the results of six- or seven- (see Table 2) phase complex Fresnel calculations for each layer, and the shift in the angle of the minimum R is due to differences in thickness and index of refraction between the layers.

corresponds to about half of a monolayer based on the size of streptavidin (54 Å × 58 Å × 48 Å).[50,51] Because avidin and streptavidin have similar dimensions, a surface coverage of 1.5×10^{12} molecules/cm^2 for half a monolayer of streptavidin is estimated based upon the previously measured surface coverage for a full monolayer of avidin of 3×10^{12} molecules/cm^2.[38] The additional thickness of the adsorbed streptavidin is probably useful for scanning SPR measurements on very small amounts of hybridized DNA, but the enhancement is even more important for fixed-angle SPR imaging experiments.

C. Imaging SPR Experiments on DNA Hybridization and Streptavidin Adsorption. Imaging SPR experiments are performed at a fixed angle and can simultaneously measure the percent reflectivity (R) across an entire surface patterned with areas of differing thickness. Since imaging SPR experiments are performed at a single fixed angle, they are less sensitive than scanning SPR experiments, which use an average over many angles to determine an effective thickness. For this reason they benefit significantly from amplifying the SPR response by streptavidin adsorption. Figure 5a shows the arrangement of P1 and P2 spots adsorbed to a mal-PL surface (mal-PL/probe surface) used in the following experiment. The sequences of P1 and P2 differ by 8 out of 16 bases; therefore, the complement to each will be an 8-base mismatch with the other. Figure 5b is an image of a mal-PL/probe surface to which BC1 and streptavidin have been adsorbed (taken in 2×SSPE buffer), and Figure 5c shows a surface to which BC2 and streptavidin are bound. Because the images in Figure 5 were taken at an angle below the SPR angle, a higher R is expected for the thicker layers, as represented by the red areas. From Figure 5, it is possible to qualitatively see that adsorption of biotinylated complements and streptavidin significantly increases the SPR response in the areas containing the corresponding perfectly matched probes. SPR images can be represented quantitatively by vertically averaging the R values measured at each pixel of the CCD camera within a rectangle which crosses the center of two of the spots, thus generating a "line profile" across the spots.[23] For example, the dashed lines in panels a and b of Figure 6 are line profiles generated through the top two spots of the images in panels b and c of Figure 5, respectively.

By quantitatively analyzing the SPR imaging data for a series of images showing biotinylated DNA hybridization and streptavidin binding, it is possible to measure the increase in sensitivity due to streptavidin adsorption. Figure 6 shows line profiles generated from such a series of images, taken in 2×SSPE. A line profile generated from an image of the initial mal-PL/probe surface is shown as the solid line in Figure 6a. The R measured for the mal-PL background and the probes are in agreement with the R expected from scanning in situ measurements and the angle at which the images were taken. The dot−dash line in Figure 6a is a line profile taken after this surface was exposed to a 2 μM BC1 solution and shows a ∼2.5% increase in the R measured from the area containing P1; essentially no change is seen in the P2 or the mal-PL areas. This indicates that BC1 is hybridizing with P1 instead of nonspecifically adsorbing to the surface. Upon exposure of the mal-PL/probe/BC1 surface to a 0.44 μM streptavidin solution (dashed line), a relatively large increase of 10% R above the probe surface in the area containing BC1 is observed with no change in R measured elsewhere. It is seen (data not shown) that the line profile returns to the R measured for the initial probes (solid line Figure 6a), if this surface is exposed to 8 M urea at 31 °C. This indicates that all of the BC1 and streptavidin layers are removed, leaving only the DNA probes on the surface. The fact that BC1 and streptavidin are completely removed by urea, which disrupts the hydrogen bonding involved in the hybridization,[52] is further evidence that it is hybridization by which BC1 is adsorbed and that the streptavidin binds specifically to the biotin on the BC1. This will allow the surface containing the DNA probes to be used for multiple hybridization experiments, which is a useful feature for DNA adsorption biosensors. After the surface was regenerated with urea, the sample was exposed to BC2 and then to streptavidin. Figure 6b shows the stepwise hybridization of BC2 onto the P2 area (dot−dash line in Figure 6b) and the subsequent binding of streptavidin to BC2 (dashed line). The adsorption of streptavidin to hybridized biotinylated DNA increases the R measured as a result of the hybridization by a factor of 4. This increase in the R is slightly less than the thickness increase measured by in situ scanning SPR because the R measured at this angle is nonlinear with thickness. However, the enhancement still increases the sensitivity of the SPR imaging experiment enough that hybridization of BC1 and BC2 onto only the perfectly matched probes is easily observed, as shown in Figure 6.

