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Accelerated Articles

Surface Plasmon Resonance Imaging Measurements of DNA and RNA Hybridization Adsorption onto DNA Microarrays

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Surface plasmon resonance (SPR) imaging is a surface-sensitive spectroscopic technique for measuring interactions between unlabeled biological molecules with arrays of surface-bound species. In this paper, SPR imaging is used to quantitatively detect the hybridization adsorption of short (18-base) unlabeled DNA oligonucleotides at low concentration, as well as, for the first time, the hybridization adsorption of unlabeled RNA oligonucleotides and larger 16S ribosomal RNA (rRNA) isolated from the microbe *Escherichia coli* onto a DNA array. For the hybridization adsorption of both DNA and RNA oligonucleotides, a detection limit of 10 nM is reported; for large (1500-base) 16S rRNA molecules, concentrations as low as 2 nM are detected. The covalent attachment of thiol-DNA probes to the gold surface leads to high surface probe density (10^{12} molecules/cm^2) and excellent probe stability that enables more than 25 cycles of hybridization and denaturing without loss in signal or specificity. Fresnel calculations are used to show that changes in percent reflectivity as measured by SPR imaging are linear with respect to surface coverage of adsorbed DNA oligonucleotides. Data from SPR imaging is used to construct a quantitative adsorption isotherm of the hybridization adsorption on a surface. DNA and RNA 18-mer oligonucleotide hybridization adsorption is found to follow a Langmuir isotherm with an adsorption coefficient of 1.8×10^7 M^{-1}.

Surface plasmon resonance (SPR) has recently gained attention as a label-free method for the detection of the binding of biological molecules onto functionalized surfaces. SPR has been used to study a variety of biological processes, including DNA/DNA,[1−4] DNA/protein,[5−10] and protein/protein interactions.[11−14] SPR imaging is a variation of the SPR technique in which multiple adsorption interactions can be monitored in an array format under identical conditions.[15] Recently, we demonstrated a multistep procedure to create DNA arrays on gold surfaces for use with SPR imaging.[5,6] These arrays can be used to study affinity interactions for a variety of target molecules, including unlabeled proteins and nucleic acids. For example, we recently reported the ability to detect single-base sequence variations by monitoring the adsorption of the mismatch binding protein MutS isolated from *Escherichia coli* onto

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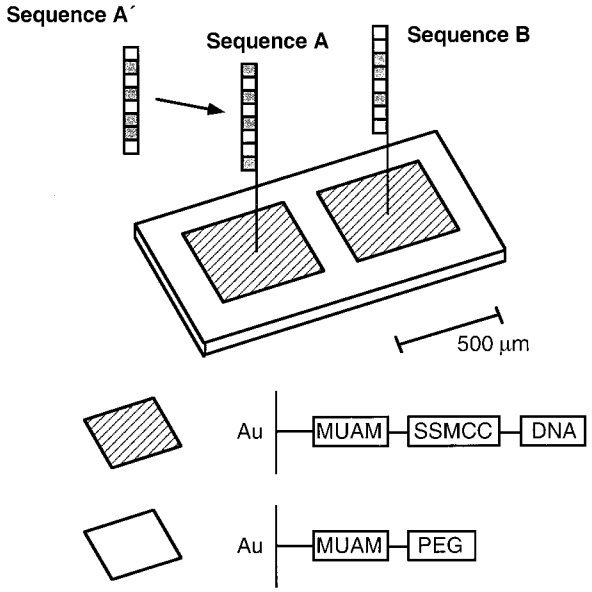

Figure 1. A simplified cartoon of the DNA arrays used for the hybridization of complement oligonucleotides onto a DNA array. DNA (18-base) probe strands, sequences A and B, were immobilized onto a chemically modified gold thin film in two separate spots. Sequence A′, the perfect complement to sequence A, will specifically hybridize onto its immobilized perfect complement. Below, a block diagram outlining the chemistry used in these DNA arrays is shown. First, the gold surface is modified with a self-assembled monolayer of MUAM. Thiol-modified DNA is chemically attached onto the amine-terminated monolayer using the heterobifunctional linker SSMCC. The amine-terminated monolayer surrounding the DNA probe spots is modified with a PEG functionality in order to prevent nonspecific adsorption interactions with the array background. DNA probe spots are 500 × 500 μm.

a DNA array with near-infrared SPR imaging.[6,15] Other biopolymer adsorption measurements have been reported in a nonarray format using SPR angle shift measurements with the commercially successful Biacore instrument.[7−9,12,16]

In an SPR imaging experiment, changes in the reflectivity from a thin gold film are used to monitor adsorption onto the surface. In this paper, we detect the hybridization adsorption of complement DNA (targets) onto surface-immobilized DNA (probes). Figure 1 is a simplified cartoon showing hybridization onto DNA arrays as conducted in the SPR imaging experiment. Two 18-base DNA sequences, A and B, have been immobilized onto a chemically modified gold thin film in two separate spots. Sequence A′ is the perfect complement to sequence A and will specifically hybridize onto the spots functionalized with sequence A. This hybridization adsorption results in an increase in the local index of refraction, which in turn leads to a change in the reflectivity from the surface that is monitored with the SPR imaging experiment.

