

Fabricating RNA Microarrays with RNA–DNA Surface Ligation Chemistry

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A novel surface attachment strategy that utilizes RNA–DNA surface ligation chemistry to create renewable RNA microarrays from single-stranded DNA (ssDNA) microarrays on gold surfaces is demonstrated. The enzyme T4 DNA ligase was used to catalyze the formation of a phosphodiester bond between 5'-phosphate-modified ssDNA attached to the surface and the 3'-hydroxyl group of unlabeled RNA molecules from solution in the presence of a complementary template DNA strand. Surface plasmon resonance imaging (SPRI) measurements were performed to characterize the ligation process as well as to verify the bioactivity of the ssRNA microarray in terms of (i) the hybridization adsorption of complementary DNA onto the RNA array to form a surface RNA–DNA heteroduplex and (ii) the hydrolysis of the RNA microarrays with either ribonuclease S or ribonuclease H (RNase H). The hydrolysis of the surface-bound RNA with RNase H required the presence of a surface heteroduplex and, upon completion, regenerated the original 5'-phosphate-terminated ssDNA array elements. These ssDNA array elements could be ligated again to create a new RNA microarray. These RNA microarrays can be used in the study of RNA–protein/RNA/apptamer bioaffinity interactions and for the enzymatically amplified SPRI detection of DNA in the presence of RNase H.

RNA is an extremely versatile biopolymer that combines the informatic storage capabilities of DNA with additional secondary structure capabilities that enhance its enzymatic and bioaffinity properties.¹ This versatility enables RNA molecules to play a central role in many basic biological systems including translation, gene expression, regulation and suppression, viruses, and biocatalysis.^{2–4} In addition, the in vitro evolution of synthetic RNA aptamers that can interact with small molecules or proteins in solution has been widely studied for a variety of diagnostic and drug discovery applications.^{5–8} RNA molecules in biological sam-

ples are often detected and identified in a multiplexed array format by sequence-specific hybridization adsorption onto single-stranded DNA (ssDNA) microarrays to form RNA–DNA heteroduplexes.^{9–11} Research studies of RNA bioaffinity, structure, and activity should also greatly benefit in principle from the use of ssRNA microarrays as a tool for the high-throughput screening of RNA–protein, RNA–DNA, and aptamer–drug interactions. However, due to the difficulty in tethering RNA molecules to a surface without loss of functionality, there are at present only a handful of reports on the fabrication of RNA microarrays in the literature.^{12–15} These fabrication strategies typically employ modified RNA (e.g., thiol-terminated or biotinylated) that are expensive and can lead to RNA degradation during both the modification and surface attachment procedures. Moreover, the sensitivity of RNA to hydrolysis at high pH and by nucleases from environmental sources suggests that RNA microarrays should be rapidly fabricated in situ and used immediately.

The ligation of unmodified RNA onto DNA attached to surfaces is a potential RNA microarray fabrication strategy that can solve some of these problems. DNA–DNA surface ligation chemistry has been previously reported by numerous researchers and applied to various biosensing applications such as the detection and discrimination of single-nucleotide polymorphisms from genomic samples^{16–20} and detection of DNA in DNA computing studies,^{21,22} as well as the creation of nanostructures and nanode-

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vices.^{23,24} The ligation of RNA to DNA using enzymes such as T4 DNA ligase is well-established in solution^{25–27} but has not yet been applied on surfaces. The creation of an RNA microarray by the simultaneous sequence-specific ligation of unmodified RNA molecules onto a ssDNA microarray should be a rapid, biocompatible method of array fabrication that minimizes the potential damage and handling of the surface RNA probe molecules.

In this paper, we demonstrate the use of surface RNA–DNA ligation chemistry to create single-stranded RNA (ssRNA) microarrays from 5′-phosphate-modified ssDNA microarrays. T4 DNA ligase is employed to form a phosphodiester bond between the juxtaposed 5′-phosphate of the surface-attached ssDNA and the 3′-hydroxyl group of unmodified RNA in the presence of a complementary ssDNA template. The RNA–DNA ligation process is followed with surface plasmon resonance imaging (SPRI) measurements,^{14,28} and the formation of the ssRNA microarray is characterized by three different experiments: (i) the hybridization adsorption of complementary DNA onto the ligated RNA array, (ii) the hydrolysis of the ssRNA microarray elements with RNase S, and (iii) the hydrolysis of RNA from surface-bound RNA–DNA heteroduplexes with RNase H. We find that the RNase H hydrolysis process actually regenerates the 5′-phosphate-modified DNA microarray elements, resulting in a ligation–hydrolysis cycle that can be used to repeatedly create RNA microarrays on a single DNA microarray substrate. As a final test, the ligated ssRNA microarrays were applied to the enzymatically amplified SPRI detection of a small amount of DNA (1 pM) in the presence of RNase H.

