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ARTICLE *in* ANALYTICAL CHEMISTRY · JUNE 2011

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Amplified On-Chip Fluorescence Detection of DNA Hybridization by Surface-Initiated Enzymatic Polymerization

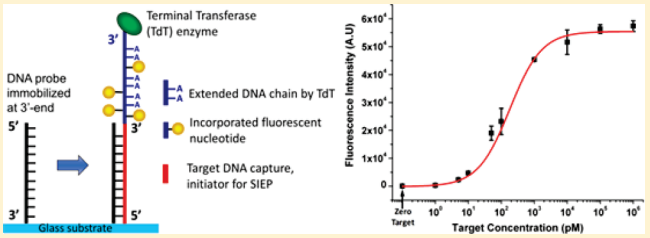
Vinalia Tjong,[†] Hua Yu,[†,‡] Angus Hucknall,[†] Srinath Rangarajan,[†,‡] and Ashutosh Chilkoti*[,†,‡]

[†]Department of Biomedical Engineering, Box 90281, Duke University, Durham, North Carolina 27708, United States

[‡]Center for Biologically Inspired Materials and Materials Systems, Box 90303, Duke University, Durham, North Carolina 27708, United States

Supporting Information

ABSTRACT: We describe the incorporation of multiple fluorophores into a single stranded DNA (ssDNA) chain using terminal deoxynucleotidyl transferase (TdT), a template-independent DNA polymerase that catalyzes the sequential addition of deoxynucleotides (dNTPs) at the 3′−OH group of an oligonucleotide primer; we term this methodology surface initiated enzymatic polymerization (SIEP) of DNA. We found that long (>1 Kb) ssDNA homopolymer can be grown by SIEP, and that the length of the ssDNA product is determined by the monomer to oligonucleotide initiator ratio. We observed efficient initiation (≥50%) and narrow polydispersity of the extended product when fluorescently labeled nucleotides are incorporated. TdT's ability to incorporate fluorescent dNTPs into a ssDNA chain was characterized by examining the effect of the molar ratios of fluorescent dNTP to natural dNTP on the degree of fluorophore incorporation and the length of the polymerized DNA strand. These experiments allowed us to optimize the polymerization conditions to incorporate up to ∼50 fluorescent Cy3-labeled dNTPs per kilobase into a ssDNA chain. With the goal of using TdT as an on-chip labeling method, we also quantified TdT mediated signal amplification on the surface by immobilizing ssDNA oligonucleotide initiators on a glass surface followed by SIEP of DNA. The incorporation of multiple fluorophores into the extended DNA chain by SIEP translated to a ∼45 fold signal amplification compared to the incorporation of a single fluorophore. SIEP was then employed to detect hybridization of DNA, by the posthybridization, on-chip polymerization of fluorescently labeled ssDNA that was grown from the 3′−OH of target strands that hybridized to DNA probes that were printed on a surface. A dose−response curve for detection of DNA hybridization by SIEP was generated, with a ∼1 pM limit of detection and a linear dynamic range of 2 logs.



INTRODUCTION

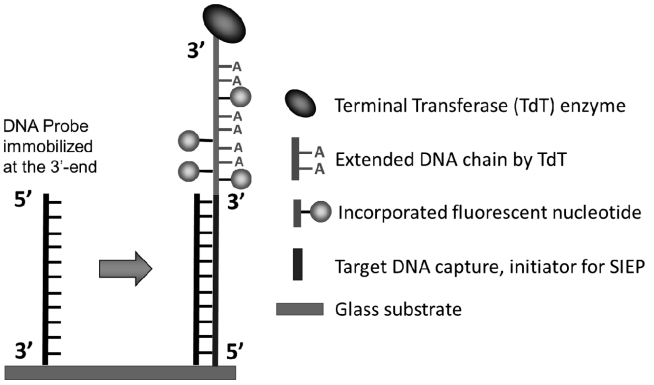
DNA microarrays have become a powerful and almost-ubiquitous analytical tool in biology and medical research, because of their massively parallel analytical power and high throughput. Microarrays are now routinely used for analysis of gene expression,[1−4] and they are also being developed for the detection of microRNA.[5−7] Fluorescence-based DNA microarrays remain the most widely adopted format, because of their high sensitivity and the easy availability of fluorescence scanners. For analysis of nucleic acids by fluorescence-based arrays, front-end processing of samples prior to microarray analysis involves (1) reverse transcription of mRNA to cDNA, (2) amplification of genomic DNA or cDNA, and (3) labeling the target DNA with fluorophores or other detection molecules prior to hybridization on the array.[8,9] The disadvantage of prehybridization labeling and amplification is that it adds significant upfront complexity to the assay. Furthermore, because labeling is carried out prior to hybridization, only a few fluorophores can be incorporated into the DNA target along the DNA chain without disrupting hybridization,[10] and all DNA from a complex clinical sample is labeled indiscriminately, potentially introducing noise in the assay, because of nonspecific binding of the target to the surface.

