Enzymatically Amplified Surface Plasmon Resonance Imaging Detection of DNA by Exonuclease III Digestion of DNA Microarrays

Hye Jin Lee, Yuan Li, Alastair W. Wark, and Robert M. Corn*

Department of Chemistry, University of California–Irvine, Irvine, California 92697

This paper describes a novel approach utilizing the enzyme exonuclease III in conjunction with 3′-terminated DNA microarrays for the amplified detection of single-stranded DNA (ssDNA) with surface plasmon resonance (SPR) imaging. When ExoIII and target DNA are simultaneously introduced to a 3′-terminated ssDNA microarray, hybridization adsorption of the target ssDNA leads to the direction-dependent ExoIII hydrolysis of probe ssDNA strands and the release of the intact target ssDNA back into the solution. Readsoption of the target ssDNA to another probe creates a repeated hydrolysis process that results over time in a significant negative change in SPR imaging signal. Experiments are presented that demonstrate the direction-dependent surface enzyme reaction of ExoIII with double-stranded DNA as well as this new enzymatically amplified SPR imaging process with a 16-mer target ssDNA detection limit of 10−100 pM. This is a 10^2−10^3 improvement on previously reported measurements of SPR imaging detection of ssDNA based solely on hybridization adsorption without enzymatic amplification.

DNA microarrays are a fast and inexpensive way to obtain sequence-specific nucleic acid information for gene analysis, viral identification, medical diagnostics, and many other biological applications. Sequence specificity is obtained by detection of the surface hybridization of target molecules from solution onto the various array elements. Fluorescence imaging is typically used to detect the hybridization adsorption of tagged DNA molecules onto the array, and SPR imaging is an alternative, "label-free" refractive index method that can be used to detect the adsorption of untagged DNA and RNA molecules. Fluorescence imaging is often limited by background to a detection limit of 1 pM, and SPR imaging typically has a detection limit of 1−10 nM. Recently, we have demonstrated that this detection limit can be lowered to 1 fM by using the enzyme RNase H in conjunction with an RNA microarray for the enzymatic amplification of the SPR imaging signal. Despite the excellent sensitivity and selectivity of this method, it has the limitation of requiring an RNA microarray as compared to a DNA microarray. RNA microarrays are stable but not yet widely used since they require special handling to avoid enzymatic or chemical destruction. A question that immediately arises is, can we use an alternative enzymatic amplification system that will work with DNA microarrays?

In this paper, we explore the use of the enzyme exonuclease III (ExoIII) in conjunction with DNA microarrays for the enzymatically amplified detection of DNA. ExoIII exhibits 3′ → 5′ exonuclease activity, which involves specific binding to double-stranded DNA (dsDNA) followed by selective hydrolysis of one strand from the DNA duplex. The direction-dependent behavior of the ExoIII activity was investigated to demonstrate that an alternative enzymatic amplification system that will work with DNA microarrays.

amplification reaction scheme is possible utilizing DNA microarrays where the probe single-stranded DNA (ssDNA) is covalently attached to the surface via its 5′-end. Figure 1 shows an overview of the ExoIII surface enzymatic amplification process. This amplification process is analogous to recently described work performed in our research group using RNase H on RNA microarrays.16,17 An ssDNA microarray is fabricated via 5′-end immobilization creating 3′-terminated ssDNA array elements that are exposed to a solution containing both complementary DNA and ExoIII. The complementary target DNA will first hybridize to the probe on the surface forming a DNA duplex to which ExoIII will then bind (step 1). ExoIII selectively hydrolyzes the 3′-terminated probe and releases the target DNA back into solution (step 2). The released DNA can then hybridize to another DNA probe on the surface, leading to removal of another probe (step 3). This cyclic reaction allows a very small number of target DNA molecules to initiate the destruction of many DNA probes on the surface. The negative change in percent reflectivity due to probe loss can be easily measured, resulting in an amplified SPR imaging signal that is considerably larger than the smaller positive signal observed from direct hybridization adsorption.