EXPERIMENTAL SECTION

Materials. T4 DNA ligase (New England Biolabs), ribonuclease H (RNase H; Takara Bio), S-Tag Grade S-protein (Novagen), S-Tag peptide (Novagen), 11-mercaptoundecylamine (MUAM; Dojindo), sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SSMCC; Pierce), 9-fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide (Fmoc-NHS; Novabiochem), and *N*-hydroxysuccinimidyl ester of methoxypoly(ethylene glycol) propionic acid MW 2000 (PEG-NHS; Nektar) were all used as received. All rinsing steps were performed with absolute ethanol and Millipore filtered water. Sterilized Tris buffer (50 mM Tris-HCl, 250 mM KCl, 10 mM MgCl₂, 0.5 mM spermidine, 10 mM DTT, pH 7.8) was used for all RNase H experiments. T4 DNA ligase reaction buffer (50 mM Tris-HCl, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, 25 μg/mL BSA, pH 7.8) was purchased from New England Biolabs and used for all T4 DNA ligase reactions. All solutions were autoclaved prior to use, and all experiments were performed at room temperature.

DNA and RNA Oligonucleotides. All of the 3′-thiol-modified DNA oligonucleotides in the presence and absence of 5′-phosphate

modification were purchased from Integrated DNA Technologies (IDT) and were deprotected and purified using binary reversed-phase HPLC. The complementary DNA sequences (HPLC purified) were also commercially obtained from IDT. The thiol-modified DNA oligonucleotides used in these experiments are as follows: D_A = 3′-S–S(CH₂)₆(A)₂₀PO₄-5′; D_N = 3′-S–S(CH₂)₆(T)₂₀; D_T = 5′-TTT TTT TTT TGC AAG CCC CA-3′; and D_C = 5′-GCA AGC CCC ACC TAG ACC GCA GAG-3′; D_{C2} = 5′-CAC TTG AGG CTA ACA C-3′, which is complementary to P₂; and D_{C3} = 5′-AGA CTC TGA CTC AGT G-3′, which is complementary to P₃. RNA oligonucleotides (RNase-free HPLC purified) were commercially obtained from Dharmacon RNA Technologies. The RNA oligonucleotides used in these experiments are R_P = P₁ = 5′-CUC UGC GGU CUA GGU GGG GCU UGC-3′, P₂ = 5′-HS(CH₂)₆(U)₈GTG TTA GCC TCA AGT G -3′, and P₃ = 5′-HS(CH₂)₆(U)₈CAC TGA GTC AGA GTC T-3′.

Surface Enzyme Reaction Conditions. *T4 RNA/DNA ligation:* The 5′-phosphorylated DNA microarray was reacted with 2.5 μL of T4 DNA ligase (400 000 units/mL) diluted in 1 mL of T4 ligase reaction buffer for 1 h along with 500 nM R_P and D_T. *RNase H reaction:* The RNA microarray was exposed to 60 units of RNase H diluted in 1 mL of RNase H buffer for 5 min unless otherwise specified. Experiments were performed both with and without D_C in the RNase H solution. *RNase S reaction:* Prior to reaction with the ligated RNA microarray, 500 μL of S-Tag Grade S-protein (50 ng/μL) and 25 μL of S-Tag peptide (0.05 pmol/μL) were mixed for 30 min to form RNase S. The RNA array was then exposed to RNase S for 5 min.

