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Photoinitiator Nucleotide for Quantifying Nucleic Acid Hybridization

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Abstract

This first report of a photoinitiator-nucleotide conjugate demonstrates a novel approach for sensitive, rapid and visual detection of DNA hybridization events. This approach holds potential for various DNA labeling schemes and for applications benefiting from selective DNA-based polymerization initiators. Here, we demonstrate covalent, enzymatic incorporation of an eosin-photoinitiator 2'-deoxyuridine-5'-triphosphate (EITC-dUTP) conjugate into surface-immobilized DNA hybrids. Subsequent radical chain photoinitiation from these sites using an acrylamide/bis-acrylamide formulation yields a dynamic detection range between 500pM and 50nM of DNA target. Increasing EITC-nucleotide surface densities leads to an increase in surface-based polymer film heights until achieving a film height plateau of 280nm $\pm$ 20nm at 610 $\pm$ 70 EITC-nucleotides/ μ m². Film heights of 10–20 nm were obtained from eosin surface densities of approximately 20 EITC-nucleotides/ μ m² while below the detection limit of $\sim$ 10 EITC-nucleotides/ μ m², no detectable films were formed. This unique threshold behavior is utilized for instrument-free, visual quantification of target DNA concentration ranges.

Keywords

Labeled nucleotides; Photoinitiator; DNA quantification; Microarrays; Signal amplification; Photopolymerization; Surface-Initiated Polymerization

Introduction

Various applications utilize nucleotides modified with detectable groups including gene expression profiling,^{1,2} pathogen identification,³ DNA sequencing reactions⁴ and single-nucleotide polymorphism (SNP) typing.⁵ Approaches for modifying DNA sequences with labeled nucleotides include using enzymatic reactions involving PCR,^{6,7} nick translation,^{6,8} primer extension⁹ and 3'-terminal tailing.¹⁰ Nucleotide reporter groups commonly include fluorophores or hapten molecules (e.g., biotin, digoxigenin) that require a secondary labeling reaction. Fluorescent nucleotides are particularly advantageous owing to the aspects of fluorescence in the context of nucleic acid detection including high sensitivity, ease of use, safety and various measurable properties.¹¹ However, because all these types of nucleotide reporter groups are only capable of a single signal output (e.g., single fluorophore or single

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Supporting Information Available. The supplemental data includes the oligonucleotide sequences utilized in this study (Supplemental A) and a graph displaying the EITC-nucleotide surface densities for capture probe dilution chips after PEX labeling (Supplemental B). This material is available free of charge via the Internet at <http://pubs.acs.org>.

biotin group), alternative approaches exist for generating enhanced signals. These include employing labeled nucleotides with rolling circle amplification,¹² high-density labeling,¹³ and catalyzed reporter deposition reactions.¹⁴ While these approaches prove highly valuable for providing large gains in signal, DNA detection assays could benefit further from investigating alternative signal enhancement techniques.

One such recent signal enhancement approach involves polymerization-based amplification (PBA) that utilizes the fundamental advantages of radical chain photopolymerization reactions¹⁵ to generate an amplified, visual response in the form of crosslinked, high molecular weight polymer films. This outcome is achieved by specifically allocating photoinitiator molecules only to locations undergoing a bio-recognition event by using dual-functional molecules designed to simultaneously bind relevant biological sites and initiate polymerization reactions. The PBA detection scheme was demonstrated on modified silicon surfaces using ultraviolet (UV) light sensitive photoinitiators conjugated with a Neutravidin protein for detecting biotinylated, surface tethered oligonucleotides.¹⁶ An additional PBA system utilized an eosin visible-light sensitive photoinitiator conjugated to a biomolecule^{17,18} (e.g., Streptavidin). This eosin-based visible-light photoinitiation system proves highly advantageous for PBA owing to the mild and inexpensive excitation source and the high amplification efficiency of UV absorbent monomer formulations.¹⁷ Eosin is part of a multi-component photoinitiation system that utilizes a co-initiator to ultimately generate the primary radical species.^{19–20} More specifically, the eosin mechanism likely proceeds by visible light irradiation promoting the eosin dye to an excited state whereupon further energy transfer with an amine co-initiator (e.g., N-methyldiethanolamine) produces photoinitiating radical species.²⁰ Recently, the eosin system was employed for the detection of hybridization events and single-base pair mismatches.^{21–22} Using another approach, He and coworkers utilized the advantages of atom transfer radical polymerization (ATRP) to detect and characterize DNA point mutations on solid surfaces.²³