Absolute surface coverages of the immobilized probe DNA can be estimated from the scanning experiments presented in section B and an additional in situ imaging experiment (data not shown). In this additional experiment, 2 μM biotinylated DNA and 2 μM

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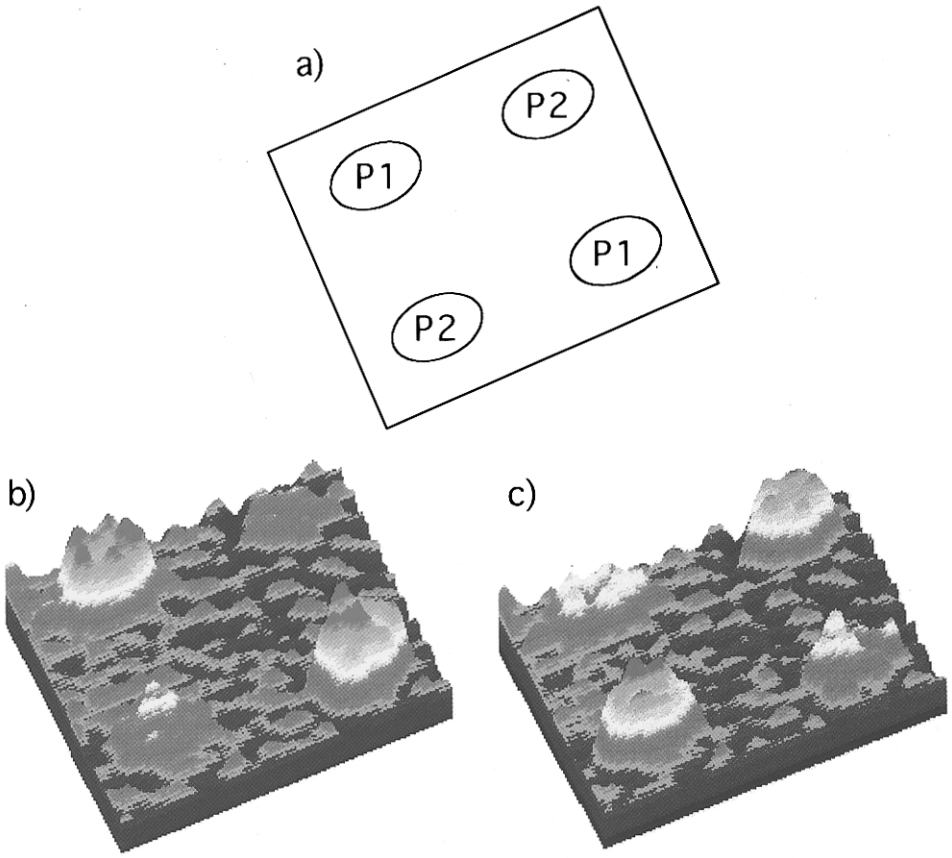

Figure 5. In situ SPR images of hybridization and streptavidin adsorption onto two different probe oligonucleotides, P1 and P2. (a) Diagram showing the placement of the probe spots on a mal-PL surface. (b) SPR image taken after hybridizing BC1 onto P1 and adsorbing streptavidin. (c) SPR image of the same surface after regenerating it with urea then hybridizing BC2 onto P2 and adsorbing streptavidin.