Hence, we believe that there is a strong rationale for the development of a DNA microarray assay that seamlessly combines labeling, amplification, and detection, and has the following attributes: (1) it can be carried out *in situ* (i.e., is on-chip); (2) labels the target with detection moieties posthybridization rather than prehybridization; (3) ensures that only the analytes of interest (targets) are labeled; (4) provides amplification by incorporating multiple fluorophores, or other labels per binding event; and (5) can be carried out under isothermal ambient conditions that are compatible with microarray analysis of DNA or RNA analytes.

Several on-chip signal amplification methods such as tyramide signal amplification,[11,12] immunoPCR,[13] rolling circle amplification (RCA),[14,15] and branched DNA technology[16] have been

Received: January 26, 2011
Accepted: May 23, 2011
Published: May 23, 2011

Scheme 1. SIEP of DNA Allows Single-Step, Isothermal, On-Chip, Post-Hybridization Fluorescence Detection of DNA Microarrays



previously used for quantification of DNA and RNA. Although these signal amplification methods are sensitive, they require customized probes and signal amplifiers that must be tailored for the target of interest, which introduces further complexity into the assay.

We report herein, a new isothermal, on-chip, post-hybridization labeling and amplification scheme for DNA microarrays. This assay uses terminal deoxynucleotidyl transferase (TdT), which is a template-independent DNA polymerase that catalyzes the sequential addition of deoxynucleotides (dNTPs) at the 3′−OH group of an oligonucleotide primer, and incorporates multiple fluorescent dNTPs into the ssDNA chain that is grown by TdT to provide post-hybridization, on-chip fluorescence detection of the hybridized DNA. We have named this methodology "surface- initiated enzymatic polymerization" (SIEP) of DNA.

As shown in Scheme 1, SIEP utilizes the target strand as the "*in situ*" signal amplifier, without prior need for a presynthesized detection probe or signal amplifier, which greatly simplifies the assay. We show that SIEP using TdT enables the facile incorporation of fluorescently labeled dNTPs directly into a growing ssDNA chain on the surface. By printing probes with their 3′-ends attached to the surface and an exposed 5′-end (Scheme 1), we ensured the on-chip detection and amplification by *in situ* growth of a DNA strand that incorporates a fluorescent dNTP only when a target DNA binds to the probe, as the only initiation sites for *in situ* polymerization of DNA on the surface by TdT are the exposed 3′−OH groups presented by target DNA bound to probes. This technology is compatible with commercial microarrays, because the orientation of the probes is the same as in many commercial microarrays.[17] We show that on-chip fluorescence amplification by SIEP has a low pM limit of detection (LOD) and a two-orders-of-magnitude dynamic range, so that it is competitive with other techniques for DNA detection on surfaces.[18,19] This method is attractive, because it does not require prelabeling of all cDNA strands prior to labeling, is highly selective (because it only labels positive "hits" on the surface), and is isothermal, so that it can be carried out directly on a DNA microarray without any need for specialized equipment.

EXPERIMENTAL SECTION

Materials. Details can be found in the Supporting Information (SI).

Incorporation of Fluorescent Nucleotides by SIEP. The reaction mixture consisted of 1 μM Cy5-labeled oligonucleotide primer initiator [5′-Cy5-dT_{10} or 5′-Cy5-dA_{10}], 1 mM dNTP monomers (dATP or dTTP), and 10 U of TdT in 10 μL of TdT buffer (1×, 100 mM potassium cacodylate, 1 mM $CoCl_2$, and 0.2 mM DTT, pH 7.2). Different amounts of fluorescent dNTPs (Cy3-dATP and Cy3-dUTP) were added to the TdT reaction mixture. The ratios of fluorescent to natural dNTPs were 1:5, 1:10, 1:20, 1:50, and 1:100 at 1 mM total dNTP concentration. The reactions were carried out in a total volume of 10 μL, which was incubated at 37 °C for 2 h, and terminated by heating the solution at 70 °C for 10 min.

Determination of the Number of Fluorescent Nucleotides Incorporated per DNA Chain via SIEP. The TdT reaction product was subjected to a purification step to remove the nonextended primer initiator (<20 bases) and unreacted monomers, especially the unreacted fluorescent dNTPs, by adding 40 μL of filtered water to the mixture and passing it through a Probe Quant G50 spin column (GE Healthcare). To determine the mole fraction of primer that was extended and the degree of fluorescent dNTPs that were incorporated to the polymerized DNA, the purified product was then diluted with 100 μL of deionized water, and its fluorescence was measured on a Victor^3 microplate reader (Perkin−Elmer Life Sciences) using two sets of excitation and emission filters for Cy3 and Cy5 fluorescence measurement. The amount of primer and fluorescent dNTPs was determined by establishing a linear standard curve. The number of fluorescent dNTPs per DNA chain can be calculated by taking the molar ratio of the fluorophores with the primer. The initiation efficiency of the primer (% primer extended) is determined relative to the control reaction with only natural dNTP present in the reaction.