DNA Array Fabrication. A chemical protection/deprotection multistep method described previously^{14,29} was used for the creation of DNA microarrays. 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 first modified with a self-assembled monolayer of an amine-terminated alkanethiol MUAM. The amine-terminated MUAM surface was then reacted with the hydrophobic protecting group Fmoc-NHS. A quartz mask containing 500 μm × 500 μm features in conjunction with UV photopatterning was used to create bare gold patches within the hydrophobic Fmoc background. The bare gold spots were reacted with an amine-terminated self-assembled MUAM monolayer, and these spots were modified with the heterobifunctional cross-linker SSMCC to form a thiol-reactive maleimide-terminated surface. Thiol-modified sequences of DNA or RNA were then manually spotted onto these SSMCC array elements using a pneumatic picopump. The Fmoc background was finally removed with a mildly basic solution to regenerate the amine-terminated surface. This surface was reacted with PEG-NHS to create a background that can prevent nonspecific adsorption of biomolecules.

SPR Imaging Measurements. An SPR imager (GWC Technologies) using near-infrared excitation from a collimated white light source was used for monitoring in situ the creation, verification, and regeneration of a renewable RNA microarray. Briefly, collimated p-polarized light is passed through a flow cell/gold chip/prism assembly at a fixed angle. The light reflected from this assembly is then collected through a 830-nm narrow-

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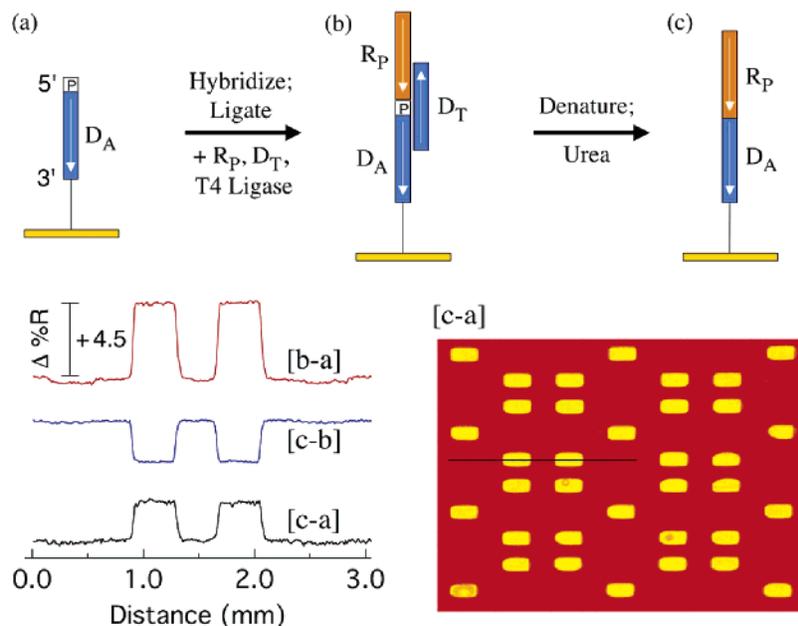


Figure 1. Schematic representation of the fabrication of an RNA microarray element via RNA ligation onto surface-immobilized ssDNA. The anchor ssDNA sequence D_A (a) is hybridized with template DNA (D_T) and probe RNA (R_P) to form a nicked duplex substrate, followed by sealing with T4 DNA ligase (b). D_T is then removed by washing with 8 M urea to generate a biologically active ssRNA surface (c). The bottom right of the figure is an SPR difference image obtained using an ssDNA microarray composed of two noninteracting sequences (D_A and D_N) with the specific ligation of R_P observed only at the D_A array elements. This image [c - a] was obtained by subtracting images acquired before hybridization/ligation and after removal of D_T from the array surface. The bottom left of the figure depicts a series of line profiles characterizing each step in the ligation process; [b - a], specific hybridization/ligation of R_P onto D_A , [c - b], the removal of D_T from the array surface, and [c - a], the presence of biologically active surface ligated RNA.

band-pass filter onto a CCD camera. The data were acquired using the software package V++ (Digital Optics). SPR difference images and line profiles were generated using NIH image software. The details of the SPR imager can also be found elsewhere.^{14,28}