streptavidin were mixed in solution and then adsorbed by hybridization onto oligonucleotides immobilized on a gold surface. The identical *R* was measured by this method and by sequentially exposing the same surface to first biotinylated DNA and then streptavidin. This indicates that the same amount of streptavidin is adsorbed to the surface by either method. Because the interaction between biotin and streptavidin is so strong ($K_{binding} = 10^{15}$),[51] when stoichiometric amounts of streptavidin and biotinylated DNA are mixed in solution and then adsorbed to immobilized probes, all of the biotinylated DNA will be bound to streptavidin. Since either method will adsorb only half of a monolayer of streptavidin, it must also be true that all of the biotinylated complements interact with streptavidin when a sequential method of adsorption is employed.

From the fact that all of the biotinylated oligonucleotides bind to streptavidin and information provided by the in situ scanning experiments, it is possible to make an order of magnitude estimate of the absolute surface coverage of thiol-terminated probes adsorbed to a mal-PL surface. In situ scanning experiments have determined the absolute surface coverage of streptavidin to be $\sim 1.5 \times 10^{12}$ molecules/cm^2, and the hybridization efficiency between the probes and the biotinylated complements to be 60 $\pm$ 20%. From the surface coverage of streptavidin and the fact that all of the biotinylated complements are bound to one of streptavidin's four binding sites, a surface coverage for the biotinylated complements is estimated to be 1.5×10^{12} molecules/cm^2. Then using the hybridization efficiency, the surface coverage of immobilized DNA is estimated to be 3×10^{12} molecules/cm^2. This is in good agreement with surface coverages measured for other methods of immobilizing DNA, which generally range from 3.0×10^{12} to 1.5×10^{13} molecules/cm^2.[10,48,53]

The adsorption of streptavidin onto biotinylated DNA duplexes immobilized at a gold surface as discussed in this section will increase the SPR signal produced as a result of the hybridization. This application of biotinylated DNA and streptavidin binding is the reverse of what most researchers have done previously. Most other applications have used surfaces to which streptavidin is bound to initially immobilize biotinylated oligonucleotides.[25−30] The fact that these researchers have used streptavidin to attach DNA to surfaces suggests that it may be possible to bind a second layer of biotinylated DNA to the streptavidin layers discussed in this paper. The following section describes experiments investigating this as a possible method of forming multiple layers of streptavidin and DNA.

D. Formation of DNA Multilayers. (a) Streptavidin/DNA Multilayers. Although one step of streptavidin adsorption causes a significant increase in the SPR signal observed due to the hybridization of biotinylated DNA, it is possible to further amplify this signal by the formation of streptavidin/DNA multilayers. The streptavidin/DNA multilayers are formed by first making a streptavidin-coated surface as discussed above and then sequentially adsorbing two strands of complementary biotinylated linker oligonucleotides (BL3 and BC3 shown in Table 1) followed by a second layer of streptavidin as shown in the inset of Figure 7. Figure 7 shows the differential *R* measured during the formation of streptavidin/DNA multilayers on a spot of P2 deposited on a