The chemistry used in the DNA array is shown in block diagram format in the lower portion of Figure 1. Our strategy for DNA immobilization onto gold surfaces has been characterized by a variety of spectroscopic methods and has been presented elsewhere.[1,17,18] Briefly, the gold surface is modified with a self-assembled monolayer of 11-mercaptoundecylamine (MUAM). Thiol-modified DNA is chemically attached to the amine-terminated monolayer using the bifunctional linker sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SSMCC). The thiol-DNA contains a 15-T spacer prior to the active sequence to provide additional spacing away from the surface. The amine-terminated monolayer surrounding the DNA probe spots is modified with a poly(ethylene glycol) (PEG) functionality in order to prevent nonspecific adsorption interactions with the array background.

In this paper, we determine the sensitivity of near-infrared SPR imaging measurements to the hybridization adsorption of label-free DNA and RNA oligonucleotides onto DNA arrays and demonstrate how to quantify the SPR imaging reflectivity signal in order to obtain relative surface coverage measurements. Sensitivity is an important aspect for the detection of biomolecules with SPR, and several amplification techniques have been devised to enhance the sensitivity of SPR measurements. For example, the use of attached colloidal particles[3,19] and amplification of hybridization signal through streptavidin have been reported.[1] We find that, with the recent improvements brought about by the use of near-infrared excitation and an incoherent light source,[20] SPR imaging of DNA arrays can be used to detect the adsorption of biological molecules at the nanomolar concentration level without the need for chemical labeling.

In addition to the detection of DNA and RNA oligonucleotides, we also demonstrate in this paper that DNA arrays can be used to detect larger RNA molecules from biological samples. Specifically, we use SPR imaging measurements to detect the sequence-specific binding of 16S ribosomal RNA (rRNA) from *E. coli* onto DNA arrays. Recently, researchers reported the use of DNA microarrays for the detection of fluorescently labeled rRNA.[21] Researchers have proposed using rRNA to monitor microbial populations since it is naturally amplified and easily isolated from natural systems. For example, fluorescently labeled oligonucleotides specific to regions of 16S rRNA have been used to differentiate bacterial phylogenetic groups in environmental samples.[22,23] By contrast, SPR imaging using DNA arrays can provide a more efficient alternative for the characterization of microbial diversity, avoiding the need for fluorescent labels. The detection of other large RNA molecules with SPR imaging may also prove to be important in the future. Previous researchers have immobilized RNA as a surface probe to monitor other binding events,[24,25] but there have been few reports of the direct detection of RNA hybridization onto a surface with SPR.[26]

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EXPERIMENTAL CONSIDERATIONS

Materials. 9-Fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide (Fmoc-NHS) (Novabiochem), *N*-hydroxysuccinimidyl ester of methoxypoly(ethylene glycol) propionic acid MW 2000 (PEG-NHS; Shearwater Polymers, Inc.), and sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Pierce) were all used as received. The 11-mercaptoundecylamine, was a generous gift from the laboratory of Professor George M. Whitesides, Harvard University. Absolute ethanol and Millipore-filtered water were used for rinsing and for aqueous solutions. Gold films (45 nm) were vapor deposited onto SF-10 glass slides (18 × 18 mm) as described previously.[27,28] A thin (<1-nm) chromium underlayer was used to enhance the adhesion of gold to the SF-10 slide. For MUAM monolayer formation, gold-coated slides were immersed in a 1 mM ethanolic solution for at least 2 h and subsequently rinsed with ethanol and water.

Preparation of Nucleic Acids. All oligonucleotides were synthesized on an ABI DNA synthesizer at the University of Wisconsin Biotechnology Center. Attachment of DNA onto the surface was accomplished through 5′-Thiol-Modifier C6 (Glen Research), with a 15-T spacer to allow space for ample interaction with target molecules. Thiol-modified oligonucleotides were deprotected as outlined by Glen Research Corp.[29] Before use, each DNA oligonucleotide was purified by reversed-phase binary gradient elution HPLC (Shimadzu SCL-10AVP), and DNA concentrations were verified with an HP8452A UV−visible spectrophotometer. Thiol-DNA immobilization via maleimide-modified SAM helps avoid nonspecific interaction of the DNA with the gold and, along with the 15-T spacer, facilitates interaction with the target molecule compared to direct attachment of the thiol-DNA to the gold surface. Probe DNA sequences were designed to avoid loops and hairpins and to maximize discrimination to each other's complements. The sequence of the surface-immobilized DNA used in our experiment to capture the 16S rRNA was based on the accessibility of regions of the RNA molecule to oligonucleotide probes[30] as well as a comparison of the probe sequence to the database of known 16S rRNA sequences available at GenBank (http://www.ncbi.nlm.nih.gov). Table 1 outlines sequences immobilized onto the surface (probes) and complementary sequences injected into the cell as analytes (targets). The table includes sequences A and B, used for the detection of complement oligonucleotides by hybridization, and the probe sequence used to capture *E. coli* 16S rRNA, listed as sequence EC. Complement sequences of DNA and RNA, where applicable, are also listed in Table 1. *E. coli* ribosomal RNA was purchased from Sigma and used as received.