EXPERIMENTAL SECTION

Materials. ExoIII (Promega; 1 unit/mL = 0.17 nM), 11-mercaptoundecylamine (MUAM; Dojindo), 9-fluorenylmethoxycarbonyl-N-hydroxysuccinimide (Fmoc-NHS; Novabiochem), sulfo-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SSMCC; Pierce), and N-hydroxysuccinimidyl ester of methoxypoly(ethylene glycol) propionic acid (PEG-NHS; Nektar; MW 2000) were all used as received. Tris buffer (pH 7.9) containing 50 mM Tris-HCl, 10 mM MgCl₂, and 250 mM KCl was used for all reported hybridization and ExoIII digestion experiments. All of the 3′- and 5′-thiol-modified DNA oligonucleotides were obtained commercially from Integrated DNA Technologies (IDT) and were deprotected and purified using binary reversed-phase HPLC. The complementary DNA (HPLC purified) was also purchased from IDT. The thiol-modified DNA oligonucleotides used in these experiments are as follows: D₁ = 5′-S(T)₁₅GTCTATCCGTGAACCTG, D₂ = 5′-S–S(T)₁₅GTCTATCCGTGAACCTG, D₃ = 5′-S–S(CH₃)₂/T₃₀, A₁ = 3′-S–S(T)₁₅GTGAACCTCGATTGTG, A₂ = 3′-S–S(T)₁₅GTCAAGTGCGTATCTG, and A₃ = 3′-S–S(CH₃)₂/T₃₀. The complementary DNA sequences used were as follows: C₁ = 5′-CAGTTCACGCATAGAC, and C₂ = 5′-CAGTTCACGCATAGAC, which is the complementary sequence of both D₁ and A₁, and C₃ = 5′-CAGTTCACGCATAG. The (T)₁₅ spacer improves the accessibility of the surface-bound DNA to both the target DNA and ExoIII. All rinsing steps were performed with absolute ethanol and MilliQ filtered water. All the experiments were performed at 37 °C.

DNA Array Fabrication. A multistep chemical modification process developed previously was used to create ssDNA microarrays for SPR imaging experiments.24 Briefly, a thin gold film (45 nm) was vapor deposited onto an SF10 glass slide (Schott Glass) with an underlayer of chromium (1 nm) using a Denton DV-502A metal evaporator. The gold slides (18 mm × 18 mm) were immersed in 1 mM ethanolic solutions of MUAM for at least 4 h. The amine terminal group of the self-assembled alkanethiol MUAM monolayer was then reacted with the hydrophobic protecting group Fmoc-NHS. By exposing the gold surface to UV radiation through a quartz mask containing patterns of 500 μm × 500 μm square features, patterns of bare gold square patches surrounded by the hydrophobic background were created. The slide was then immersed in MUAM for a further 2 h, and the resulting MUAM patches were reacted with the heterobifunctional cross linker SSMCC to form a thiol-reactive maleimide-terminated surface. Thiol-modified sequences of DNA were then spotted onto the SSMCC array elements using a manually controlled pneumatic picopump. The Fmoc background was removed, and the regenerated MUAM background was reacted with PEG to prevent any nonspecific adsorption of target biomolecules. The surface coverage of the ssDNA monolayer was estimated to be ~5 × 10¹² molecules/cm². The DNA arrays fabricated in this manner demonstrated excellent reproducibility with a chip-to-chip variation of ~5% for repeated SPR imaging measurements of hybridization and ExoIII digestion.

Kinetic Flow Cell Design. A serpentine PDMS microfluidics system was used to continuously deliver small volumes of samples across the DNA microarray surface for kinetics measurements.25,26 Briefly, a PDMS microchannel featuring a serpentine pattern (670-μm width, 9.5-cm length, 200-μm depth, total volume ~10 μL) was
first treated with oxygen plasma for 10 s to enhance the hydrophilicity of the channel, which can in turn help the flow of aqueous samples inside the channel and also minimize any sample adsorption onto the walls of the channels. The microchannel/gold chip/prism assembly was then encased in a specially designed water-jacketed cell allowing the system temperature to be controlled to within 0.1 °C. As a result, any fluctuations in SPR signal over time owing to room-temperature variations are also reduced. A syringe pump at a constant flow rate of 30 μL/min was used to deliver all solutions through the microchannel.

**Real-Time SPR Imaging Measurements.** An SPR imaging apparatus (GWC Technologies) was used for the real-time monitoring of the DNA hybridization and ExoIII reaction. Briefly, collimated p-polarized light is reflected through a microchannel/gold chip/prism assembly at a fixed angle and then passed through a narrow band-pass filter and sent to a CCD camera. The image and kinetic data were collected using the software package V++ (Digital Optics, NZ). Custom macros were written using this software so that data could be collected with simultaneous processing of several specific user-designated regions of interest (ROIs) on the array surface. For real-time measurements, a five-frame averaged image was saved and analyzed with the calculated change in average pixel intensity resulting in a data point collected for each ROI. During hybridization experiments, a time interval of 10 s was typically set between data point acquisitions, whereas for the ExoIII reaction, a 2-s time interval was applied. The difference in percent reflectivity (Δ%R) for each array element was normalized with respect to the average Δ%R measured for the negative control and PEG background ROIs. Kinetic data from multiple identical array elements were averaged to obtain the final SPR response curve. Microsoft Excel and Igor Pro were used for all data processing and kinetic model fitting in these experiments.