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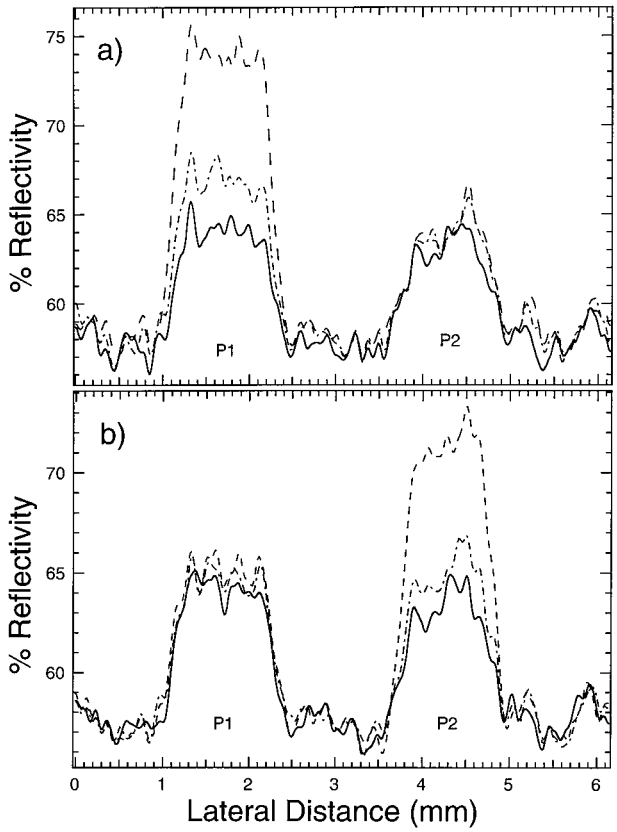

Figure 6. Line profiles showing hybridization and streptavidin adsorption onto areas of P1 and P2 immobilized at a mal-PL surface. (a) The solid line is the *R* measured from a mal-PL surface to which the two DNA probes have been attached. The dot−dash line is from the same surface after exposing it to a solution of BC1 and shows a small increase in *R* only in the P1 area. The dashed line is a line profile taken after exposing the surface to streptavidin and shows a relatively large increase in *R* in the P1 area. (b) The solid line is the *R* measured from the same surface shown in (a) after removing all of the BC1 and streptavidin with urea. The dot−dash and dashed lines in (b) are respectively the *R* measured after exposing the surface to BC2 and streptavidin. The comparatively large increase in *R* observed after exposing a surface having hybridized biotinylated complements on it to streptavidin shows that the adsorption can be used to significantly amplify the SPR imaging signal produced by the hybridization.

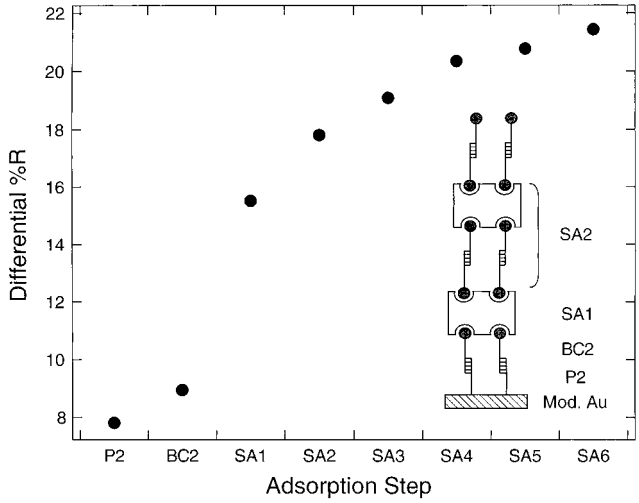

Figure 7. The differential percent reflectivity measured between a spot of DNA probe, P2, and a mal-PL surface as streptavidin/DNA multilayers are built up on the P2 spot. The inset is a schematic diagram showing how the streptavidin/DNA multilayers are deposited. After the first streptavidin adsorption step the increase in *R* for the biotinylated DNA binding and hybridization is so small that only the total differential *R* after depositing two biotinylated oligonucleotides and streptavidin is shown for the adsorption steps, SA2−SA6. This provides a method for further increasing the SPR imaging signal above that observed for a single streptavidin layer.

