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Accelerated Articles

Enzymatically Amplified Surface Plasmon Resonance Imaging Method Using RNase H and RNA Microarrays for the Ultrasensitive Detection of Nucleic Acids

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A novel surface enzymatic amplification method that utilizes RNA microarrays in conjunction with the enzyme RNase H is developed for the ultrasensitve detection and analysis of target DNA molecules. The enzyme RNase H is shown to selectively and repeatedly destroy RNA from RNA−DNA heteroduplexes on gold surfaces; when used in conjunction with the label-free technique of surface plasmon resonance imaging, multiple DNA targets can be detected at a concentration of 10 fM on a single chip. In addition, this method is utilized for the sequence-specific detection of the TSPY gene in both purified and unpurified PCR products. Finally, in a series of kinetics measurements, the initial rate of hydrolysis is shown to depend directly on the surface concentration of DNA−RNA heteroduplexes.

DNA microarrays provide a powerful platform for the analysis of nucleic acids in a multiplexed format and have become an indispensable tool for gene expression analysis,[1−3] viral[4,5] and microbial identification,[6,7] and clinical diagnostic studies.[8,9] For many of these applications, however, the target nucleic acid concentration is too low for direct detection by any spectroscopic method. To increase sensitivity, either enzymatic amplification methods such as PCR are employed prior to exposure to the array[4,7] or coupled enzymatic labeling methods such as an ELISA sandwich assay are used after target adsorption.[10,11] Unfortunately, both of these enzymatic amplification methods are not easily adapted to a multiplexed array format. For example, PCR often will not faithfully reproduce the relative concentrations of nucleic acid sequences from complex mixtures, and the enzymatic product detected in ELISA measurements is a solution species that can diffuse into adjacent array elements.

To replace these enzymatic methods with a process more amenable to the microarray format, we recently introduced a novel surface enzymatic amplification process using the enzyme RNase H to greatly enhance nucleic acid detection using RNA microarrays.[12] This new enzymatic amplification method is especially useful when utilized in conjunction with the technique of surface plasmon resonance (SPR) imaging, which has a 1 nM direct

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detection limit for DNA when used in a microarray format.[13] Using this enzymatic amplification technique, the sensitivity of SPR imaging was enhanced to a 1 fM detection limit. In addition, the RNase H amplification method could be used to detect sequences in longer DNA fragments and was shown to have sufficient sensitivity to directly identify and detect sequences in genomic DNA samples.[12]

In this paper, we describe experiments which demonstrate in detail that this RNase H surface enzymatic amplification method can be used in a multiplexed array format for the direct detection of DNA oligonucleotides with SPR imaging at femtomolar concentrations, and we extend the use of this method to the detection of unpurified PCR products. Moreover, we show that the RNase H surface enzyme kinetics in this amplification process can be directly related to the target nucleic acid concentration through the adsorption isotherm of the DNA−RNA surface heteroduplex.

EXPERIMENTAL SECTION

Materials. 11-Mercaptoundecylamine (MUAM; Dojindo), *N*-hydroxysuccinimidyl ester of methoxypoly(ethelene glycol) propionic acid MW 2000 (PEG-NHS; Nektar), sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1carboxylate (SSMCC; Pierce), 9-fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide (Fmoc-NHS; Novabiochem), and ribonuclease H (RNase H; Takara) were all used as received. All rinsing steps were performed with absolute ethanol and Millipore filtered water. Sterilized Tris buffer (50 mM Tris-HCl, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM DTT, pH 7.8) was used for all RNase H experiments.