Surface-Initiated Enzymatic Polymerization (SIEP). SIEP was performed on the glass slide spotted with Cy5-labeled oligonucleotide primers by incubating each well on the slide with 10 U of TdT, 100 μM dATP monomer, and 0.5 μM, 1 μM, 2 μM, or 5 μM Cy3-dATP nucleotide or a dye-terminator, Cy3-dideoxynucleotides (Cy3-ddATP) in 100 μL 1× TdT buffer. Details of immobilization of probes to the glass surface by their 3′-end are given in the SI. Wells incubated with Cy3-ddATP served as a reference, because the extended oligonucleotides initiator incorporated only a single Cy3-ddATP "dye-terminator" per chain. SIEP was carried out for 1 h at 37 °C, after which the slides were washed three times with 1× SSC buffer containing 0.1% Tween 20 to facilitate the removal of any nonspecifically bound reactants.

Analysis of Fluorescence Signal Amplification from DNA Spots. Cy5 and Cy3 fluorescent signals were analyzed using GenePix 6.0 (Molecular Devices) by localized background subtraction; only signals that had a signal-to-noise ratio (SNR) of > 3 were included in the analysis. Cy5 fluorescent signal from the immobilized oligonucleotide initiators was used to normalize the amount of immobilized primer across the different DNA spots. This step is necessary to exclude the increase in fluorescent signal, because of the variations in the printing volume. The ratio of fluorescent signal intensity of Cy3 to Cy5 was then used as the measure of the fluorescent dNTP incorporation. Via comparison of the ratio of Cy3/Cy5 signal intensities of the well incorporating multiple Cy3-dATP into each primer by SIEP with the reference wells that can only incorporate a maximum of a single Cy3-ddATP per DNA primer, we could determine the magnitude of signal amplification from the incorporation of multiple fluorophores in the DNA chain extended from the 3′−OH end of the primer by TdT.

On-Chip Fluorescent Labeling of DNA Hybridization by TdT. The 25-mer target sequence [5′-GAG CTT CTG TGG AGT TAC TCT CTT T-3′] was selected from the core protein of the Hepatitis B virus. The fully complementary probe [5′-AAA GAG AGT AAC TCC ACA GAA−$(CH_2)_6$−NH_2−3′] was spotted using a noncontact printer (Piezzorray, Perkin−Elmer, Inc.) to covalently attach the probe to the glass surface. The slides spotted with the probes were then incubated overnight in a humidified chamber and rinsed in 1× SSC buffer with 0.1% Tween 20 followed by rinsing with filtered water and spun dry. A dose−response curve of the hybridized target was generated by incubating the printed probes to a range of target DNA concentrations (from 1 pM to 1 μM) for 4 h at 37 °C in a 3× SSC buffer, followed by SIEP using TdT to incorporate Cy3-dATP (0.1 U/μL TdT, 100 μM dATP, and 0.5 μM Cy3-dATP in 1× TdT buffer) for 1 h at 37 °C. The slides were then rinsed in a 1× SSC buffer with 0.1% Tween 20 for 30 min and scanned immediately on a GenePix scanner. The average signal intensity of the spot was then plotted as a function of the target DNA concentration.

Instrumentation. The incorporation of fluorescent nucleotides in solution was determined by a Victor^3 microplate reader (Perkin−Elmer Life Sciences). For Cy5 fluorescence measurement, an excitation band filter of 650 ± 4 nm and an emission band filter of 680 ± 5 nm was used, whereas, for Cy3 fluorescence measurement, a 560 ± 4 nm excitation band filter and a 590 ± 10 nm emission band filter was used. On-chip TdT labeling was determined by scanning the glass slides for the Cy5 (635 nm) and Cy3 (532 nm) fluorescent signal using an Axon GenePix Pro 4200 scanner (Molecular Devices) at 10-μm resolution with optimized PMT and gain settings.

RESULTS AND DISCUSSION

Homopolymerization of DNA in Solution by TdT. Despite extensive characterization of TdT in the early 1960s by Bollum et al.,[20] we decided to revisit the DNA extension catalyzed by TdT in solution, prior to SIEP of DNA, because the enzyme used in these studies is a recombinant protein, compared to the enzyme used in those studies, which was isolated from calf thymus gland.[21] In addition, although TdT has been used in molecular biology as a reagent for end labeling,[22] homopolymeric tailing in rapid amplification of cDNA ends (RACE),[23] and detection of apoptosis by the TUNEL assay;[24] for the optimization of these applications, characterization of DNA polymerization using TdT focused on the polymerization of short (15−30 bases) homopolymeric DNA tail.[25,26] The absence of reports on the TdT-mediated polymerization of long DNA chains (>1 Kb) also prompted us to carry out experiments in solution to optimize the synthesis of long DNA chains using TdT.