Here, we expand the PBA utility by presenting a novel eosin photoinitiator-modified nucleotide that contains a unique reporter function based upon a radical chain polymerization reaction. Unlike conventional nucleotide detectable groups including biotin, digoxigenin and fluorophores, the reporter function of the eosin photoinitiator nucleotide is the subsequent polymerization reaction that directly generates a signal amplification resulting in the generation of visible, macroscopic amount of material. This DNA modification is unique in that a single modified nucleotide may rapidly generate a substantial signal enhancement by enabling the polymerization of a large number of monomers (i.e., detectable moieties). For signal amplification, this approach is beneficial since polymerization solely relies upon inexpensive, non-enzymatic reagents such as monomer. Moreover, this novel reporter function holds potential for alternative nucleic acid detection schemes given the general versatility and value of employing functionalized nucleotides (e.g., PCR, nick translation). Using these traditional labeling schemes, this photoinitiator-nucleotide conjugate could potentially be incorporated into any relevant DNA sequences to readily generate unique DNA-based radical chain initiators with all the corresponding benefits associated with sequence-directed assembly and detection. This ability offers an innovative approach for generating radicals and initiating polymerization from any desired DNA sequence.

To this end, we introduce an eosin photoinitiator conjugated directly to 2'-deoxyuridine-5'-triphosphate (EITC-dUTP) and describe its covalent, enzymatic incorporation into surface immobilized DNA sequences and subsequent radical chain initiation from these sites. Here, the EITC-dUTP is specifically utilized for detecting surface-based nucleic acid hybridization events between surface-immobilized, oligonucleotide capture probes and complementary, solution-phase DNA targets. In particular, this approach streamlines and improves the PBA assay by eliminating an assay step used in previous reports (e.g., avidin-biotin) and covalently

incorporates the eosin photoinitiator directly into a surface bound DNA capture sequence during a hybridization event (Figure 1). Using the EITC-dUTP, we further detail an improved dynamic detection range and the subsequent visual, quantitative responses from polymer films obtained on glass slides using oligonucleotides spanning a common mutation in the *p53* gene.

Experimental Section

Synthesis and Purification of Photoinitiator-Labeled Nucleotide

The eosin-5-isothiocyanate (EITC) (Invitrogen) was stored desiccated at -20°C until use. The coupling of EITC to 5-[3-aminoallyl]-2'-deoxyuridine 5'-triphosphate sodium salt (AA-dUTP) (Sigma) occurred using a one-step synthesis similar to described protocols.²⁴ Briefly, EITC in anhydrous DMSO was combined with AA-dUTP in bicarbonate buffer to achieve a final concentration of 10mM EITC, 5mM AA-dUTP, 25vol% DMSO and 100mM sodium bicarbonate, pH 8.3. The solution was agitated for approximately three hours at room temperature and protected from light. The solution was purified using reverse-phase HPLC with a Beckman Coulter Ultrasphere C-18 column (10mm $\times$ 250mm). The sample was eluted using a 0–50% acetonitrile (ACN) gradient at a flow rate of 3.3mL/minute for at least 75-minutes. The product peaks collected from HPLC were analyzed using MALDI-TOF mass spectrometry (with 2',4',6'-trihydroxyacetophenone matrix). The products were further analyzed using UV-Vis spectroscopy to determine concentrations based on known eosin standards and stored at -20°C until use.

Microarray Fabrication and DNA Hybridization

The in-house fabrication of the DNA microarrays (i.e., biochips) occurred using a VersArray ChipWriter Pro (Bio-Rad) and a 375 μm diameter solid pin to deposit 5'-hydrazide-modified capture sequences in a spotting buffer (3X saline-sodium citrate (SSC), 0.05% sodium dodecyl sulfate (SDS)) onto epoxy functionalized glass slides (ArrayIt). The printed microarray slides were incubated in a humid environment for ~ 24 hours at ambient temperature and subsequently washed for two minutes in 2X SSC, two minutes in water and two minutes in cold ethanol. Biochips containing a dilution series of capture probes were fabricated by printing *p53*-248 oligonucleotide probes spanning nearly two orders of magnitude in surface density, ranging from 6500 ± 400 capture probes/ μm^2 to 78 ± 5 capture probes/ μm^2 .