Multistep Array Fabrication. The DNA microarrays on gold surfaces were fabricated using a multistep procedure to create DNA arrays on gold surfaces as described by Brockman et al.[5] With this technique, multiple DNA sequences can be immobilized onto a gold surface while reducing the possibility of cross-contamination between spots. Briefly, a bare gold surface was modified with a self-assembled monolayer of the amine-terminated alkanethiol, MUAM. The amine-terminated surface was then modified with the hydrophobic protecting group, Fmoc. Samples were then photopatterned using a mercury xenon arc lamp (Oriel) for 1 h at 400 W with a mask on a quartz substrate (500 × 500 μm squares). Samples were rinsed thoroughly with ethanol after photopatterning. Exposed square spots of bare gold were functionalized with MUAM. Hydrophilic, maleimide-modified "wells" were created by covalently attaching a bifunctional linker, SSMCC, to the MUAM spots. Small quantities (~40 nL) of 1 mM solution of thiol-DNA were delivered separately to these wells using a PV830 Pneumatic Pico Pump (World Precision Instruments). After immobilization of the DNA surface probes, the reversible protecting group Fmoc was removed with a mild base, exposing the MUAM-modified surface, and replaced with PEG-NHS. The PEG-modified surface surrounding the DNA spots resists nonspecific adsorption during in situ experiments. After PEG modification, the array was assembled in the imaging system and the surface exposed to complementary strands of DNA.

Table 1. Oligonucleotide Sequences for Probe (Surface Immobilized) and Target (Complement) DNA[a].

symbol	sequence (5′ → 3′)
A (surface probe)	GCC GAA GCC ACC TTT TAT
A′ (DNA complement)	ATA AAA GGT GGC TTC GGC
A″ (RNA complement)	AUA AAA GGU GGC UUC GGC
B (surface probe)	GCC AGC TTA TTC AAC TAG
B′ (DNA complement)	CTA GTT GAA TAA GCT GGC
B″ (RNA complement)	CUA GUU GAA UAA GCU GGC
EC (surface probe)	GTC CCC CTC TTT GGT CTT GC
EC″ (16S rRNA, *E. coli*)	1.5-kb rRNA

[a]Probe DNA is modified with a 5′-thiol modifier C6 and chemically attached to the amine-terminated monolayer using the bifunctional linker SSMCC. A 15-T spacer is appended on the 5′ end of the probe DNA to allow for better hybridization. Note the slight difference between the DNA and RNA complements, where the thymine is replaced by a uracil. The sequence for the EC probe was designed to capture 16S rRNA isolated from *E. coli* based on a study that measured probe accessibility of various parts of the molecule.

Fresnel Calculations. Fresnel calculations were performed with an N-phase Fresnel calculation as outlined by Hansen[31] and available on our website, http://corninfo.chem.wisc.edu.

Near-Infrared SPR Imaging Apparatus. The details of SPR imaging using near-infrared excitation from an incoherent source were described previously.[20] Briefly, p-polarized light from a collimated white source (GWC Instruments) was passed through a narrow-band interference (band-pass) filter, illuminating the sample. An inexpensive CCD camera (GWC Instruments) was used to collect the images. Images were examined using the software package NIH Image on a microcomputer.

Hybridization Conditions. All hybridization experiments were conducted at 27 °C in a buffer of 20 mM phosphate, pH 7.7, 300 mM NaCl, 1 mM EDTA, and 100 mM urea.