The two parameters that we focused on to characterize TdT-catalyzed DNA polymerization in solution were the DNA primer that acts as an "initiator" for the enzymatic polymerization of DNA and the nucleotide "monomer"; we use this terminology to place the mechanism of TdT catalyzed DNA growth in the context of polymer chemistry. Because TdT polymerizes mononucleotides in a distributive manner and does not carry out the reverse reaction, the chain length in TdT-catalyzed DNA polymerization is equal to the input monomer (M) to initiator (I) ratio (M/I),[27] assuming that all primer molecules that serve as an initiator are extended, and that the reaction goes to completion. To investigate the effect of this variable on the length and polydispersity of the extended DNA product, three M/I ratios of 100, 1000, and 10 000 were examined. Homooligomer DNA initiators—dA_{10} and dT_{10}—and the preferred substrates—dATP and dTTP[28]—of TdT were chosen as the monomers. We found long polymer chains up to ∼8 Kb were polymerized from the short, 10 nt long primers after a 24 h reaction, while ∼0.1 Kb and ∼1 Kb were obtained after 2 h (Figure S-1a in the Supporting Information). The time-dependent growth of DNA chain is the result of nonprocessive properties of TdT,[29] which adds a single nucleotide in a stepwise fashion during polymerization (Figure S-3 in the Supporting Information). For both initiators −dA_{10} and dT_{10}, using dATP and dTTP as the monomer, respectively, the polymerization efficiency approached a remarkable 100%, as no band that corresponds to the residual primer was observed. A second observation of interest is that the length of the DNA chain grown from the 3′−OH end of the primer was proportional to the M/I ratio (Figure S-1b in the Supporting Information), so that the product length can be controlled simply using a higher M/I ratio and a longer reaction time to allow the reaction to go to completion. This experiment demonstrates that it is possible to generate long DNA chains (hundreds to thousands of bases long).

Incorporation of Fluorescent Nucleotides. Having shown that a long DNA chain could be facilely polymerized from a short oligonucleotide initiator, we next turned our attention to determining whether multiple fluorophores could be incorporated into the DNA chain by incorporation of fluorescently modified dNTPs. This experiment was also necessary, because, despite previous studies on unnatural dNTPs that can be polymerized by TdT, such as fluorescently labeled ribonucleotides[30] or dideoxynucleotides (ddNTPs),[31] amine allyl dNTPs,[32] biotin-labeled dNTPs,[33] digoxigenin-dUTP,[34] and modified triphosphate dNTPs,[35] to the best of our knowledge, the ability of TdT to directly incorporate fluorescent dNTPs had heretofore not been explored.

Hence, we examined the ability of TdT to directly incorporate fluorescent dNTPs into a ssDNA chain. We selected conditions of M/I = 1000, 2 h reaction, and three initiator−monomer combinations (Figure S-1c in the Supporting Information) that resulted in efficient initiation and a low polydispersity of the DNA product: Cy5-dA_{10}/dATP, Cy5-dT_{10}/dATP, and Cy5-dT_{10}/dTTP. We found that TdT did not catalyze DNA polymerization efficiently when a fluorescent dNTP was used as the sole monomer in the reaction. Therefore, we examined the effect of varying the ratio of fluorescent dNTPs [Cy3-dATP and Cy3-dUTP] to their corresponding natural dNTPs [dATP and dTTP] on the incorporation of the fluorescent dNTP in the polymerized DNA. As shown in Figure 1a, the extent of incorporation of Cy3-dNTP in the extended chain increased as a function of the ratio of the fluorescent dNTP to natural dNTP. For a Cy3-dATP/dATP ratio of 0.2, up to 50 fluorophores were incorporated into the ∼1 kb long ssDNA chains that was grown by TdT. When we compared the incorporation of Cy3-labeled dNTP using TdT with the incorporation of Cy3-labeled dUTPs obtained using PCR (*Tli* or *Taq* DNA polymerase),[36] TdT can incorporate 4−50 dye/kb with the relative efficiency of primer extension decreasing from 95% to 50% while PCR is only able to incorporate 2−20 dye/kb with lower yields (80%−15%). Taking the same reaction efficiency as a comparative measure (e.g., 80%), TdT can incorporate up to ∼15-fold more dye/kb than PCR. Figure 1a also shows that the initiator−monomer combination that was most successful at incorporation of Cy3-labeled