All hybridization reactions employed oligonucleotide targets spanning a frequent mutation in codon-248 of the *p53* gene²⁵ (Supplemental A). The hybridization reactions occurred by spiking an appropriate concentration of target sequence into bovine serum (i.e., representing a complex sample), combining the target with the hybridization solution for a final concentration of (0.2X SSC, 0.04X phosphate buffered saline (PBS), 0.2X Tris-EDTA (TE), 4.4X Denhardt's) and adding the target solutions to the arrays from between 17.5 to 18.5 hours at 45°C . A series of post-hybridization washes (2 minutes per wash) was performed as previously described.²¹ The capture probe surface densities were determined using a 3'-Cy3 labeled positive control capture probe and the identical aforesaid printing procedures. A Cy3 scanner calibration slide (Full Moon Biosystems) was employed to convert the fluorescence readings, obtained using an Agilent Technologies microarray scanner, into surface densities of the fluorophore labeled capture DNA.

EITC-dUTP Surface Labeling

The DNA hybrids on the microarray were labeled using a primer extension (PEX) reaction consisting of 500 U/mL of 3'-5' exo- Klenow fragment (NEB), 10 $\mu\text{g/mL}$ Extreme Thermostable Single Stranded Binding Protein (NEB), 50 μM each of dATP, dCTP, dGTP (NEB), either 0.5 or 0.75 μM EITC-dUTP in a buffer containing 10 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl_2 , 1mM dithiothreitol, pH=7.9. For the PEX reactions with thermophilic

polymerases, the reactions contained 1 μM EITC-dUTP with either 1000 Units/mL of Taq DNA Polymerase (NEB) with a buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl_2 , pH = 8.3 at 25°C) or 400 Units/mL of Vent_R (exo-) (NEB) with a buffer (20 mM Tris-HCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 10 mM KCl, 2 mM MgSO_4 , 0.1% Triton X-100, pH=8.8 at 25°C). The PEX reactions were performed at 37 °C for 30 minutes using the Klenow enzyme (or 55°C for 30 minutes using the thermophilic enzymes) and subsequently washed for 10 minutes in TNT buffer solution (1 M NaCl, 0.1 M Tris, 0.1wt/vol% Tween-20) and briefly rinsed with water. The quantity of photoinitiators/ μm^2 was calculated using an Agilent Technologies microarray scanner (100% PMT, green channel) prior to the polymerization reaction as the eosin photoinitiator is readily quantified through its fluorescence following excitation at 532nm.

PBA Reactions and Post-Polymerization Surface Characterization

A 300 μL volume of a monomer precursor solution containing 5.2M acrylamide (Aldrich), 126mM bis-acrylamide (Aldrich), 218mM methyl diethanol amine (MDEA) (Sigma), and 36mM 1-vinyl-2-pyrrolidinone (VP) (Sigma) was added to the EITC-nucleotide functionalized biochip slides that were secured in an incubation chamber (Whatman) and placed within an argon purged environment. The slides were irradiated for 30 minutes at 40 mW/cm² using an Acticure (Exfo) high pressure mercury lamp with an internal 350–650 nm filter (in-house) and an external 490 long pass filter placed at the end of a collimating lens. After irradiation, the arrays were gently rinsed with water to remove unreacted monomer. The surface polymer films were measured using a Dektak 6M profilometer system with a 12.5 μm diameter diamond stylus tip. To enhance image acquisition, the staining of polymer films occurred by adding 1mL of hematoxylin solution (Aldrich) to each array for one hour and subsequently washing with water. The amplified biochip images were acquired using an Epson Perfection 1650 flatbed desktop scanner.