RESULTS AND DISCUSSION

Fabrication of RNA Microarray Using Surface Ligation Chemistry. The procedure for creating an RNA microarray by ligating unmodified RNA molecules onto a DNA microarray is outlined in Figure 1. A ssDNA microarray is first created by the chemical attachment of 3'-thiol-modified, 5'-phosphate-terminated ssDNA to a maleimide-terminated alkanethiol monolayer (Figure 1a). These "anchor" DNA array elements (D_A) are then exposed to a solution containing T4 DNA ligase and both 24-mer probe RNA (R_P) and a 20-mer template DNA (D_T), which will hybridize to the D_A surface. The 20-mer D_T sequence is composed of two 10-mer binding sequences complementary to D_A and R_P . Exposure of the D_A array to this solution leads to the formation of a phosphodiester bond between the juxtaposed 5'-phosphate of D_A and the 3'-hydroxyl group of R_P (Figure 1b). On completion of the ligation reaction, the array surface is thoroughly rinsed with 8 M urea in order to denature and remove D_T and any T4 DNA ligase, resulting in the creation of biologically active ssRNA (R_P) array elements (Figure 1c).

Each step in the surface ligation reaction was characterized using in situ SPRI measurements. A two-component DNA array containing D_A , which is 5'-phosphate modified, and D_N , with no 5'-phosphate modification, was first exposed for 1 h to T4 ligase reaction buffer solution containing T4 DNA ligase (1000 units/mL) and 500 nM R_P and D_T . The line profile labeled [b-a] in Figure 1 shows a significant increase ($+4.5 \pm 0.4\%$) in percent

reflectivity ($\Delta\%R$) due to the hybridization adsorption of R_P and D_T onto the D_A array elements. The array was then exposed to 8 M urea to remove the template sequence (D_T). The line profile labeled [c-b] in Figure 1 depicts a decrease of $-2.5 \pm 0.3\%$ in ΔR due to the removal of D_T . This decrease is equivalent to approximately half of the increase observed during hybridization, which suggests that the ligated RNA remained on the surface. Furthermore, a measured ΔR of $2.2 \pm 0.3\%$ in the line profile labeled [c-a] taken from the SPR difference image shown in Figure 1 confirms that the RNA (R_P) probe is ligated onto D_A array elements. Note that it is extremely important to thoroughly remove the template D_T from the surface, as it could effect any subsequent RNA bioaffinity measurements.

Verification of RNA Microarray Activity. Three different approaches were used in conjunction with in situ SPRI measurements to verify the biological activity of the ligated RNA microarrays:

(i) Hybridization Adsorption. The hybridization of the 24-mer DNA (D_C) that is complementary to the 24-mer R_P sequence was accomplished by exposure of the RNA microarray to a 500 nM solution of D_C for 20 min, followed by rinsing with buffer solution. The SPR difference image shown in Figure 2a verifies that a $+1.5 \pm 0.1\%$ increase in ΔR was observed due to the hybridization adsorption of D_C onto the R_P array elements. This SPRI signal is only slightly smaller than the value of $+1.8 \pm 0.2\%$ measured under identical hybridization conditions with an RNA microarray fabricated using our previous method of chemically attaching thiol-modified RNA.^{13,14} Assuming a similar hybridization efficiency for the two RNA microarrays, the surface ligation efficiency can be roughly estimated at $80 \pm 20\%$.

