A novel surface enzymatic amplification method that utilizes RNA microarrays in conjunction with the enzyme RNase H is developed for the ultrasensitive detection and analysis of target DNA molecules. The enzyme RNase H is shown to selectively and repeatedly destroy RNA from DNA–RNA 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 viral4,5 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 array5,6 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 detection limits.

A summary of the kinetic measurements and the results of multiple TSPY DNA amplifications are shown in Table 1. The addition of RNase H to the RNA target concentration Table 1. Kinetic Measurements and Results

<table>
<thead>
<tr>
<th>DNA Concentration (fM)</th>
<th>Hydrolysis Rate (M/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.015</td>
</tr>
<tr>
<td>100</td>
<td>0.15</td>
</tr>
<tr>
<td>1000</td>
<td>1.5</td>
</tr>
<tr>
<td>10 fM (4°C)</td>
<td>0.0015</td>
</tr>
</tbody>
</table>

The results in Table 1 indicate that the rate of hydrolysis is directly proportional to the target DNA concentration. This suggests that the enzyme RNase H is acting in a sequence-specific manner, selectively destroying the RNA component of the DNA–RNA heteroduplexes on the gold surface.

The successful performance of the enzymatic amplification technique in conjunction with surface plasmon resonance imaging suggests that this method could be used for the ultrasensitive detection of many other nucleic acid sequences in a multiplexed format. The potential applications of this technique are numerous, including the analysis of many of the applications mentioned earlier, as well as the detection of viral, bacterial, and fungal nucleic acids in environmental monitoring and diagnostic studies.

detection limit for DNA when used in a microarray format. 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.

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 (MUA; Dojindo), N-hydroxysuccinimidy ester of methoxypoly(ethylene glycol) propionic acid M W 2000 (PEG-NHS; Nektar), sulfo succinimidy 4(N-maleimidomethyl)cyclohexane-1-carboxylate (SSMCC; Pierce), 9-fluorenylmethoxy carbonyl-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 MgCl2, 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, 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 MUA. 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 protecting group Fmoc-NHS was removed and deprotected. The RNA oligonucleotides used in these experiments 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: R1 = P3 = 5′HS(CH2)6(U)8GUU CUC CGC UUC GAU AAC UC, R2 = P4 = 5′HS(CH2)6(U)8AAG GGG CAG CAA UCA CAC UC, R3 = P5 = 5′HS(CH2)6(U)8UCU UUU GGG GCA UCU UUU UG, P1 = 5′HS(CH2)6(U)8GCA AGC CCC ACC UAG ACC GCA GAG, and P2 = 5′HS(CH2)6(U)8UGC CCC CGU CCC CGU AAA CUA. U8 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 continuous delivery of small volumes of sample RNA onto a chip surface for kinetics measurements is described previously. 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

were delivered to the array surface using a syringe pump at a constant flow rate of 20 \( \mu \)L/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 \( \text{V}++\) (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 \( \sim 5 \) 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.nlm.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

![Figure 2](image-url)
TaqBead (Promega), 5 mM MgCl₂, 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 D₁ and D₂ were studied with an RNA array that contained the three noninteracting complementary RNA sequences denoted as R₁, R₂, and R₃. Figure 2a shows the SPR difference image obtained by subtracting the images taken before and after the array was exposed 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.
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 $8 \text{ M urea}$ to denature any remaining complementary DNA from the array elements. The array was then exposed to a $10 \text{ fM}$ solution containing DNA $D_2$ and RNase H. The removal of the $R_1$ 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 ($< 0.03 \Delta\%R$) of residual RNase H activity was observed for the previously hydrolyzed $R_1$ array elements. With $50\mu\text{M}$ 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 $544\text{ bp}$ 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 $100 \text{ pM}$ solution of purified PCR product, which was heated to $95 ^\circ \text{C}$ for $5 \text{ min}$ 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 $100 \text{ pM}$ 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 affect 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 $10 \text{ nM}$, $1 \text{ nM}$, $100 \text{ pM}$, and $10 \text{ pM}$ complementary DNA solutions ($D_2$) is shown in Figure 4. For all concentrations, an immediate decrease in $\Delta\%R$ 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 $10 \text{ pM}$); 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, $\theta$, as predicted by the Langmuir isotherm model ($\theta = \frac{K_{\text{Ads}}C}{1 + K_{\text{Ads}}C}$). A fit of the data to this equation (solid line) yields an adsorption coefficient ($K_{\text{Ads}}$) of $6.6 \times 10^7 \text{ M}^{-1}$. This number agrees with previously reported literature values for
oligonucleotide hybridization adsorption.\textsuperscript{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.\textsuperscript{19} Novel spectroscopic methods such as a modulated SPR technique\textsuperscript{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,\textsuperscript{21} but this technique requires a sandwich assay format similar to that used by fluorescence-based methods.\textsuperscript{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 $1 \times 10^{-6}$, 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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\textsuperscript{22} Nam, J.; Thaxton, C. S.; Mirkin, C. A. Science \textbf{2003}, \textit{301}, 1884–1891.