**RESULTS AND DISCUSSION**

**(A) Exonuclease III Reactivity with 5′- and 3′-Terminated DNA Microarrays.** Our first task is to verify and quantitate the reactivity of ExoIII with the surface-bound DNA arrays. ExoIII is known to have 3′- to 5′-exonuclease activity for dsDNA in solution. Single-stranded DNA microarrays were fabricated by covalently attaching thiol-modified oligos to a maleimide-terminated alkanethiol monolayer via either the 3′- or 5′-end as shown schematically in Figure 2. For the case of 3′-end attachment (scheme a), the ssDNA array element is 5′-terminated. Hybridization with a complementary DNA oligomer from solution will result in a dsDNA array, and the 3′-terminated target DNA strand from the duplex will be digested by ExoIII; only the complementary DNA strand will be destroyed, leaving the original ssDNA still attached to the surface. In contrast, when the ssDNA array element is attached via the 5′-end, creating a 3′-terminated ssDNA element (scheme b in Figure 2), the exonuclease activity of ExoIII should result in the destruction of the surface-attached ssDNA and the release of the complementary DNA back into solution.

A series of real-time SPR imaging measurements were performed to verify the directional behavior of the surface ExoIII reaction with dsDNA. Two three-component ssDNA arrays were constructed using either 5′-terminated (scheme a) or 3′-terminated ssDNA (scheme b) monolayers. The surfaces were then exposed to a 100 nM solution of the 16-mer complement of one of the three DNA sequences (C1), and the resultant change in percent reflectivity (Δ%R) was measured as a function of time. Figure 3 plots these data and shows that a rapid rise in Δ%R was observed at the appropriate ssDNA elements due to hybridization adsorption of the complement onto the surface, reaching a steady-state Δ%R value of +2% after ~250 s. Both the 3′-terminated and 5′-terminated

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ssDNA monolayers exhibited very similar hybridization adsorption kinetics. Not shown in Figure 3 are the ΔR curves obtained at the array elements containing the two noncomplementary DNA array sequences; these are used as controls for nonspecific adsorption and showed no change in ΔR during the experiment.

Following hybridization, the array was rinsed with buffer solution and then exposed to ExoIII (80 nM). For the case of the 5′-terminated array, exposure to the ExoIII solution resulted in a ΔR decrease of ~2% back to the original reflectivity level (curve a in Figure 3). In contrast, for the case of the 3′-terminated array, a decrease of ~4% was observed corresponding to the loss of both the target and probe DNA from the surface (curve b and see also Figure 3 inset). This is exactly what we expected to see for our two reaction schemes in Figure 2. As additional confirmation, we were able to readsort complementary 16-mer ssDNA onto the 5′-terminated ssDNA monolayer and observe a ΔR of +2%, but no readsortion or change in ΔR was observed for the 3′-terminated ssDNA monolayer.

These experiments allow us to draw the following conclusions about the ExoIII surface enzymatic reaction: (i) with either 5′-terminated or 3′-terminated ssDNA monolayers, ExoIII can completely digest the dsDNA monolayer formed by hybridization adsorption; (ii) for the case of the 3′-terminated ssDNA monolayer, the entire ssDNA can be removed by ExoIII digestion. This second point is important because, in principle, ExoIII could possibly digest the complementary strand as shown in the alternative reaction path of scheme b in Figure 2 (the reaction path containing a question mark). However, this would result in the incomplete digestion of the 3′-terminated ssDNA monolayer, which was not observed.

A final caveat to the use of ExoIII for the digestion of 3′-terminated DNA monolayers is that, on rare occasions, ExoIII activity was observed for ssDNA array elements without hybridization (data and sequences not shown). This unusual ssDNA activity was identified with sequences that formed hairpins on the surface, to which the enzyme was able to bind and interact. No ExoIII digestion of the 3′-terminated ssDNA array elements (D1, D2, D3) reported in this paper was observed in the absence of duplex formation.