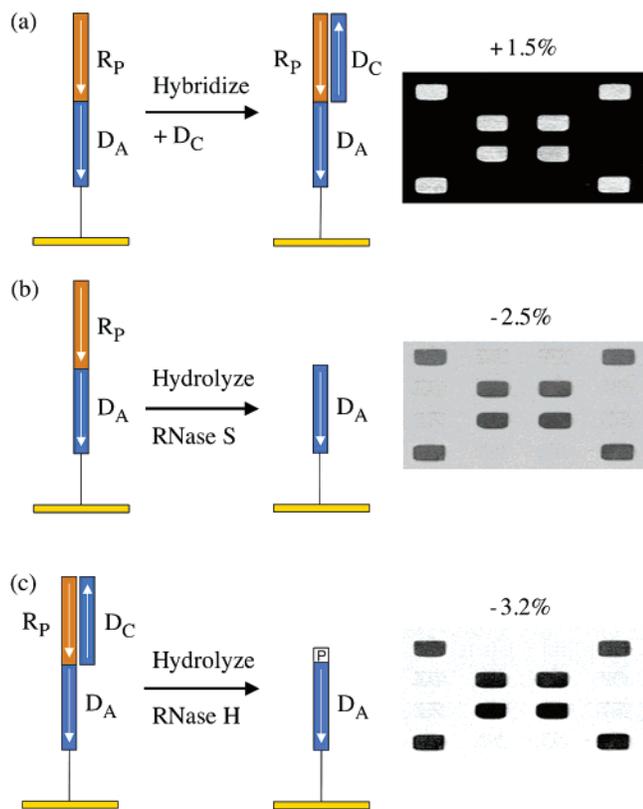


Figure 2. Schematics and accompanying SPR difference images depicting a series of experiments performed to verify the bioactivity of the ligated RNA microarray. (a) Hybridization adsorption of complementary DNA (D_C) to the RNA microarray element (R_P), (b) hydrolysis of single-stranded R_P array elements using RNase S, and (c) the selective RNase H hydrolysis of the RNA component of D_C – R_P heteroduplexes formed by hybridization–adsorption of 500 nM D_C to R_P array elements. In each case, the ssRNA microarray was created by the selective ligation of R_P onto D_A array components as described in Figure 1.

(ii) RNase S Hydrolysis. In a separate experiment, the ligated ssRNA (R_P) microarray was exposed to a solution of RNase S, which selectively hydrolyzed all of the R_P array elements. A ΔR loss of $-2.5 \pm 0.3\%$ was observed in an SPR difference image (Figure 2b) due to the removal of R_P from the surface. This value is approximately equal to that we have observed previously for the removal of chemically attached RNA microarray elements by RNase S.

(iii) RNase H Hydrolysis. In an additional enzymatic study, the ligated R_P microarray was first hybridized to D_C to form a R_P – D_C heteroduplex and then subsequently hydrolyzed with a 60 units/mL (16 nM) solution of RNase H as shown in the SPR difference image in Figure 2c. A ΔR loss of $-3.2 \pm 0.3\%$ in the differential SPR image was observed due to the removal of both R_P and D_C from the surface; this loss is approximately twice the $\Delta\%R$ increase measured in Figure 2a for D_C hybridization adsorption only.

It is also worth noting that no nonspecific adsorption was observed onto either the D_N control array elements or the poly(ethylene glycol) (PEG) background in each of the three experiments shown in Figure 2. These three results establish unequivocally that surface ligation can be used to create functional RNA microarrays that can be readily applied to various bioaffinity and biosensing measurements.

Regeneration of the RNA Microarray. The verification of RNA microarray activity using RNase H hydrolysis described in Figure 2c resulted in the discovery that the surface ligation process can be repeated to create a new RNA microarray from the same anchor ssDNA microarray. This is because RNase H specifically cleaves the phosphodiester bonds in RNA to produce 5'-phosphate and 3'-hydroxyl termini.^{30,31} Figure 3 illustrates a simplified schematic of the renewable RNA microarray fabrication