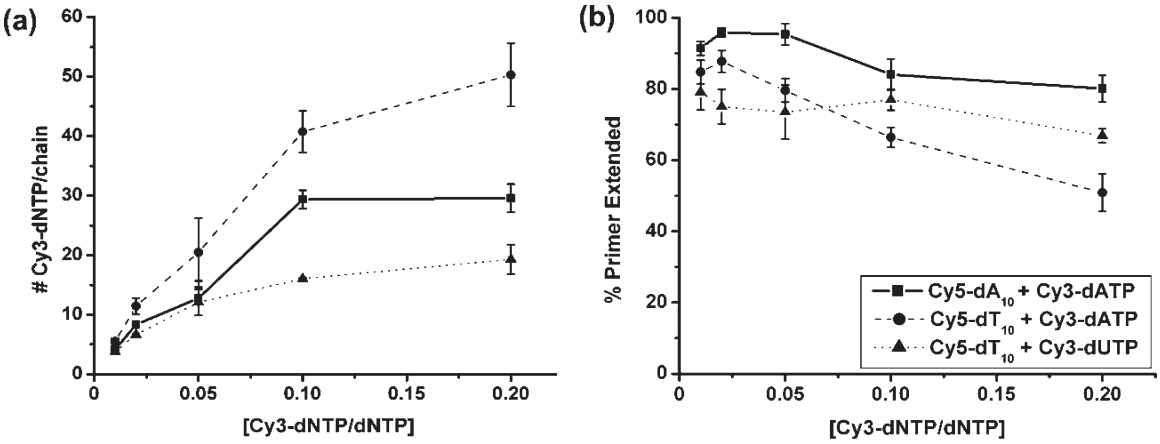


Figure 1. The inclusion of fluorescent dNTPs affects the polymerization DNA by TdT, as shown by the polymerization products of different molar ratios of fluorescent dNTPs to natural dNTPs (0, 0.01, 0.02, 0.05, 0.1, and 0.2): (a) as the mole fraction of Cy3-dNTP increases in the TdT reaction mixture, the number of Cy3-dNTP incorporated per DNA chain increases, while (b) the fraction of extended oligonucleotide initiator [Cy5-dA_{10} and Cy5-dT_{10}] decreases. The data points in panels a and b were obtained from the average of three independent TdT reactions.

dNTP showed the following trend: Cy5-dT_{10}/dATP > Cy5-dA_{10}/dATP > Cy5-dT_{10}/dTTP. On the other hand, as the ratio of fluorescent dNTP to natural dNTP increased in the reaction, the amount of primer (initiator) that was extended decreased (Figure 1b). This trend is consistent with other studies on fluorescent dNTP incorporation using Taq and other DNA polymerases, in which the yield of the PCR product was inversely related to the ratio of fluorescent dNTP to unmodified dNTP in the reaction.[36] Figure 1b shows that polymerization initiation in the presence of fluorescent nucleotide was >80% efficient for the primer−initiator combination of Cy5-dA_{10}-$(dATP)_n$, while a significant drop in polymerization initiation to ~50% was observed for Cy5-dT_{10}-$(dATP)_n$.

In addition, we studied the effect of fluorescent dNTP on the DNA chain length, by visualizing the length of the DNA product by gel electrophoresis. We found the DNA product was shorter when the ratio of Cy3-dNTP to dNTP was ≥0.1 (Figure S-4 in the Supporting Information). This result showed the effect of the inclusion of fluorescent dNTP on the TdT reaction kinetics. As the fraction of fluorescent dNTP increased in the reaction mixture, a shorter and more polydisperse product was generated. The greatest effect of fluorescent dNTP on TdT reaction kinetics was shown by the initiator/monomer combination of Cy5-dT_{10}/dTTP/Cy3-dUTP, which suggests that dUTP is not a preferred substrate for TdT, as compared to dATP and dTTP.[28]

Overall, our results show that the presence of fluorescent dNTP in the reaction affects the initiation step and the kinetics of TdT reaction, so that as the molar ratio of fluorescent dNTP to natural dNTP is increased, the fraction of DNA primer that is initiated during polymerization decreases and the length of extended product also decreases. We determined that up to ~50 Cy3-dATP per kb can be incorporated into extended ssDNA chain within a 2-h reaction time under experimental conditions that are optimized for the incorporation of fluorescent nucleotides.

Incorporation of Fluorescent Nucleotides Using SIEP. After optimizing the polymerization conditions for TdT-catalyzed DNA growth in solution, we selected two optimal combinations from these solution studies for subsequent SIEP: dA_{25} and dT_{25} as the initiators and a mixture of dATP and Cy3-dATP as the monomers. Although previous work by our group[37] has

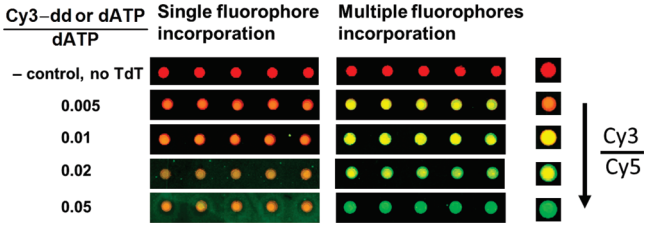


Figure 2. Incorporation of fluorescent dNTPs into ssDNA on the surface from immobilized oligonucleotide initiators by SIEP after 1 h at 37 °C. Fluorescence image of spots of immobilized oligonucleotide initiator incubated with the TdT reaction mixture, visualized by the Cy-5 fluorescence (red) that was incorporated within the initiator sequence and Cy3 fluorescence (green) that was incorporated into the extended DNA chain. The left panel shows Cy3/Cy5 fluorescence images of spots, wherein SIEP was carried out with mixtures of Cy3-ddATP and dATP (molar ratios of 0.005 to 0.05, from top to bottom); the incorporation of Cy3-ddATP causes chain termination, resulting in the incorporation of only a single fluorophore per DNA chain grown by SIEP from an immobilized initiator. The panel on the right shows the Cy3/Cy5 fluorescence from spots, wherein SIEP was carried out using mixtures of Cy3-dATP (green) and dATP. The color transition from red (Cy5) to green (Cy3) verifies the incorporation of multiple Cy3 dye molecules per DNA chain.