RESULTS AND DISCUSSION

A. Hybridization Adsorption of DNA 18-Mer Oligonucleotides. SPR imaging can be used for the sequence-specific detection of small, unlabeled DNA molecules at low concentration using hybridization adsorption onto a surface array. Measurements of DNA hybridization have been performed previously using SPR

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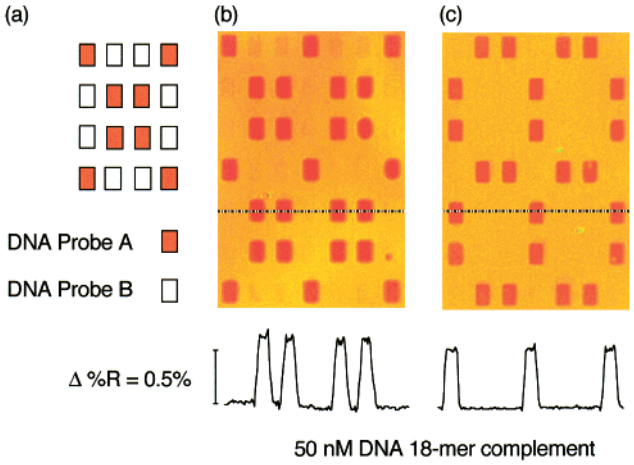

Figure 2. SPR image showing hybridization adsorption of 50 nM DNA 18-mer oligonucleotides onto a DNA-modified surface array. Hybridization adsorption onto the array is indicated by a change in the percent reflectivity of incident light. The pattern used for immobilization of single-stranded DNA probe sequences A and B is shown in (a). Hybridization adsorption occurs at perfect match spots after exposure to a 50 nM solution of DNA complement A′ for 30 min, as shown in (b). The surface is briefly denatured with 8 M urea, and exposed for 30 min to a 50 nM solution of DNA complement B′, resulting in the image seen in (c). Arrays were denatured and hybridized up to 25 cycles without a significant loss in signal or specificity. Plot profiles (below) taken across the dotted line in the fifth row show the excellent specificity and signal-to-noise ratio for both interactions. The resulting change in reflectivity (%*R*) upon hybridization for both arrays is ∼0.6%.

angle scan techniques,[2,4,26] and SPR imaging with DNA arrays,[1,3,5,32] but have previously required high concentrations and/or amplification of the target DNA to achieve sufficient signal. In previously published measurements of the hybridization of unlabeled DNA, target DNA concentrations of close to 1 μM are common.[2,4,5,26,32] Our measurements take advantage of the benefits of near-infrared excitation, which results in a sharper SPR minimum on gold and subsequently greater contrast in SPR imaging.[20] This is demonstrated in Figure 2, which shows the label-free detection of low concentration target DNA 18-mer oligonucleotides using a functionalized array. After exposing the array to a 50 nM solution of the DNA complement to the surface probe for 30 min, hybridization to the perfectly matched spots is clearly evident. This is shown in Figure 2b. Hybridization on the array is indicated by a change in the percent reflectivity of light at these spots only. After exposure to one complement, the surface is completely denatured by rinsing with a solution of 8 M urea. Exposure of the array to the other complement for 30 min results in the SPR image shown in Figure 2c. In this image, hybridization is again observed to the perfectly matched spots with excellent specificity.

To give quantitative information regarding binding onto each spot on the array, a line profile, like that shown in Figure 2c, can be produced by integrating the values of percent reflectivity across a linear or rectangular region. A line profile taken across the fifth row of the array (shown as a dotted line) shows quantitatively the change in percent reflectivity for both hybridization events. These plot profiles can be correlated with changes in percent reflectivity. Hybridization from a 50 nM solution results in an ∼0.6% change in the percent reflectivity. This value changes slightly (±0.1%) depending on surface chemistry and the exact angle at which the experiment is conducted. Note the excellent signal/noise ratio[33] at this low concentration of unlabeled target DNA, particularly for a short (18-base) complement oligonucleotide. Concentrations of DNA 18-mer as low as 10 nM were easily detected on this array with a signal/noise ratio close to 5 (data not shown). The number of molecules required to make a 0.6% change in the percent reflectivity, as seen in Figure 2, can be calculated from a separate fluorescence wash-off experiment. Briefly, fluorescently labeled target DNA is hybridized onto a surface from a 50 nM DNA solution and denatured, and the denatured DNA is quantified using solution fluorescence measurements. Based on this measurement, the density of target DNA hybridized onto the surface is found to be $\sim 1 \times 10^{12}$ molecules/cm^2 at 50 nM. This corresponds to 4 fmol of material adsorbing onto each 500 × 500 μm spot.

A major advantage of SPR imaging over conventional SPR is the ability to create built-in controls to distinguish between specific and nonspecific surface interactions. For example, the amount of DNA mismatch binding (e.g., binding of target A′ to probe spot B) can be used to monitor nonspecific interactions. With this in mind, one can easily optimize such things as buffer composition and surface chemistry for maximum discrimination. The results of this optimization are shown in Figure 2c, where the plot profile shows that nonspecific adsorption to the mismatched spot is undetectable for the hybridization of both sequences. Even at high concentrations of complement DNA, binding to mismatch spots is negligible. The degree of hybridization specificity, or stringency, can be affected by temperature, salt concentration, pH, and the presence of denaturants such as urea or formamide. For our experiments, a solution of 300 mM NaCl, 20 mM phosphate buffer at pH 7, 20 mM EDTA, and 100 mM urea resulted in the highest amount of hybridization without sacrificing specificity. At this stringency, increasing salt concentration resulted in more adsorption to both the matched and mismatched probe spots for DNA hybridization. A decrease in salt concentration resulted in less hybridization to the perfect match. Where discrimination is needed between more closely matched sequences, more stringent conditions may be necessary. A major advantage of the covalent attachment of thiol-DNA to our surfaces is the robust response to multiple DNA hybridization and urea denaturing cycles without loss of signal or specificity. These surfaces were hybridized and denatured for more than 25 cycles without a significant loss in signal or specificity.