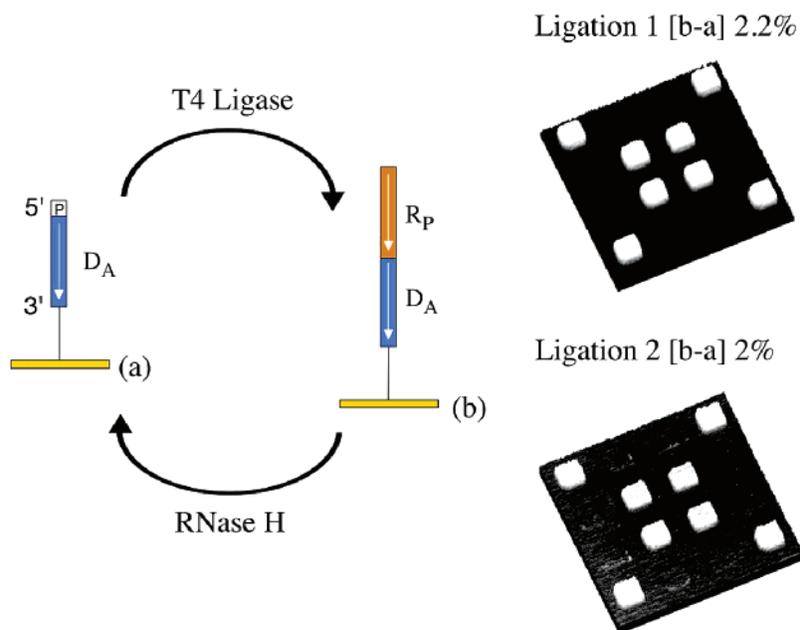


Figure 3. Simplified schematic for the fabrication of a renewable ssRNA microarray via RNA–DNA ligation chemistry and RNase H hydrolysis. Hybridization of complementary DNA (D_C) onto the ligated ssRNA followed by the selective hydrolysis of RNA using RNase H regenerates the original 5'-phosphorylated ssDNA surface. The ssRNA microarray was fabricated by the selective ligation of R_P onto the D_A elements of a two-component DNA microarray as described in Figure 1. The in situ SPR difference images were obtained for a series of two repeated RNA ligation steps [b – a] on the same ssDNA microarray. The first image (ligation 1) was acquired by subtracting images before hybridization/ligation and after removal of D_T from the array surface. This ligated RNA microarray was then exposed to a 500 nM solution of D_C complement followed by hydrolysis of the R_P – D_C heteroduplex with a 16 nM solution of RNase H. The ligation was repeated with a fresh solution of R_P , D_T , and ligase enzyme. On removal of D_T , a second SPR difference image (ligation 2) was obtained in a manner identical to the first image.

process in conjunction with RNase H hydrolysis. The 24-mer RNA sequence R_P is ligated to the 5'-phosphate-terminated D_A anchor microarray elements with T4 DNA ligase as described above. Hybridization adsorption of the complementary 24-mer DNA sequence D_C forms a surface DNA–RNA heteroduplex that can then be hydrolyzed by RNase H to regenerate the original ssDNA microarray. Specifically, the RNA strand of the heteroduplex, R_P , is hydrolyzed, releasing D_C back into solution and returning the anchor DNA element D_A to its original 5'-phosphate-terminated state. These regenerated DNA elements can be reacted again with either the same or a different R_P sequence in the presence of T4 DNA ligase to create a new RNA microarray.

To demonstrate the ability to renew the RNA surface, SPR difference images were acquired for a series of two RNA ligations to the same DNA microarray. The first image in Figure 3 (ligation 1) shows a ΔR increase of $2.2 \pm 0.3\%$ and the second SPR difference image (ligation 2) measured a very similar ΔR increase of $2 \pm 0.3\%$. The ligation–hydrolysis cycle was repeated up to 3 times using the same DNA microarray with virtually no degradation of the measured SPRI signal.

This renewable ligation–hydrolysis cycle depends crucially upon the RNase H cleavage of the RNA phosphodiester bond to restore the 5'-phosphate-terminated DNA microarray. In contrast, single-stranded specific RNA exonucleases such as RNase A or RNase S leave a 5'-hydroxy-terminated DNA fragment.³² To verify the uniqueness of RNase H hydrolysis for array regeneration, religation of RNA was attempted with the RNA microarray that had been exposed to RNase S as described above. As expected, no religation was observed due to the formation of a 5'-hydroxyl-terminated DNA array elements.

Enzymatically Amplified SPRI Measurements. As a final demonstration of the utility of the RNA–DNA surface ligation chemistry, a ligated RNA microarray element was used for the ultrasensitive detection of DNA with enzymatically amplified SPRI measurements. In this methodology,^{13,14} a small concentration of DNA can be detected by the repeated cycle of hybridization–adsorption on a complementary RNA microarray element followed by RNase H hydrolysis and release of the target DNA (see Figure 4a). A three-component RNA microarray was exposed to a solution containing both RNase H and 1 μM of the DNA sequence (D_C) complementary to the ligated RNA probes P_1 . After only 3 min, a significant decrease in percent reflectivity of $-0.6 \pm 0.06\%$ in ΔR was observed in the SPRI image (see Figure 4b) due to the repeated removal of P_1 from the surface. No change in SPRI signal was observed for the P_2 and P_3 RNA probe elements due to either nonspecific adsorption or enzymatic activity. A loss of $-0.6 \Delta R$ in 3 min is ~ 20 times faster than that observed previously with a thiol-modified RNA microarray.^{13,14} A slightly higher buffer ionic strength and RNase H concentration was used in these experiments as compared to our earlier efforts, but the superior performance of the ligated probe RNA array is primarily attributed to an increase in RNase H surface enzymatic activity that occurs because the 20-mer anchor DNA (D_A) is an excellent spacer that places the probe RNA further away from the gold surface. This