RNA and DNA Array Fabrication. A multistep fabrication process, described previously,[14] was used in the creation of RNA microarrays for SPR imaging experiments. Thin gold films (45 nm) with an underlayer of chromium were deposited onto SF-10 glass (Schott Glass) using a Denton DV-502A metal evaporator. The gold substrates were modified with a self-assembled monolayer of an amine-terminated alkanethiol MUAM. The amine-terminated surface was then modified with the hydrophobic protecting group Fmoc-NHS. A quartz mask containing 500 μm × 500 μm features was used in conjunction with UV photopatterning to create areas of bare gold within the hydrophobic background. The areas of bare gold were filled with an amine-terminated self-assembled monolayer, and these spots were reacted with the heterobifunctional cross-linker SSMCC to form a thiol-reactive maleimide-terminated surface. Thiol-modified sequences of RNA were then spotted into these hydrophilic array elements using a pneumatic picopump. Next, the Fmoc background was removed with a mildly basic solution to regenerate the amine-terminated surface. This surface was then reacted with PEG-NHS to create a background that is resistant to the nonspecific adsorption of biomolecules. The surface coverage of the RNA monolayer was estimated to be ~1×10^{12} molecules/cm^2. The RNA microarrays were used immediately and were active throughout the course of the experiments. The RNA arrays were shown to be stable upon exposure to air, but their long-term stability was not tested.

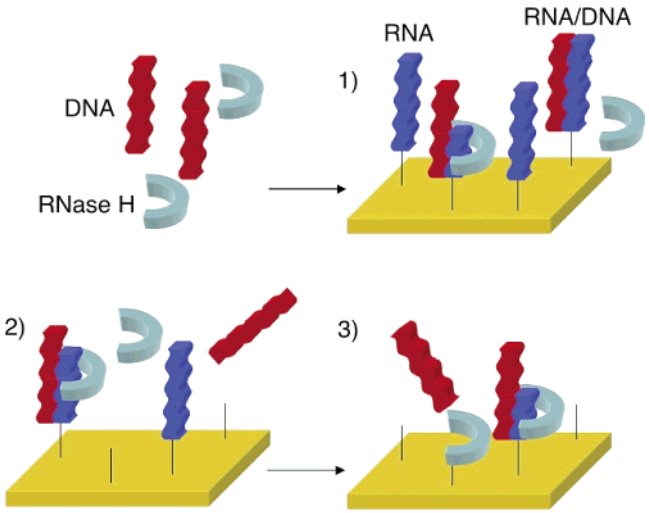

Figure 1. Schematic representation of a surface enzymatic reaction for the amplification of SPR signal by the selective removal of RNA probes from the array surface.

RNA and DNA Oligonucleotides. All RNA oligonucleotides were commercially obtained from Dharmacon RNA Technologies. These 5′-thiol-modified RNA oligonucleotides were received gel-purified and deprotected. The RNA oligonucleotides used in these experiments are as follows: $R_1 = P_3 =$ 5′HS$(CH_2)_6$(U)$_8$GUU CUC CGC UUC GAU AAC UC, $R_2 = P_4 =$ 5′HS$(CH_2)_6$(U)$_8$AAG GGG CAG CAA UCA CAC UC, $R_3 = P_5 =$ 5′HS$(CH_2)_6$(U)$_8$CGU UUU GGG GCA UCU UUU UG, $P_1 =$ 5′HS$(CH_2)_6$(U)$_8$GCA AGC CCC ACC UAG ACC GCA GAG, and $P_2 =$ 5′HS$(CH_2)_6$(U)$_8$UCG CCU CCC CGU CCC CGU AAA CUA. U_8 serves as a spacer to make the RNA sequence accessible to DNA hybridization and RNase H reaction. All complementary DNAs used in these experiments were RNase-free HPLC purified and obtained commercially from Integrated DNA Technologies (IDT).

Kinetic Flow Cell Design. A microfluidics system designed for the continuous delivery of small volumes of sample RNA onto a array surface for kinetics measurements has been described previously.[15] Briefly, a flow cell was created by placing a PDMS microchannel (670-μm width, 9.5-cm length, 200-μm depth) with a 13-μL total volume in direct contact with the sample surface. Careful alignment of the microchannel onto the chip surface was necessary in order to cover each 500 μm × 500 μm array element. Prior to assembly onto the array surface, the PDMS microchannels were exposed to oxygen plasma for 15 s to improve their hydrophilicity and reduce biomolecular adsorption to the walls of the channels. A constant-temperature sample holder was mated to the microfluidics in order to limit fluctuations in SPR signal over time due to temperature variations. The constant-temperature cell was fabricated in two halves: (a) Half contained an inlet and outlet system, which was mated to the microfluidics for sample delivery to the array surface, and (b) the other half was used to secure the prism to the backside of the array surface with enough pressure to seal the PDMS microfluidics to the array surface. Temperature control of the flow cell was achieved by recirculating water from a constant-temperature bath through a water jacketing system fabricated into both halves of the sample holder. Solutions