B. Hybridization Adsorption of RNA. 1. Synthetic RNA 18-Mer Oligonucleotides. In addition to the detection of DNA, SPR imaging can also directly detect the hybridization of RNA onto a DNA surface array. One possible application for this is the measurement of the expression levels of specific genes in an organism. Here, we show that SPR imaging can detect the specific hybridization adsorption of small unlabeled RNA oligonucleotides onto DNA arrays at low concentrations under the same conditions used for the detection of DNA. This is shown in Figure 3, in which hybridization adsorption of 25 nM RNA 18-mer oligonucleotides to a three-component array is demonstrated. The complements

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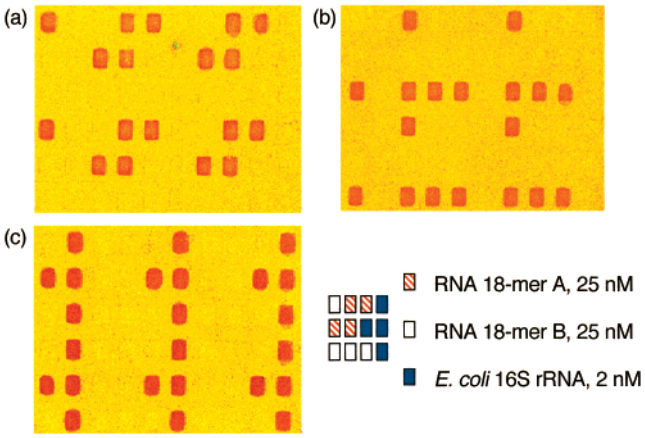

Figure 3. SPR image showing the hybridization adsorption of 25 nM RNA 18-mer oligonucleotides onto a three-component array using RNA sequences (A″ and B″) analogous to the complement DNA used in Figure 2. This is shown in (a) and (b). Probe DNA was spotted onto the surface in geometric patterns for easy recognition of a specific sequence (inset). Figure 3c shows the hybridization adsorption of *E. coli* 16S rRNA from a 2 nM solution onto the same oligonucleotide array. In this case, rRNA (containing 16S, 23S, and 5S rRNA) isolated from *E. coli* was boiled in buffer solution for 10 min and exposed to the array for 30 min. The approximate size of the 16S rRNA is 1.5 kilobases.

of the RNA 18-mers (sequences A″ and B″) are analogous to the DNA complements used in the experiment shown in Figure 2. The exact sequences are outlined in Table 1. For the results shown in Figure 3, probe DNA was spotted onto the surface in geometric patterns for easy recognition of hybridization of a specific sequence (patterns are shown in the inset of Figure 3). Even at 25 nM, the signal/noise ratio is high enough for easy recognition of hybridization adsorption. Note also the high degree of specificity for the hybridization adsorption of the RNA complements. Unlike the target DNA oligonucleotides, RNA samples were not purified by HPLC in order to keep the handling of these samples at a minimum before injection into the SPR cell. Both RNA 18-mer and DNA 18-mer binding experiments were conducted at the same solution stringency; however, RNA 18-mer binding onto the DNA array showed a higher degree of specificity than the binding of the DNA 18-mers when the solution stringency was decreased. To our knowledge, this is the first demonstration of the label-free detection of RNA oligonucleotides using DNA probes on a surface. Previous authors have demonstrated the direct detection of the binding of RNA molecules to a surface using peptide nucleic acid (PNA) probes with SPR in a nonarray format.[26]