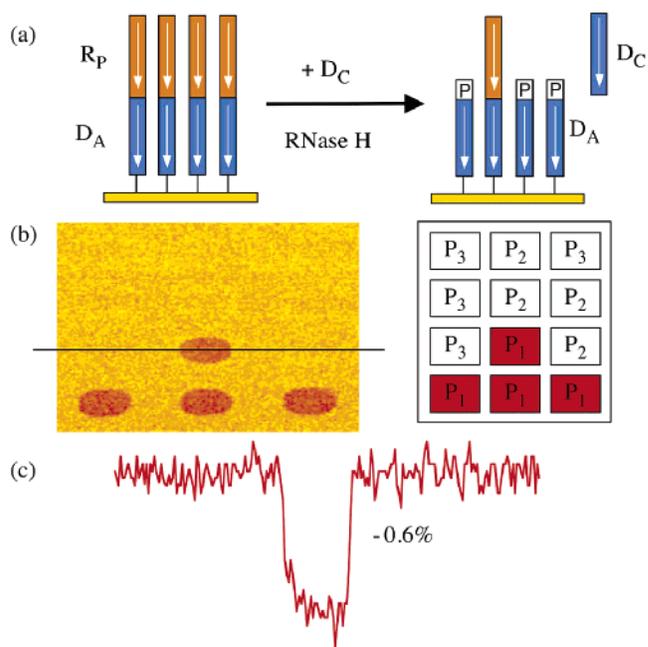


Figure 4. (a) Schematic showing the amplification mechanism for the SPRI detection of target complementary DNA (D_C) by the selective removal of RNA probes (P_1) attached to the surface by ligation. A three-component RNA microarray was used, which was composed of one set of ligated RNA–DNA microarray elements (P_1) and two sets of RNA microarray elements formed by the chemical attachment of thiol-modified RNA to maleimide-terminated alkanethiol monolayers (P_2 and P_3). (b) An SPR difference image obtained on subtracting images taken before and after exposure of an RNA microarray to a solution containing both 1 μM D_C (complementary to P_1) and 16 nM RNase H for 3 min. (c) The line profile taken across the difference image shows a decrease in percent reflectivity associated with the selective removal of probe RNA, while no changes at the other RNA array elements (P_2 and P_3) were observed. Schematic representation of the pattern of the three-component RNA array is shown where RNA probe P_1 is designed to bind to D_C while RNA probes P_2 and P_3 serve as negative controls.

DNA spacer is about over twice the length of the U8 spacer employed with the previous thiol-modified RNA microarrays.^{13,14}

CONCLUSIONS

In this paper, we have demonstrated that robust and biologically active RNA microarrays can be created from unmodified RNA and a DNA microarray using RNA–DNA surface ligation chemistry. Although DNA–DNA ligation on surfaces has been used previously by other researchers,^{17,19,21} the use of the RNA–DNA ligation activity of T4 DNA ligase on surfaces has not been reported. There are a number of benefits of using this new RNA–DNA ligation methodology for the creation of renewable RNA microarrays. The ligation chemistry described here can in principle be used with any DNA microarray, which is rapidly becoming a well-established and cost-effective biosensor technology. The ability to fabricate the RNA microarray in situ using unmodified RNA also means that there is very little ex situ handling or chemical exposure that can cause RNA degradation. In addition, regeneration of the RNA microarray in situ after RNase H hydrolysis can be used to ensure the reliability and fidelity of a biosensing measurement. One limitation in the RNA–DNA ligation strategy described here is that for each different RNA

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probe sequence a unique DNA template is required, which must also be thoroughly removed from the array prior to application. However, there are a number of strategies for creating DNA word arrays³³ or DNA zip code arrays^{16,34} that can be used to create noninteracting sets of DNA templates; use of these multitemplate strategies means that multielement RNA microarrays can be fabricated without any need for spotting or microfluidic delivery of the probe RNA sequences. The creation of multielement RNA

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microarrays using this methodology will be demonstrated in a future paper. We hope that this new fabrication strategy will accelerate the application of RNA microarrays to the study of RNA–RNA, RNA–DNA, RNA–protein, and other bioaffinity interactions.

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