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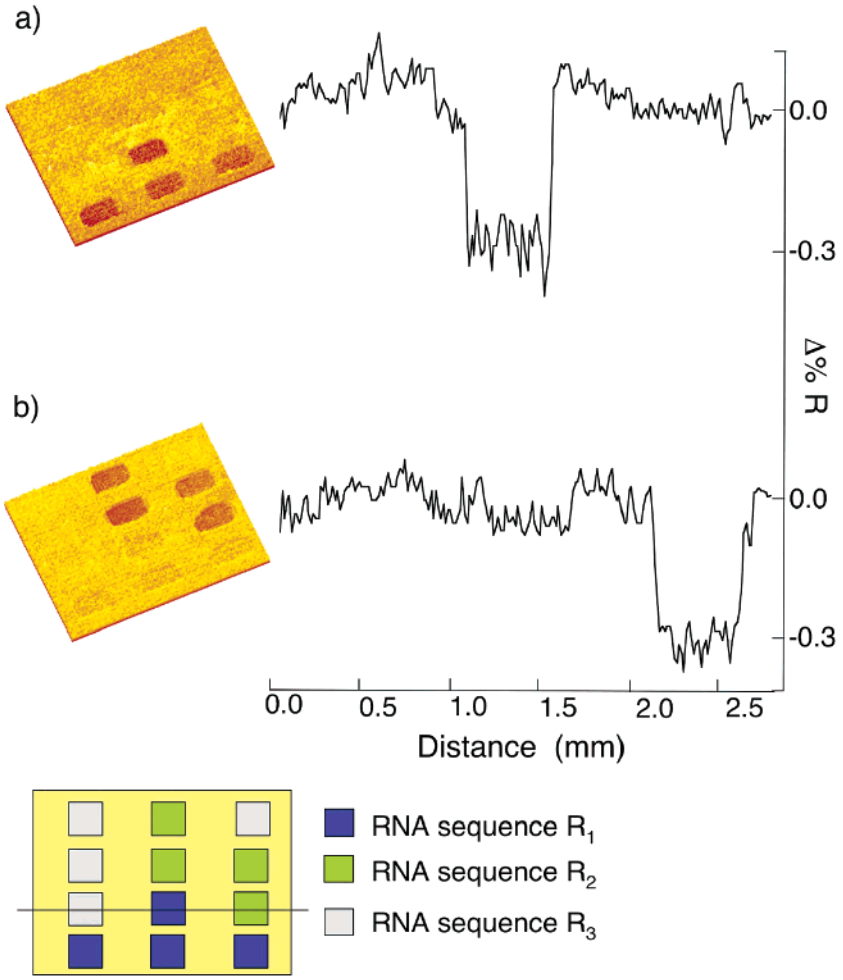

Figure 2. Three-component array created using noninteracting thiol-modified RNA sequences R_1, R_2, and R_3. (a) An SPR difference image was obtained by subtracting images taken before and after the array had been exposed to a 10 fM DNA solution complementary to R_1 and the enzyme RNase H. A line profile was taken across the image as drawn on the pattern in the bottom part of the figure. The line profile shows a decrease in percent reflectivity corresponding to DNA hybridization and the subsequent hydrolysis of the complementary RNA probes by RNase H, while no changes for the other array elements are observed. The array was then washed with 8 M urea to denature the surface. (b) An SPR difference image showing the sequential detection of a different 10 fM DNA solution (D_2) onto the same array surface. A line profile taken across the image shows a decrease in percent reflectivity is obtained for this binding event. Approximately 2 h was required to achieve a change in percent reflectivity of −0.3% in both (a) and (b).

were delivered to the array surface using a syringe pump at a constant flow rate of 20 μL/min.