Results and Discussion

Dynamic range and sensitivity of detecting DNA targets using EITC-dUTP

The covalent, enzymatic incorporation of a photoinitiator-nucleotide conjugate into nucleic acid sequences foremost requires molecular recognition by DNA polymerases as certain nucleotide modifications potentially diminish, or nearly eliminate, polymerase enzyme activity.^{6,13} Here, we prepared the EITC-dUTP conjugate (Figure 2) by conjugating the eosin-5-isothiocyanate photoinitiator to 2'-deoxyuridine-5'-triphosphate (dUTP) to assist in maintaining molecular recognition by DNA polymerases. During hybridization, this C5 site is positioned within the major groove of the double-stranded DNA, thus reducing possible disturbances in base pairing²⁶ and potentially facilitating photoinitiator accessibility for the subsequent radical chain polymerization reaction.

These studies utilized a primer extension (PEX) labeling reaction wherein the surface-based DNA hybrids serve as a 'primer' for a DNA polymerase enzyme to add nucleotides onto the 3'-termini of the surface-tethered capture probes (Figure 1). Using this approach, PEX enzymatic labeling of complementary hybridization reactions were implemented on biochip surfaces fabricated with arrays containing *p53*-248 capture probes (at a capture density of 6500 $\pm$ 400 DNA capture probes/ μm^2). Figure 3A displays the surface density of the EITC-nucleotide resulting from the hybridization with DNA targets at differing concentrations (spiked into serum to represent a complex sample) and subsequent PEX labeling of the surface-based complementary DNA hybrids. These PEX reactions yielded an increased incorporation of EITC-nucleotide after hybridization with a range of target from 0.5nM of target DNA (20 ± 10 EITC-nucleotides/ μm^2) to 1000nM of target DNA (1230 ± 30 EITC-nucleotides/ μm^2). The results in Figure 3 also demonstrate successful molecular recognition of EITC-dUTP by the Klenow fragment of DNA polymerase I (*E. coli*). In addition to the mesophilic Klenow

fragment, the EITC-dUTP was also recognized and incorporated into surface-tethered capture probes by using certain thermophilic polymerases at 55°C including Vent_R (exo-) DNA polymerase and Taq DNA polymerase. For example, after hybridization with 1nM of complementary *p53*-248 target, the EITC-dUTP was covalently incorporated into surface-tethered capture probes (at a capture density of 6500 ± 400 DNA capture probes/ μm^2) at 100 ± 20 EITC-nucleotides/ μm^2 using the Taq enzyme and 40 ± 10 EITC-nucleotides/ μm^2 using the Vent_R (exo-) enzyme. The variations in EITC-nucleotide levels from each enzyme are likely associated with the differing enzymatic activities illustrating that, in general, nucleotide incorporation into DNA sequences using PEX greatly depends upon enzymatic reaction conditions (e.g., enzyme activity, pH, temperature).

After the PEX labeling reactions (using the Klenow fragment), these EITC-nucleotide functionalized chips were further amplified by the PBA reaction for 30 minutes using a 40mW/ cm^2 visible light source. This amplification reaction yielded visible polymer films from hybridization with a range of DNA target solution concentrations from 0.5nM to 1000nM. Analysis of these polymer film thicknesses (Figure 3B) indicates an increase in the film heights, associated with a two order of magnitude increase of DNA target solution concentrations from 0.5nM to 50nM. The PBA response achieved a plateau resulting in average polymer film thickness between 250nm and 300nm above 50nM of target DNA. Taken together, Figure 3A and 3B verify the expected trend that increasing EITC-nucleotide surface density yields a monotonic increase in polymer film thicknesses. Notably, the polymer film heights become independent of the EITC-nucleotide surface densities above 610 ± 70 EITC-nucleotides/ μm^2 . This EITC-nucleotide surface density independence likely arises due to the limitations in radical initiator diffusivity through the growing surface polymer.