mal-PL surface. The differential reflectivity for these experiments is measured as the difference in the *R* for the mal-PL background and the P2 spot observed after each adsorption step. The additional *R* measured for the adsorption of BL3 and BC3 is very small and so only the total increase in *R* after the adsorption of BL3, BC3, and streptavidin is shown as the streptavidin adsorption step (SA2), in Figure 7. These three adsorption steps can be repeated to form multiple streptavidin/DNA layers as has been demonstrated for six streptavidin adsorption steps (SA1−SA6). It has also been shown that no measurable adsorption occurs to the mismatch P1 and that the streptavidin/DNA multilayers can be completely removed from the surface by exposing it to 8 M urea at 31 °C (data not shown). Several factors indicate that multilayer formation occurs by a combination of DNA hybridization and biotin−streptavidin binding. These include the ability to remove the multilayers with urea, the observation that no nonspecific adsorption occurs on the mismatch, P1, or the mal-PL background, and the fact that multilayer formation does not occur unless the streptavidin surface is exposed to both BL3 and BC3 before depositing a second layer of streptavidin. The ability to form streptavidin/DNA multilayers using this scheme indicates that at least some of the biotin sites on the adsorbed streptavidin are active, which is an important point as adsorbing biological molecules to surfaces sometimes changes their activity.[54]

Figure 7 shows that the increase in *R* measured after six streptavidin adsorption steps is about twice as much as that measured for a single streptavidin amplification. This indicates that the SPR imaging signal can be further increased by streptavidin/DNA multilayer formation; however, the change in *R* becomes smaller for each subsequent streptavidin layer. Part of this smaller change in *R* is due to the nonlinear relationship between *R* and thickness at the angle the experiment was performed. However, this will not account for all of the change in *R*, indicating that there is a real decrease in additional thickness between subsequent streptavidin adsorption steps. Hybridization efficiencies between complementary DNA oligonucleotides of less than 100% and the possibility of some biotin binding sites being inactive are both expected to contribute to the deposition of less streptavidin with each adsorption step. Despite this decrease in additional thickness, it is possible to form at least six streptavidin/DNA multilayers which further improves the SPR imaging signal that can be produced from the initial hybridization of biotinylated DNA.

The combination of DNA hybridization and streptavidin−biotin binding has been used previously to increase the amount of signal observed from hybridization adsorption or to form macroscopic structures. Caruso et al. have shown that multilayer formation will occur via the successive deposition of avidin and poly(stryenesulfonate) and that once these multilayers are formed it is possible to bind a biotin-labeled oligonucleotide to the avidin

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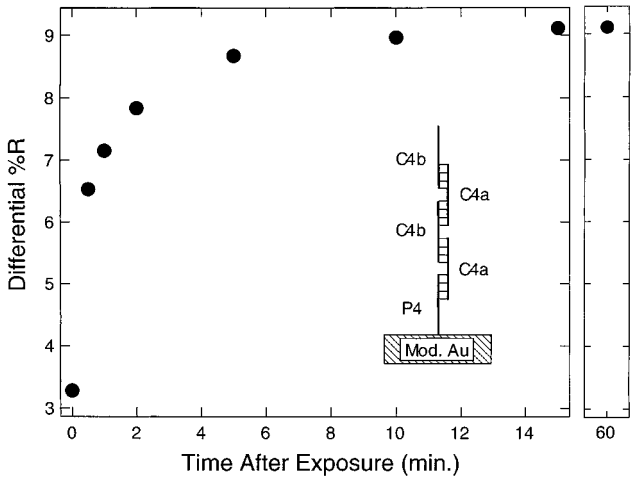

Figure 8. Differential percent reflectivity between a spot of DNA probe, P4, and a mal-PL surface measured as a function of time after exposing the surface to a solution containing a mixture of the oligonucleotides C4a and C4b. The inset is a schematic diagram showing the formation of DNA/DNA multilayers. The differential *R* increases rapidly for the first 5 min and then slowly levels out until it reaches a constant value after ~10 min. DNA/DNA multilayer formation is expected to depend on the efficiency and kinetics of the hybridization and may provide a novel method for investigating these quantities by SPR imaging.

in these multilayers and subsequently hybridize another oligonucleotide to this immobilized DNA.[32] DNA hybridization has also been used to reversibly form macroscopic aggregates of colloidal gold. This has been done by coating the particles with two different thiol-terminated oligonucleotides, then, if an oligonucleotide duplex is added that has one end complementary to each of the immobilized DNA sequences, the particles will aggregate.[55] These colloidal gold particles aggregate in a single solution, suggesting that perhaps streptavidin/DNA multilayers at gold surfaces could also be formed from a single adsorption solution. However, attempts to form streptavidin/DNA multilayers from a solution containing a mixture of BL3, BC3, and streptavidin were unsuccessful. For this reason, an alternative method of forming multilayers from a single adsorption solution has been developed.