shown that SIEP using TdT can lead to a 50-nm-thick poly(A) homopolymer on the surface, those studies did not attempt to incorporate of a fluorescent nucleotide into the DNA chain grown by SIEP. We immobilized an oligonucleotide initiator on a glass substrate, followed by polymerization of nucleotides using a mixture of dATP and Cy3-dATP. All initiators were modified at their 5′-end for covalent immobilization on the surface and labeled internally with Cy5 in order to visualize the microspots of the printed primers on the surface and provide an independent estimate of the spotting density of the probe. The Cy5 dye on the immobilized initiator was placed 15 bases away from the 3′-end, to prevent any energy transfer from Cy3 to Cy5.[36] We selected ratios of Cy3-dATP to dATP of 0.005, 0.01, 0.02, and 0.05 for SIEP. Two types of control experiments were also carried out; (1) SIEP in the absence of TdT, to examine the effect of nonspecific binding of fluorophores to the surface; and (2) SIEP

with Cy3-dideoxynucleotides (Cy3-ddATP) instead of Cy3-dATP. Because ddATP is a chain terminator, the incorporation of Cy3-ddATP only allows a single fluorophore to be incorporated into the DNA strand grown by TdT. Comparison of the Cy3 fluorescence from spots amplified with Cy-dATP and with Cy3-ddATP hence provided a metric to compare the level of fluorophore incorporation by SIEP using Cy3-dATP, and thereby a measure of the degree of fluorescence signal amplification possible with SIEP.

As shown in Figure 2, spots of immobilized Cy5-labeled DNA initiator [5′-NH_2-$(CH_2)_{12}$-dT_{10}-Cy5-dT_{15}] were successfully extended by SIEP and incorporated Cy3-dATP into the DNA chain, as seen by the high level of Cy3 fluorescence intensity from the spots after SIEP. Furthermore, the spots exposed to Cy3-ddATP (left panel) showed some Cy3 fluorescent intensity, albeit with significantly lower intensity than spots that were exposed to the reaction mixture containing Cy3-dATP (right panel). The inclusion of Cy3-ddATP into the reaction mixture ensured that a maximum of only one fluorophore could be incorporated into the DNA chain polymerized by SIEP, because the incorporation of a single Cy3-ddATP causes chain termination. Comparison of the Cy3/Cy5 fluorescence intensities in the left panel with those in the right panel in Figure S-5 in the Supporting Information also clearly indicates the incorporation of mutiple fluorophores when Cy3-dATP is used, and it shows the potential signal amplification that can be achieved by SIEP. In addition to the greater Cy3 fluorescence intensity from spots that were exposed to Cy3-dATP, we also observed an increase in the diameter of the spots, compared to the Cy-5 signal that emanates solely from the immobilized primer, which could be due to the dispersion of a large fluorescence signal or an increase in the footprint of the DNA spots, because of the polymerization of long DNA chains that spill over the margin of the printed spots of the covalently immobilized oligonucleotide primer.

The control spots that were exposed to the TdT reaction mixture without any enzyme in the reaction mixture did not show any detectable Cy3 fluorescence signal, which demonstrates that nonspecific adsorption of free fluorescent dNTPs was below the detection limit and that the Cy3 fluorescent signal is generated exclusively from fluorescent dNTPs that are incorporated into the DNA chains whose polymerization is catalyzed by TdT.

TdT-Catalyzed SIEP Signal Amplification on a Surface. In order to quantify the ability of TdT to add multiple fluorophores into a DNA chain that is polymerized from a short DNA initiator and is immobilized on a surface, DNA spots with a single fluorophore Cy3-ddATP per chain were used as the reference to determine the Cy3 signal amplification. We inferred the average number of Cy3-dATP incorporated per ssDNA on the surface by comparing the fluorescence intensity ratio of Cy3-dATP to Cy5-tagged oligonucleotide initiator from DNA spots that incorporated multiple Cy3-dATP with the ratio of Cy3-ddATP to Cy5-tagged initiator from control spots that incorporated only a single Cy3-tagged "dye-terminator" nucleotide into the extended chain (see Figure S-5 in the Supporting Information). We also compared two surface initiators, dT_{25} and dA_{25}, to study the effect of initiator−monomer composition on signal amplification. In addition, we spotted 2 μM and 10 μM DNA initiator onto a glass substrate to examine the effect of surface initiator concentration on the DNA polymerization and overall signal amplification after multiple fluorophores incorporation. We were only able to test a limited range of