In the present studies, employing the EITC-dUTP and an acrylamide monomer formulation improved the dynamic range of DNA hybridization detection by an order of magnitude, as compared to a previous report using a streptavidin photoinitiator conjugate.²¹ This dynamic range improvement may occur, in part, due to the ability to incorporate higher levels of photoinitiators on the surface as associated with the potential incorporation of multiple EITC-nucleotide moieties within a single extended DNA strand. Moreover, the utilization of the acrylamide/bis-acrylamide monomer formulation may facilitate the production of thicker polymer films as previously demonstrated using eosin-modified surfaces.²⁷

Effect of capture probe density on EITC-dUTP PBA assay

An important design parameter in DNA microarrays involves capture probe density owing to the direct effects on hybridization efficiency and kinetics.^{28,29} Here, the effects of capture probe densities on the PBA assay using the EITC-dUTP were explored by using biochips containing a dilution series of *p53*-248 capture probes. The biochips (each containing a *p53*-248 capture probe dilution series) were separately hybridized with differing concentrations of complementary DNA target and subsequently labeled covalently with EITC-dUTP (Supplemental B). These EITC-nucleotide functionalized chips were further amplified by PBA using a 40mW/ cm^2 visible light source for 30 minutes. The resultant film heights (Figure 4A) illustrate the general trend that an increase in capture probe surface density leads to an increase in polymer film thicknesses for each fixed DNA target concentration. For example, an approximate one order of magnitude increase in capture probe density (from 460 ± 40 capture probes/ μm^2 to 4500 ± 200 capture probes/ μm^2) results in a film thickness increase from 100 ± 20 nm to 250 ± 10 nm for hybridization with 50 nM of DNA target. Additionally, polymer film heights increase with increasing target DNA concentration, saturating above 50 nM of target DNA for all capture probe densities as demonstrated by the similar film thickness profiles after hybridization with 50 nM and 1000 nM of target DNA (Figure 4A), in accordance with Figure 3B.

Notably, all acquired polymer film heights (resultant of various capture probe densities and solution target concentrations in Figure 4A) collapse onto a single curve when plotted as a function of the EITC-nucleotide surface density (Figure 4B), likely independent of the means of achieving that eosin surface density. This behavior highlights a crucial and unique aspect of polymerization-based amplification, namely the fundamental dependence of the assay response on photoinitiator level. Given identical polymerization conditions, this PBA assay primarily depends on the photoinitiator surface density (i.e., EITC-nucleotide density) to generate predictable film heights. The graph in Figure 4B also demonstrates that under the current assay conditions, an insignificant initiator surface density (i.e., below approximately 10 EITC-nucleotide molecules/ μm^2) results in a negative response with no detectable polymer films. This low EITC-nucleotide surface density results from the combination of low target DNA solution concentration and low surface-based capture probe densities. For example, at 78 ± 5 capture probes/ μm^2 , hybridization with 5nM of target resulted in a surface density of 3 ± 1 EITC-nucleotides/ μm^2 and no subsequent, detectable polymer films. This absence of polymer films likely results from trace inhibitors present during the polymerization that prevents low initiator levels from generating macroscopically observable polymer.

On-chip, visual quantification of DNA targets

Detecting and quantifying the PBA assay response (i.e., surface-based polymer films) occurs through a variety of techniques. In general, PBA detection exploits the appearance of macroscopic, crosslinked polymeric materials at the relevant analyte surface location. For example, as discussed with Figure 4, the nanomechanical height characterization of surface polymer films (i.e., via profilometry) demonstrates a dependence of surface polymer films on DNA concentrations from solution. Other detection approaches have employed microscopy to quantify fluorescent nanoparticles entrapped within polymer films.³⁰ Additionally, the PBA assay also allows instrument-free detection simply through visual observation of the surface-based polymers. Such visual detection successfully served to identify yes/no qualitative responses for base-specific DNA detection²¹ and antigen detection.³¹

Here, for the first time, this visual detection characteristic was combined with the unique threshold behavior of PBA to design an instrument-free assay capable of visual quantification of target DNA levels. Key to this assay design is the unequivocal negative response (i.e., no polymer films) at inadequate EITC-nucleotide surface densities, here, when the EITC-nucleotide density is below ~ 10 EITC-nucleotides/ μm^2 . Therefore, it would be expected that inadequate initiator density, as a function both of low solution DNA target concentrations and of low capture probe surface density, would result in the absence of polymer films as illustrated in Figure 4. Using this idea, biochips containing a dilution series of capture probes were used to evaluate the unique polymer film response for differing DNA targets from solution.