(B) Surface Amplification Process for DNA Detection.

Having characterized the reaction of ExoIII on 3′-terminated DNA monolayers, we are now in a position to use the enzyme amplification scheme depicted in Figure 1 for the detection of low concentrations of DNA target molecules. Before demonstrating the enzymatically amplified detection of DNA with ExoIII, we first wish to show the detection limit without amplification, i.e., SPR imaging measurements of direct DNA adsorption. The detection of DNA by hybridization adsorption with SPR and SPR imaging has been demonstrated previously by a number of research groups; for DNA monolayer adsorption, typically a detection limit in the range of 1–10 nM is reported.20−22,28−32 Figure 4 plots the SPR imaging response upon exposure of a ssDNA monolayer to 1, 10, and 100 nM solutions of complementary 16-mer DNA (C2). Note that, at short times (<150 s), the adsorption kinetics are linear for all three concentrations. The adsorption rate, as determined from the initial slopes, decreases by factors of 10 as the target DNA concentration is reduced from 100 to 10 to 1 nM as expected from simple Langmuir kinetics. The slope of the 1 nM data is almost zero, and the SPR difference image shows virtually no change after 500 s. We have reported previously the detection limit of SPR imaging to be in the range of 1–10 nM.21,23 It is important to recount the reasons for this detection limit: (i) with SPR imaging, we can typically sense changes in DNA surface coverages (θ) down to ~2% of a monolayer, or ~1011 molecules/cm2; (ii) the Langmuir adsorption coefficient Kθ for 16-mer DNA hybridization adsorption is ~2 × 104 M−1, and since θ = Kθ/C, 1 nM is the detection limit; and (iii) as seen in Figure 4, at low concentrations the adsorption kinetics become rather slow, so that competition with nonspecific adsorption becomes fairly significant, often limiting the detection of the specific hybridization adsorption of DNA. All of these points have also been noted by other researchers when discussing the detection limits of various SPR and other label-free DNA hybridization adsorption measurements.20−28

As stated previously, we have recently employed RNA microarrays and the enzyme RNase H to increase the sensitivity of DNA detection with SPR imaging from 10 nM to 1 fM.16,17 Figure 5 shows our experiments using DNA microarrays and the enzyme ExoIII for enzymatically amplified SPR imaging using the reaction scheme in Figure 1. ExoIII and target DNA are simultaneously introduced into the microfluidic reaction channel containing the 3′-terminated ssDNA array elements. Adsorption of the target DNA onto the monolayer followed by ExoIII digestion results in

a decrease in $\Delta R$. In the experiment shown in Figure 5, two complements were introduced into the reaction channel with ExoIII when a solution containing a mixture of 80 nM ExoIII, 100 nM C1, and 1 nM C2 was introduced onto a three-component ssDNA array consisting of D1, D2, and D3. The starting time of 0 s refers to when the ssDNA array surface was first exposed to the solution mixture. 

(b) An SPR difference image obtained by subtracting images taken before and after the ssDNA array has been exposed to the solution mixture for 60 min. The arrows represent the flow direction of the target DNA in the serpentine microchannel flow cell. (c) Schematic representation of the pattern of the three-component ssDNA array. Probes D1 and D2 are designed to bind to the target DNA C1 and C2, respectively, and probe sequence D3 is a negative control. All experiments were carried out at 37 °C.

CONCLUSIONS

The ability to sensitively detect multiple DNA sequences simultaneously on a single chip without the use of labeling is of importance in many ongoing biosensing applications. Through the use of ExoIII in conjunction with DNA microarrays, a $10^2$–$10^3$ improvement in the detection limit for the multiplexed SPR imaging detection of 16-mer oligonucleotides was achieved. This enhancement is not as great as RNase H amplification with RNA microarrays; however, the major advantage of ExoIII amplification compared to RNase H is that the additional difficulty in preparing and handling RNA microarrays is avoided. The ExoIII enzymatic amplification process can be used with the more robust and cost-effective DNA microarrays that are currently applied in research areas such as gene analysis and medical diagnostics. As in the case of RNase H amplification, most DNA sequences can be detected since the activity of ExoIII is not sequence dependent, although caution is required to avoid using probe DNA that may form hairpin structures resulting in signal loss without hybridization adsorption. Furthermore, this enzymatic amplification method can in principle be used in conjunction with other detection methods such as fluorescence and electrochemical measurements, as will be demonstrated in future papers.

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