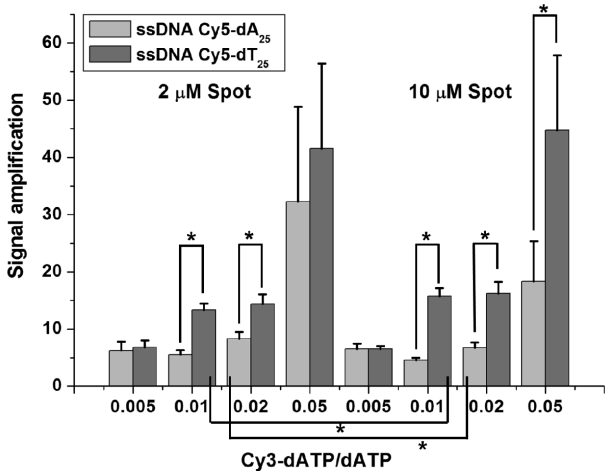


Figure 3. Effect of the composition of oligonucleotide initiator (Cy5-dA_{25} or Cy5-dT_{25}), its concentration, and the mole fraction of fluorescent Cy3-dATP (when mixed with dATP) on the number of Cy3-dATP moieties incorporated into the DNA chain grown by SIEP. Data for the level of signal amplification (mean ± standard deviation (std)) for each sample were averaged from five replicate spots from three independent SIEP reactions. The asterisk (*) represents $P < 0.05$ (Student's t-test), comparing the effect of the type of oligonucleotide initiator and the spotting concentration to the signal amplification generated from different TdT reaction conditions.

immobilized initiator concentrations, because of the limited ability to control the surface density of the initiator. When we spotted <2 μM DNA solution, DNA spots with an inconsistent morphology and fluorescence intensity were observed, whereas spotting >10 μM DNA solution resulted in saturated fluorescence intensity after TdT reaction with Cy3-dATP. As shown in Figure 3, there was no significant change in the signal amplification as the DNA spotting solution concentration increased, which indicates that the DNA polymerization is independent of the initiator concentration on the surface in the range that we tested. However, we did observe differences in the level of signal amplification, as a function of the initiator and monomer combination. Figure 3 shows that the immobilized dT_{25} initiator resulted in higher signal amplification, compared to immobilized dA_{25} as an initiator. The higher signal amplification observed when using dT_{25} as an initiator is contributed mainly by the lower Cy3/Cy5 value of the reference spots. This observation is consistent with the solution experiment where a greater number of Cy3 were incorporated when oligodT was used as the initiator.

The difference in the signal amplification when dA_{25} and dT_{25} were used as the initiator may imply that different initiators will result in a different labeling efficiency. However, we believe that the difference in the signal amplification is caused by the higher degree of Cy5 quenching by the multiple adenosine moieties of the dA_{25} initiator,[38] which resulted in a higher Cy3/Cy5 value of the reference spots and thus lower signal amplification when dA_{25} was used as the initiator. To study the effect of the initiator sequence in greater detail, we carried out an extension in solution using initiators with random sequences. We found that the product of TdT extension is identical, especially when the preferred monomers—dATP or dTTP—were added as monomers to the reaction mixture (see Figure S-2 in the Supporting Information). Since most initiators in a DNA microarray are unlikely to consist of long stretches of a single nucleotide, these

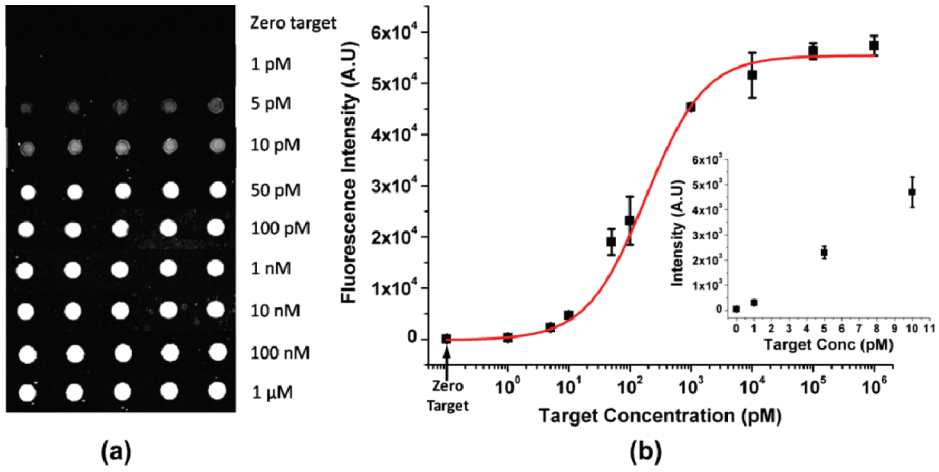


Figure 4. Dose−response of SIEP mediated on-chip fluorescence detection of DNA hybridization in a DNA microarray: (a) fluorescence image of target DNA after hybridization with printed probes on glass, and in situ, TdT-catalyzed SIEP of fluorescent DNA from hybridized target; (b) fluorescence signal intensity, as a function of target DNA concentration, generates a dose response curve with a LOD of ~1 pM and a dynamic range of 2 logs. The inset shows the signal intensity at target concentrations of ≤10 pM.

results suggest that the effect of initiator sequence on the DNA extension by TdT is likely to be minimal.