To test this assay design, separate biochips (each containing a dilution series of capture probes) were separately hybridized with various DNA target concentrations, PEX labeled with the EITC-dUTP and amplified using PBA. Figure 5 displays an example of reverse-grayscale images of surface-based polymer films obtained using a simple desktop flatbed scanner post-amplification. Although polymer films were visible by the unaided eye immediately post-amplification, the polymer films were further stained by immersion in a hematoxylin colorimetric dye to enhance the polymer film contrast and facilitate image collection. Each column represents individual biochips (each biochip containing a capture probe dilution series) that were separately hybridized with a single concentration of *p53-248* complementary target DNA.

The images in Figure 5 clearly illustrate that the appearance of polymer films at each capture probe level directly depends upon the solution DNA target concentrations. Polymer films were visibly apparent for all present capture probe densities at DNA target levels above 10nM

whereas polymer films were only visible at high capture probe densities below 10nM of target DNA concentration. For example, hybridization with 1nM of target DNA results in a polymer film at 1200 ± 100 capture probes/ μm^2 but no polymer film at 340 ± 20 capture probes/ μm^2 . However, hybridization with 100nM of target produced polymer films even at the spot locations containing the lowest density of capture probes (i.e., 78 ± 5 capture probes/ μm^2). Importantly, in all locations void of polymer films, the EITC-nucleotide levels were below the threshold concentration of ~ 10 EITC-nucleotides/ μm^2 . This assay design represents a straightforward approach for visually determining DNA target levels present in solution simply by observing the appearance of polymer films at particular surface locations (i.e., counting the number of spots present post-amplification on an individual array with a capture probe dilution series). This quantitative approach holds potential for applications benefiting from minimal instrumentation for characterizing biomolecule levels.

Overall, utilizing the EITC-dUTP conjugate with the current assay design results in the capacity to visually identify target DNA in solution at a 500pM detection limit. Attempts to detect lower DNA target concentrations resulted in no polymer films under the conditions evaluated here. Improving the surface PBA assay sensitivity likely requires increasing the EITC-nucleotide surface deposition, potentially through alternative labeling approaches. For example, the combination of PBA with methods aimed at increasing the quantity of surface nucleotide levels, such as microarray-based rolling circle amplification, may improve sensitivity in both DNA³² and antibody detection schemes.³³ Moreover, since the current findings demonstrate the capacity to incorporate the EITC-nucleotide with thermophilic DNA polymerases, such as Taq and Vent_R (exo⁻), novel possibilities exist for combining PBA with thermal cycling PCR reactions for target amplification. For example, the incorporation of eosin-nucleotide into fluorescence in situ hybridization (FISH) probes during enzymatic labeling reactions could be useful for new cytogenetic detection methods. This possibility is highly promising as similar cytochemical PBA-based detection schemes are currently being investigated in our laboratory.

Additionally, enhancements in EITC-dUTP assay sensitivity should be readily achieved by combining PBA with the benefits of sensitive fluorescence detection. For example, recent studies utilized fluorescent nanoparticles entrapped within surface-based polymer films to achieve highly sensitive antibody detection.³⁰ Currently, fluorescence nucleic acid detection techniques may demonstrate higher sensitivity relative to the current EITC-dUTP PBA approach. However, the unique attributes of the PBA assay (i.e., visual detection, no instrumentation, and inexpensive signal amplification reagents) combined with unexplored novel EITC-dUTP assay designs (i.e., PCR, rolling circle amplification) may offer innovative approaches for enhanced sensitivities and benefits. Overall, the various existing DNA labeling schemes may benefit from this innovative reporter function of a photoinitiator-labeled nucleotide.