Real-Time SPR Imaging Measurements. An SPR imaging apparatus (GWC Technologies)[16] using near-infrared excitation from an incoherent light source was used for the real-time monitoring of RNA hydrolysis by RNase H. Briefly, collimated p-polarized light impinges on a sample assembly at a fixed angle. The light reflected from this sample assembly is passed through a narrow band-pass filter centered at 830 nm and collected using a CCD camera. The data are collected using the software package V++ (Digital Optics). Custom macros were written using this software so that data could be collected at specific user-designated regions of interest (ROIs) on the array surface. The processes for collecting time-dependent data utilizing these ROIs can be found elsewhere.[15] All kinetics experiments presented in this paper were obtained by collecting one data point every ~5 s that was the average of 30 camera frames. The difference in percent reflectivity for each of the probe areas was normalized to the average change in percent reflectivity measured for the PEG background and negative control ROIs. Kinetic data from multiple array elements were averaged to obtain the final kinetic curves. Microsoft Excel and Igor Pro were used for all data processing and kinetic model fitting in these experiments.

PCR Product Preparation. PCR primers were commercially obtained from IDT and purified using reversed-phase binary gradient elution HPLC. These primers were selected to amplify the TSPY gene located on the human Y chromosome using the sequence obtained from Genbank with accession number AF106331. The selected primers are located at positions 1206 and 1749 of the TSPY gene, and their binding specificity was confirmed using the BLAST program (http://www.ncbi.nl.nih.gov/blast/), against the Human Genome Sequence Database. These sequences are as follows: 5′GCA AGC CCC ACC TAG ACC GCA GAG and 5′TCG CCT CCC CGT CCC CGT AAA CTA. PCR was performed with 250 ng of male genomic DNA (Promega), 0.2 mM dNTP, 50 pmol each of TSPY gene primer, one Hot Start Polymerase

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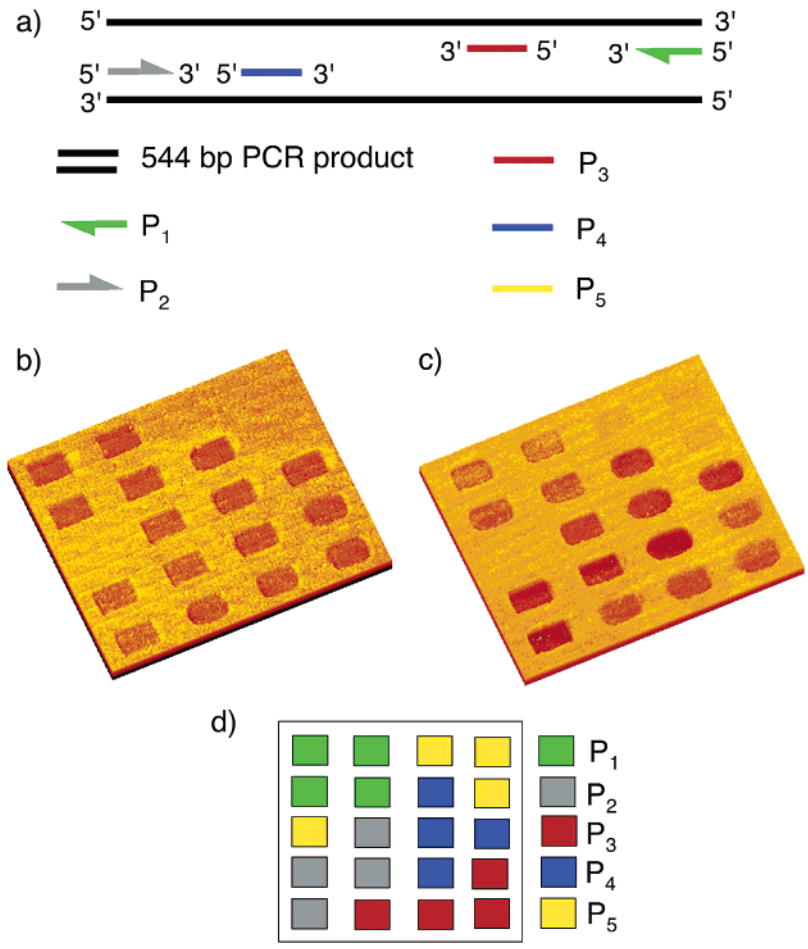