(b) DNA/DNA Multilayers. The formation of DNA/DNA multilayers without streptavidin present has been investigated as a means of amplifying the SPR signal produced by hybridization using a single adsorption solution. This scheme involves assembling multilayers of two DNA oligonucleotides (C4a and C4b), as shown in the inset of Figure 8. The hybridization occurs as shown in Table 1, where half of C4a at the 5′ end is complementary to half of C4b at the 3′ end and the other half of C4a is complementary to the 5′ end of C4b. In preliminary experiments, a spot of the thiol-terminated oligonucleotide P4, which is complementary to half of C4a at the 3′ end, was attached to a mal-PL surface as described in section A. This surface was then exposed to a 2×SSPE solution having a 4 μM concentration of each C4a and C4b. Figure 8 shows the differential *R* between the P4 spot and the mal-PL background measured as a function of time after exposing the surface to the C4a and C4b solution. From the figure it can be seen that the differential *R* increases quickly for the first 5 min and then after ~10 min levels off to a constant *R* which is ~9% higher than the mal-PL background. This increase is due to the adsorption of multiple strands of C4a and C4b onto each P4 oligonucleotide. The imaging experiments shown in Figure 8 were performed at a fixed angle different from that for the experiments shown in Figures 5−7, so no direct comparisons between the increase in *R* produced by streptavidin/DNA and DNA/DNA multilayers can be made. Although the amount of adsorption due to DNA/DNA multilayer formation has not been quantified, it can be seen that this provides a means of increasing the *R* due to hybridization using a single amplification solution. It is also expected that the rate at which this multilayer formation occurs is related to the hybridization rate for C4a and C4b and that this could provide a novel method of measuring DNA hybridization kinetics with in situ SPR imaging. Further investigation of the formation of DNA/DNA multilayers and their possible applications to the study of hybridization efficiency and kinetics are left for future experiments.

CONCLUSIONS

Hybridization adsorption of biotinylated oligonucleotides on gold surfaces has been characterized by in situ scanning and imaging SPR, and the subsequent adsorption of streptavidin as a method of amplifying the SPR signal produced by the hybridization has been investigated. In situ scanning SPR experiments have measured a hybridization efficiency of 60 ± 20% for immobilized oligonucleotide probes and perfectly matched biotinylated complements. The adsorption of streptavidin has been shown to occur specifically to biotinylated DNA, and both streptavidin and biotinylated DNA can be removed by disrupting the hybridization. The specific adsorption of streptavidin to biotinylated DNA allows streptavidin adsorption to be used as a method of amplifying the SPR imaging signal produced by the hybridization of biotinylated DNA to oligonucleotides immobilized at gold surfaces. This amplification improves the detection limit for DNA hybridization using SPR imaging by a factor of ~4. Using a combination of the in situ scanning and imaging experiments, it is possible to estimate surface coverages for the thiol-terminated probe, biotinylated complement, and streptavidin.

The formation of hybridization dependent multilayers has also been investigated. It has been shown that streptavidin/DNA multilayers, up to at least six streptavidin layers, can be formed and that these multilayers grow by a combination of both DNA hybridization and streptavidin−biotin binding. This multilayer formation can be used to further amplify the signal produced by the initial hybridization above that observed from a single streptavidin layer. DNA/DNA multilayers without streptavidin have also been deposited. Future experiments will investigate the formation of these multilayers as a novel method for measuring DNA hybridization efficiency and kinetics by SPR imaging.

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