2. Detection of Naturally Occurring 16S rRNA. One potential application for the label-free detection of RNA is the identification of species based upon 16S rRNA. Researchers have shown that variable regions of microbial 16S rRNA can be used as genetic markers for specific species.[22] These same genetic markers can be used on DNA arrays for the detection of microbial 16S rRNA with SPR imaging. Here, we describe the binding of 16S rRNA isolated from *E. coli* using a specially designed DNA probe. In Figure 3c, the SPR image of the hybridization adsorption of *E. coli* 16S rRNA onto a DNA array is shown. In this experiment, a solution of rRNA containing a mixture of 16S, 23S, and 5S rRNA was used, with the concentration of 16S rRNA estimated to be 2 nM. In the case of 16S rRNA and other large target nucleic acids, special considerations must be made to deal with secondary structure, slower hybridization kinetics, and sequence accessibility. The sequence for the probe was chosen on the basis of both accessibility to hybridization[30] and its uniqueness to *E. coli* according to a database of rRNA sequences at GenBank (http://www.ncbi.nlm.nih.gov). The 16S rRNA molecule isolated from *E. coli* is ~1500 bases in length and is replete with secondary structure, which complicates hybridization to DNA probes. The most common approach to this problem is fragmentation of the target molecule, preferably to a size close to that of the oligonucleotide sequence on the array.[33] We have found that denaturing the rRNA prior to introduction to the array can also alleviate problems caused by secondary structure. In this case, ribosomal RNA isolated from *E. coli* is boiled in buffer solution for 10 minutes and exposed to the array for 30 min. Gel electrophoresis indicates the molecule remains intact after boiling. The adsorption of larger molecules onto the surface produces a greater change in the index of refraction and increases the change in the percent reflectivity as measured by SPR imaging. Note that the signal generated for a 2 nM solution of the large molecule is comparable or greater than that generated by a 25 nM solution of RNA 18-mer (a 2 nM solution of the RNA 18-mer oligonucleotide complement to the probe DNA was found to be under our S/N threshold of 3). Experiments with rRNA fragmented in the presence of Mg^{2+} also show binding to the surface with no need for boiling the sample prior to its introduction. No binding was observed when unfragmented rRNA was introduced to the DNA array without boiling. Hybridization of the large RNA molecules could also be controlled on the array by changing the stringency of the solution. Reduced salt concentration resulted in a reduction of binding, and increasing the salt concentration resulted in nonspecific binding to the surface.

C. Fresnel Calculations. While SPR imaging has been shown adept at detecting the presence or absence of an analyte, a complete discussion of its ability to quantify adsorption is now presented. Here, we use a five-phase Fresnel calculation to show that changes in the surface coverage relate linearly to changes in the percent reflectivity. Fresnel calculations have been shown to closely match data for a variety of wavelengths and surface modifications.[2,18,20,34,35] In these calculations, small changes in the thickness or index of refraction of a thin film at the gold surface can be used to accurately model the adsorption of molecules onto the surface. To quantitatively model DNA hybridization at a surface, we first assume that the change in the index of refraction due to hybridization adsorption is proportional to the number of molecules adsorbing onto the surface.[2,36] Next, we use a five-phase Fresnel calculation to show that these changes in the index of refraction at the surface result in a linear change in the percent reflectivity over a limited range for excitation at 794 nm. The structure of the five-phase model used in the calculation is shown in Figure 4. Each theoretical phase is denoted by its index of refraction label and listed as follows: SF-10 glass (n_1), a 45.0-nm gold layer (n_2), a 2.0-nm self-assembled monolayer (SAM)

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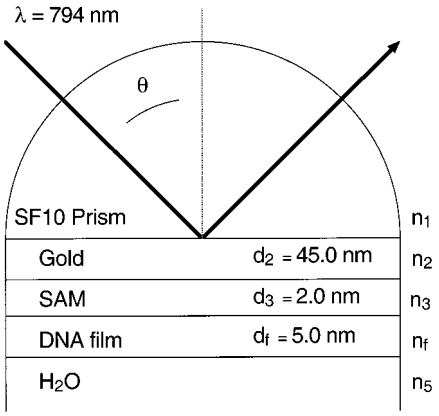

Figure 4. Five-phase model used for Fresnel calculations. Each theoretical phase is denoted by its index of refraction label and listed as follows: SF-10 glass (n_1), a 45.0-nm gold layer (n_2), a 2.0-nm self-assembled monolayer (SAM) consisting of a packed monolayer of the maleimide-modified alkanethiol (n_3), a 5.0-nm layer of immobilized DNA where hybridization occurs (n_f), and water (n_5). Adsorption onto the surface, such as hybridization of DNA on the surface, can be modeled by changing the index of refraction of the DNA thin film (n_f).