Figure 3. Five-component array created using thiol-modified RNA sequences (P_1, P_2, P_3, P_4, P_5). Probes P_1, P_2, P_3, and P_4 were designed specifically to bind to the TSPY PCR product, and probe sequence P_5 was designed as a negative control. (a) Schematic showing the relative binding sites for probe molecule hybridization onto the PCR product. (b) SPR difference image obtained after ~30 min exposure to a 100 pM solution of purified PCR product and RNase H. A decrease in percent reflectivity was observed for the array elements designed to bind to the PCR product, while no changes in percent reflectivity were observed at the negative control array elements. (c) SPR difference image obtained by subtracting images taken before and after the array was exposed to a 100 pM unpurified PCR product solution and the enzyme RNase H for 30 min. A decrease in percent reflectivity was observed for the array elements designed to bind to the PCR product.

TaqBead (Promega), 5 mM $MgCl_2$, and 1× thermophilic DNA polymerase reaction buffer (Promega) in a 50-μL reaction volume. The amplification reaction consisted of a 5-min cycle at 95 °C followed by 30 cycles of 1 min at 95 °C, 1 min annealing at 58 °C, 1 min of primer extension at 72 °C, and a final extension at 72 °C for 5 min performed on a thermal cycler (MJ Research, model PTC-100). PCR products were purified using the Wizard PCR Prep PCR purification system (Promega).

RESULTS AND DISCUSSION

Surface Amplification Process. An overview of the RNase H surface enzymatic amplification method is shown in Figure 1. A single-stranded RNA (ssRNA) microarray is exposed to a solution containing both complementary DNA and RNase H. The DNA will bind to its RNA complement on the surface and form an RNA−DNA heteroduplex (step 1). RNase H will then bind to this heteroduplex, selectively hydrolyze the RNA probe, and then release the DNA complement back into solution (step 2). The released DNA molecule is then free to bind to another RNA probe on the surface, so that a single DNA molecule can initiate the destruction of many surface-bound RNA probes (step 3). If this process is allowed to proceed for a sufficient amount of time, all of the RNA probe molecules will be destroyed and removed from the surface. The irreversible loss of RNA probe molecules from the surface can be detected with SPR imaging by a decrease in percent reflectivity at array elements where RNA−DNA heteroduplexes are formed. This enzymatic process does not affect array elements that contain ssDNA, dsDNA, ssRNA, or dsRNA as they are not suitable substrates for RNase H. The sequence selectivity of this amplification technique thus arises from the hybridization of the RNA−DNA heteroduplex.

Sequence-Specific Ultrasensitive Detection of DNA Oligonucleotides. The detection of two different sequences of DNA at concentrations of 10 fM was demonstrated on a single three-component RNA array to illustrate the potential use of this technique for multiplexed analysis. Two target DNA sequences D_1 and D_2 were studied with an RNA array that contained the three noninteracting complementary RNA sequences denoted as R_1, R_2, and R_3. Figure 2a shows the SPR difference image obtained by subtracting the images taken before and after the array was exposed to a 10 fM D_1 DNA solution and RNase H. A decrease in percent reflectivity of −0.3% was observed for the R_1 RNA array

elements due to the removal of probes from the surface. Nonspecific adsorption of the enzyme to the background or other array elements was not observed. This array was then exposed to a solution of 8 M urea to denature any remaining complementary DNA from the array elements. The array was then exposed to a 10 fM solution containing DNA D_2 and RNase H. The removal of the R_2 probes from the array surface was observed as a decrease in percent reflectivity of −0.3% in the SPR difference image shown in Figure 2b. A very small decrease (< 0.03 Δ%*R*) of residual RNase H activity was observed for the previously hydrolyzed R_1 array elements. With 50-μm array elements, this experiment can be scaled up to a DNA array density of 10^4 sequences/cm^2.