We also observed an increase in signal amplification as the concentration of fluorescent dNTP increased in the reaction mixture, which again corresponds well with results obtained in solution. The highest signal amplification achieved in this experiment corresponded to an ~45-fold increase in fluorescent signal (equivalent to ~45 Cy3-dATP/chain), compared to reference spots with a single fluorophore per DNA chain. When we compared the fluorescence signal from DNA spots with multiple fluorophores to reference DNA spots with a single fluorophore along the extended DNA chain, the increase in fluorescence signal clearly demonstrated the capacity of TdT to incorporate multiple fluorophores and the possibility of using TdT-mediated DNA polymerization as a method for on-chip labeling and signal amplification.

Although the large number of incorporated dyes has the potential to lead to self-quenching, and if self-quenching between Cy3 dye did occur, its effect was negligible at the reaction conditions that we tested, because we observed significant enhancement in the fluorescence signal upon TdT amplification with fluorescent nucleotides, compared to the control sample, which incorporated only a single fluorescent nucleotide. Furthermore, self-quenching—should it become a problem—can be ameliorated by controlling the average spacing between the fluorescent nucleotides via control of the length of the extended product and by controlling the ratio of fluorescent dNTP and unmodified dNTP in the reaction mixture.

SIEP of Fluorescent DNA Enables on-Chip Detection and Signal Amplification of Hybridization on a Surface. Next, we demonstrated that the ability of TdT to incorporate fluorescent dNTPs could be used to carry out on-chip labeling of target DNA on a surface, as shown in Scheme 1. A 25-base-long target DNA was captured by an immobilized probe and selectively extended by TdT using a ratio of 0.005 (Cy3- dATP to dATP) in the TdT-catalyzed extension of DNA. Multiple Cy3-dATP were incorporated into the extended target and the presence of the target was detected by quantification of the fluorescence intensity from each spot by a fluorescence scanner. The dose−response curve show a sigmoidal response of the background corrected fluorescence signal intensity, as a function of increasing DNA target concentrations, with a linear range that spanned two orders of magnitude in fluorescence intensity (see Figure 4). The dose−response curve also provided a 1 pM limit of detection (LOD), which is comparable to, and, in some instances, better than other DNA target detection and amplification techniques, such as SPR-based detection with Au nanoparticle enhancement,[39] microsphere-based rolling circle amplification (RCA),[14] and fluorescent conjugated polymers.[40]

We also examined the specificity of TdT reaction to the target strand by exposing the probe to the TdT reaction mixture containing fluorescent dNTP in the absence of a bound target. We observed undetectable fluorescence signal from the surface, indicating that the TdT-catalyzed extension of DNA solely occurs from a bound target. However, as the ratio of fluorescent dNTP to its corresponding natural dNTP increased, we found that the nonspecific signal from immobilized probes also increased, for reasons that we do not yet understand.

In terms of sensitivity, the LOD of a sensing technique is generally limited by two factors: the nonspecific signal and the binding constant between the probe and the target strand. Although we expect that the incorporation of multiple fluorophores labeling is one factor in controlling the detection sensitivity, the LOD, in practice, is often not limited by the signal at low concentration, but by the noise, which largely arises from nonspecific binding or extension. The second factor that fundamentally limits the sensitivity of DNA detection is the hybridization affinity of the DNA strands. In this study, we used a DNA probe to capture a DNA target from solution. Using a probe that has a higher affinity for the target, such as locked nucleic acid (LNA) or peptide nucleic acid (PNA), could possibly improve the LOD of SIEP mediated DNA detection.[41] In terms of dynamic range, TdT labeling generated a linear dynamic range that spanned across two orders of magnitude (from 1 pM to 100 pM in the log−log plot), which is typical of fluorescence-based detection technique with a standard DNA microarray scanner.[18] We believe that this dynamic range can be further extended by optimizing the scanner gain settings or using a scanner that can combine signals scanned at two different gain settings for different regimes of target concentration.[42]

dx.doi.org/10.1021/ac200946t |*Anal. Chem.* 2011, 83, 5153–5159

CONCLUSIONS

This paper presents a new methodology for signal amplification on a surface by direct incorporation of multiple nucleotides bearing fluorophores by terminal deoxynucleotidyl transferase (TdT)-catalyzed DNA polymerization. Our results clearly show multiple fluorescent nucleotiodes can be incorporated into the extended ssDNA chain on a surface, a finding that we exploited to develop a post-hybridization, on-chip detection and amplification of DNA hybridization in a microarray format. This methodology has attractive attributes, in that it is both isothermal and on-chip, because the fluorophores are covalently incorporated into a ssDNA chain that is grown from a tethered DNA strand at 37 °C. Compared to other amplification techniques, surface-initiated enzymatic polymerization (SIEP) offers an alternative signal amplification methodology that is straightforward and can be achieved via a one-step isothermal reaction under conditions that are compatible with commercial microarrays.

ASSOCIATED CONTENT

Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Tel.: 919-660-5373. Fax: 919-660-5409. E-mail: chilkoti@duke.edu.

ACKNOWLEDGMENT

A.C. acknowledges the support of the Coulter Foundation, DARPA (Grant No. N66001-09-C-2082) and the NSF (Grant No. CBET-1033621). V.T. acknowledges the support of Duke University through a Nanoscience Graduate Fellowship.

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