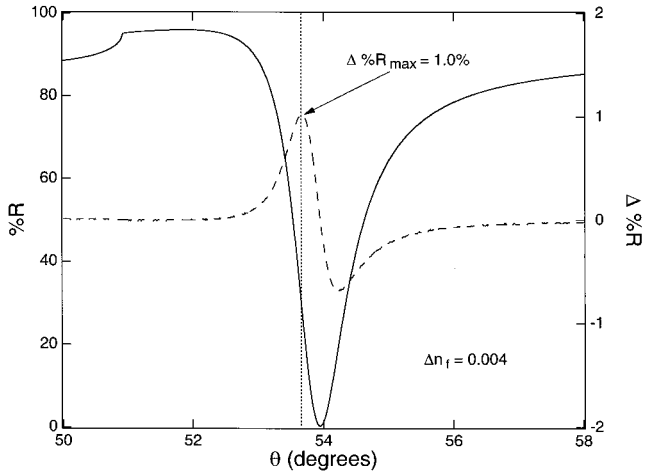

Figure 5. Theoretical reflectivity versus angle plot for a five-phase Fresnel calculation with 794-nm excitation prior to adsorption (solid line). Fresnel calculations have been shown to closely match data for a variety of wavelengths and thin films on gold. After adsorption, the curve shifts slightly, and the difference between the two theoretical curves is plotted on an expanded scale as a dashed line. The vertical-dotted line marks the angle where the maximum change in percent reflectivity (%R) occurs; this is the optimal angle for SPR imaging.

composed of a packed monolayer of the maleimide-modified alkanethiol (n_3), a thin 5.0-nm layer of immobilized DNA and water where hybridization occurs (n_f), and bulk water (n_5). Adsorption onto the surface by the hybridization of complementary DNA molecules is modeled as a change in n_f, the index of refraction of the thin DNA film. The initial value of n_f is chosen to be 1.4, but results do not change significantly if an initial value between 1.3 and 1.5 is chosen. Specifically, the hybridization adsorption event observed by SPR imaging in Figure 2 is modeled by a change in n_f of 0.004, or 0.3%. This change in n_f results in changes in the reflectivity. Figure 5 shows a theoretical plot of reflectivity versus angle for the five-phase model system with 794-nm excitation prior to adsorption (solid line). After adsorption, this curve shifts slightly, and the difference between the two theoretical curves is

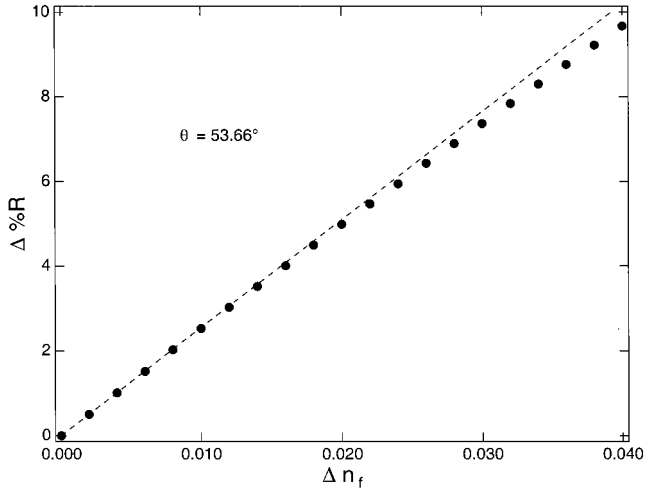

Figure 6. Theoretical change in percent reflectivity (Δ%R) for a range of changes in the index of refraction, n_f, of the DNA thin film at the optimal imaging angle, 53.66°. For (Δ%R) of less than 5%, a linear relationship to changes in the index of refraction is evident. The linear response of Δ%R over a limited range of Δn_f means that changes in %R can also be related to the number of molecules adsorbing onto the surface. This linear relationship is due to the shape of the SPR minimum close to the optimal imaging angle (Figure 5), which is very close to a straight line. Actual data from the hybridization adsorption of DNA and RNA molecules result in a maximum 1% change in the percent reflectivity, well within the linear range of this sensor.

plotted on an expanded scale as the dashed line in Figure 5. Two major changes are observed: (i) the angle of the surface plasmon resonance minimum, θ_{SPR}, shifts by 0.007°, and (ii) the change in percent reflectivity ($\Delta\%R_{max}$) as a function of θ (the dashed line in Figure 5) reaches a maximum of 1% at 53.66° (denoted by the vertical dotted line in Figure 5). This maximum always occurs at an angle below θ_{SPR} and is the optimal position for the fixed angle SPR imaging measurements.

To use SPR imaging as a quantitative adsorption sensor, it is important to show that changes in percent reflectivity are linearly proportional to changes in the index of refraction, n_f and, thus, to the amount of molecules adsorbed on the surface. Figure 6 shows the calculated change in percent reflectivity for a range of changes in n_f at the optimal imaging angle, 53.66°. For changes in reflectivity (Δ%R) of less than 5%, a linear relationship to changes in the index of refraction is evident. This linearity is due to the shape of the SPR minimum close to the optimal imaging angle (Figure 5), which is very close to a straight line. A similar linear relationship can be demonstrated for SPR instruments that rely on measurements of the shift in θ_{min}. For example, Fresnel calculations show that angle shift measurements taken at 794 nm are linear for changes in θ_{min} of less than 0.05°.