Direct Detection of PCR Products. In addition to short oligomers, this enzymatic amplification technique can be applied to the analysis of DNA sequences present in longer DNA molecules. In our previous report, we showed that sequences in fragmented human genomic DNA samples could be detected directly with the RNase H enzymatic amplification method. Here we use this method for the multiple sequence detection and identification of a PCR-amplified 544-bp portion of the testis-specific protein Y (TSPY) gene taken from a human genomic DNA sample.

A five-component RNA array (P_1, P_2, P_3, P_4, P_5) was constructed with probe sequences P_1, P_2, P_3, and P_4 designed to specifically bind to the TSPY PCR product and P_5 designed as a negative control sequence. The binding positions for each of these probe molecules onto the PCR product is shown in Figure 3a. The RNase H enzymatic amplification process was utilized to detect a 100 pM solution of purified PCR product, which was heated to 95 °C for 5 min and then mixed with RNase H prior to exposure to the array surface. The SPR difference image in Figure 3b shows a decrease in percent reflectivity for all of the probes specific to the PCR fragment, with negligible nonspecific adsorption to the negative control probe or the background surface. No change in the percent reflectivity was observed for any of the array elements in the absence of RNase H.

The same five-component array format was then used to detect a 100 pM solution of *unpurified* TSPY PCR product. Figure 3c shows the resulting difference image. As in the case of the purified PCR product, a significant decrease in percent reflectivity was observed for the four probes P_1−P_4, along with a small amount of nonspecific adsorption to the negative probe P_5 and the background. The presence of polymerase and other reagents in the unpurified sample did not significantly effect the identification of the PCR fragment.

Concentration Dependence and Surface Enzyme Kinetics. The SPR signal detected in this experiment is due to the hydrolysis and removal of surface-bound RNA molecules from a given array element. The rate of this hydrolysis depends on both concentration and time. A series of kinetics measurements were performed to demonstrate that the initial rate of hydrolysis depends directly on the surface concentration of DNA−RNA heteroduplexes. This surface concentration is linked to the target DNA concentration in solution by the Langmuir adsorption isotherm.

A plot of the real-time SPR signal obtained for the enzymatic hydrolysis of surface-bound RNA probes by RNase H in the presence of 10 nM, 1 nM, 100 pM, and 10 pM complementary DNA solutions (D_2) is shown in Figure 4. For all concentrations,

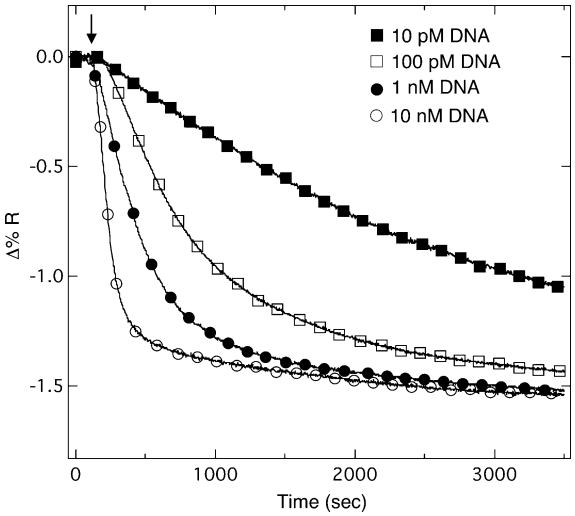

Figure 4. Time-dependent SPR data obtained for the hydrolysis of RNA array elements by RNase H at various concentrations of complementary DNA D_2. Starting at the time denoted by the arrow, a solution containing RNase H at a concentration of 0.06 unit/μL and DNA at concentrations of 10 nM (○), 1 nM (●), 100 pM (□), and 10 pM (■) were placed in contact with the array surface. The solid lines are the data, and the symbols are for identification of the different concentrations. The initial linear slope of these curves was used to extract an initial reaction rate for the enzymatic process.