D. Adsorption Isotherms. Since the changes in percent reflectivity in the SPR imaging experiment are proportional to the amount of molecules adsorbed onto the surface, data from these measurements can be used to quantitatively determine the concentration dependence of DNA hybridization on a surface. This is accomplished by integrating the change in reflectivity on each probe spot due to hybridization at various DNA concentrations. These data are plotted in Figure 7 (top), where the signal resulting from the hybridization of a DNA 18-mer oligonucleotide onto the array is plotted as a function of the solution DNA concentration

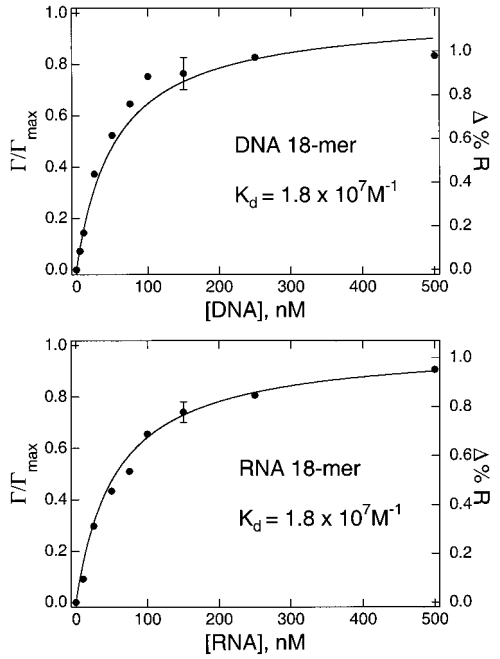

Figure 7. Experimental results showing an adsorption isotherm for the hybridization adsorption of DNA (top) and RNA (bottom) 18-mer oligonucleotides onto a DNA array for concentrations ranging from 5 to 500 nM complement DNA or RNA. The signal upon hybridization is calculated by integrating line profiles for spots containing the perfect match and normalizing the signal. After each hybridization at a given concentration, the surface is denatured with 8 M urea. A solid line shows the result of the calculated Langmuir adsorption isotherm. The calculated adsorption coefficient for the hybridization adsorption of both DNA and RNA onto the surface was found to be 1.8×10^7 M^{-1}. Error bars represent uncertainty for all data points.

from 5 to 500 nM. The signal upon hybridization was calculated by integrating line profiles for several of the spots containing the perfect match and normalizing the signal to reflect the change in the percent reflectivity upon adsorption. After each hybridization at a given concentration, the surface was denatured with 8 M urea and a solution of new concentration was introduced. The data in Figure 7 (top) was fit using a Langmuir adsorption isotherm and yielded an adsorption coefficient, K_{ads}, of 1.8×10^7 M^{-1}. From this constant, the concentration that corresponds to 50% relative surface coverage is found to be 55 nM. This result equates well with measurements made by Jensen et al., who reported a value of 25 nM from kinetic measurements of the k_a and k_d of unlabeled DNA 15-mers at 20 °C using angle shift SPR.[26] At higher concentrations, the signal levels off due to filling of adsorption sites on the surface, not from saturation of the SPR signal. A slight variation from ideal Langmuir behavior in the DNA isotherm is noted near 100 nM. This variation has been shown to be repeatable in several experiments.

Adsorption isotherms were also constructed for the RNA 18-mer oligonucleotides under the same conditions as for the DNA isotherms and are shown in Figure 7 (bottom). The calculated adsorption coefficient for RNA hybridization adsorption was found to be approximately the same as for DNA hybridization, 1.8×10^7 M^{-1}. The similar results for DNA and RNA hybridization here may be due to the use of identical solution stringency for both experiments. RNA hybridization could be increased without increasing the amount of nonspecific adsorption, yielding a higher adsorption coefficient for the RNA/DNA interaction while maintaining an excellent degree of specificity.

CONCLUSIONS

Detection of the hybridization adsorption of short (18-base), unlabeled RNA and DNA oligonucleotides onto DNA arrays at concentrations as low as 10 nM has been observed using near-infrared SPR imaging. This detection limit corresponds to less than 1 fmol of adsorbed oligonucleotides for each 500×500 μm square spot on an array. This makes detection of as low as 1 pmol of target DNA possible in a 100-μL SPR imaging cell. Preliminary results in our laboratory show that detection of DNA hybridization adsorption is possible by individually spotting array elements with as little as 40 nL of target DNA. This permits the label-free detection of femtomole quantities of DNA for 500×500 μm spots. For smaller features, such as 50×50 μm squares, detection of attomole quantities is possible with this technique.

The detection of the hybridization adsorption of 16S ribosomal RNA onto a DNA array with SPR imaging has also been demonstrated using a surface probe specific to *E. coli*. The direct detection of 16S rRNA is currently being investigated in our laboratory as an efficient tool for the identification and differentiation of microbial populations from environmental samples on a single array. Studies of other large RNA molecule interactions using SPR imaging may also prove to be important in the future.

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