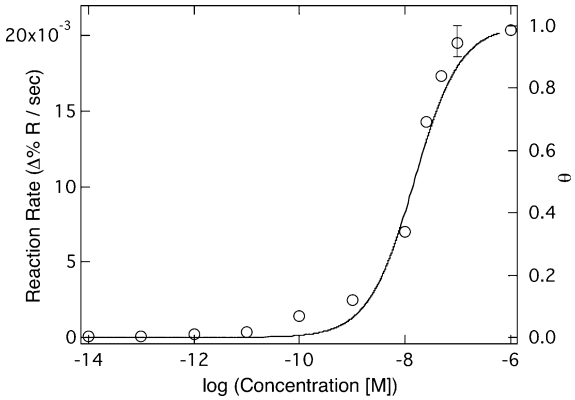

Figure 5. Initial reaction rates obtained from the time-dependent measurements as a function of DNA concentration. These data were normalized and fit to a Langmuir isotherm (solid line). From this fit, a K_{Ads} of 6.6×10^7 M^{-1} was determined.

an immediate decrease in Δ%*R* is observed due to the removal of RNA probes from the surface. In all cases, the final change in percent reflectivity is −1.5% (not shown for 10 pM); however, the rate of reaction slows significantly as the solution concentration of DNA is decreased. A full kinetic analysis of the RNase H surface reaction can be obtained from this time-dependent data. However, in this paper, we will only look at the concentration dependence of the initial reaction rate as determined by the initial slope of the data in Figure 4.

A plot of this initial reaction rate as a function of complementary DNA concentration is shown in Figure 5. The form of the concentration dependence appears to have the same shape as the concentration dependence of the fractional surface coverage for an adsorbing species, θ, as predicted by the Langmuir isotherm model ($\theta = K_{Ads}C/(1 + K_{Ads}C)$). A fit of the data to this equation (solid line) yields an adsorption coefficient (K_{Ads}) of 6.6×10^7 M^{-1}. This number agrees with previously reported literature values for

oligonucleotide hybridization adsorption.[16−18] We can therefore assert that the initial surface enzyme reaction rate is directly related to the fractional surface coverage of the RNA−DNA heteroduplexes.

CONCLUSIONS

The surface amplification process based on RNase H and RNA microarrays is a simple yet extremely sensitive method for the detection of multiple DNA targets on a single chip. Any DNA sequence can be detected with this method, since the activity of RNase H is not significantly sequence-dependent.[19] Novel spectroscopic methods such as a modulated SPR technique[20] have been implemented recently to improve the SPR detection limit, but these methods cannot be used in an array format. In addition, a nanoparticle amplification method has been used to increase the sensitivity of SPR imaging of DNA microarrays to 1 pM,[21] but this technique requires a sandwich assay format similar to that used by fluorescence-based methods.[22,23] While in this paper we have used SPR imaging to detect the RNase H activity, other methods such as fluorescence or nanoparticle labeling can be incorporated into this amplification method.

A key component of the RNase H enzymatic amplification process is that a very small number of target DNA molecules can destroy a large number of surface RNA molecules. For example, at a DNA solution concentration of 10 fM, the Langmuir isotherm for DNA hybridization adsorption predicts a fractional surface coverage of 1×10^{-6}, corresponding to only ∼2500 DNA molecules adsorbed to an array element. Given sufficient time, this small number of molecules can hydrolyze enough RNA probes (1 fmol) to generate an observable SPR imaging signal.

The kinetics measurements described in this paper clearly demonstrate that this amplified detection process can be quantitatively related to the solution concentration of DNA target molecules through the RNA−DNA heteroduplex surface concentration. It should be noted that since this enzymatic method does not deplete the concentration of DNA target in solution, the reaction rate is not limited by DNA diffusion to the surface. At low concentrations, the rate of RNA−DNA hybridization will determine the overall reaction rate. A full analysis of the surface enzyme kinetics will be presented in a future paper.

Finally, a major strength of the RNase H enzymatic amplification process is that it can be used to detect DNA sequences in longer target molecules such as fragmented genomic DNA and PCR products. This method can be used for the rapid detection and identification of multiple sequences from unpurified PCR products, replacing the need for gel electrophoresis measurements of PCR product purity. We expect that the RNase H detection methodology will find many biosensing applications in the areas of biowarfare detection, gene expression analysis, and drug discovery.

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