Conclusion

These results convey the first synthesis and utilization of a photoinitiator-nucleotide conjugate and offer a novel approach for the rapid, robust and visual detection of nucleic acid hybridization events on biochip surfaces. Unlike certain traditional nucleotide reporter function (e.g., fluorescence, haptene), the assay response using a photoinitiator-nucleotide involves the formation of macroscopic visual polymer films specifically at surface locations containing the relevant hybridization events, thereby potentially minimizing the necessity for detection instrumentation. The EITC-dUTP conjugate was covalently incorporated into surface immobilized DNA sequences on glass microarrays (i.e., biochips) using DNA polymerases. After the enzymatic incorporation, the EITC-nucleotide maintained initiation capacity yielding visible acrylamide polymer films from 500pM of target DNA present in a serum-based solution. The resultant polymer film heights displayed a dynamic detection range spanning two orders

of magnitude of DNA targets in solution from 500pM to 50nM. The unique threshold behavior of the EITC-nucleotide was evaluated and further employed for an instrument-free, visual quantification of target DNA levels from complex biological samples between 500pM and 10nM of DNA targets. The recognition of the EITC-dUTP by both mesophilic and thermophilic polymerases illustrates the potential for alternative DNA-labeling schemes that could benefit from this novel polymerization-based reporter function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Using the photoinitiator nucleotide (EITC-dUTP) conjugate for detecting nucleic acid hybridization events with the PBA scheme. As shown here, a biochip containing two covalent surface tethered capture probes (A & B) is incubated with a solution that contains oligonucleotide target A (step 1). After the complementary hybridization between capture probe A and target A, the DNA polymerase enzyme incorporates both the EITC-dUTP (represented by *) and unlabeled dNTPs (represented by dashed line) onto the 3'-terminus of the DNA capture probe (step 2). Upon exposure to monomer and visible light (step 3), polymerization occurs solely at biochip locations labeled with the eosin-nucleotide. Ultimately, a positive response is visually observed by the formation of a macroscopic polymer film over the biochip locations that contained complementary hybridization.

Figure 2.
Molecular structure of the photoinitiator comprised of eosin-modified 2'-deoxyuridine-5'-triphosphate (EITC-dUTP).

Figure 3.

Detecting and quantifying *p53*-248 DNA targets from solution using EITC-dUTP and a single capture probe density (6500 ± 400 DNA capture probes/ μm^2). (A) Biochips were separately hybridized with differing *p53*-248 target solution concentrations (from 0.5 nM to 1000 nM of target DNA) and subsequently labeled using the PEX reaction with EITC-dUTP. The graph illustrates an increase in EITC-nucleotide surface density with increasing DNA target concentrations after this PEX labeling reaction. (B) The slides were then irradiated with $>480\text{nm}$ light at $40\text{mW}/\text{cm}^2$ in the presence of 5.2 M acrylamide, 126 mM bis-acrylamide, 218 mM MDEA and 36 mM VP for 30 minutes. The resulting polymer film heights display a logarithmic dependence on DNA target concentrations from 0.5 nM to 50 nM, whereupon the polymer film heights saturate between ~ 250 and 300 nm.

Figure 4.

(A) The dependence of polymer film heights for various capture probe surface densities after hybridization with (∇) 1×10^{-6} M, ($\circ$) 5.0×10^{-8} M, (Δ) 1.0×10^{-8} M, ($\bullet$) 5.0×10^{-9} M, ($\square$) 1.0×10^{-9} M, ($\blacklozenge$) 5.0×10^{-10} M of complementary *p53*-248 DNA target (present in serum-based solution). After PEX labeling, EITC-nucleotide functionalized biochips were amplified by PBA using visible light (40mW/cm²) for 30 minutes. (B.) All acquired polymer film heights, resultant of various capture probe densities and target concentrations, collapse onto a single logarithmic-dependent line, indicating the PBA assay's strict dependence on the EITC-nucleotide surface density. No detectable polymer films were generated from surface locations containing less than ~10 EITC-nucleotides/ μm^2 .

Figure 5.

Visually quantifying DNA target solution concentrations using the EITC-dUTP conjugate. Each column represents an individual biochip containing a capture probe dilution series. Each individual biochip was separately hybridized with a single concentration of *p53-248* complementary target DNA in solution (i.e., 1000, 100, 10, 1, or 0.5nM of DNA target). After EITC-dUTP labeling and a 30 minute PBA reaction, a specific number of polymer spots appear on each biochip, indicative of the DNA levels present in solution. The reverse-contrast images were acquired using a desktop flatbed scanner after staining the polymer films with hematoxylin. (Biochip hybridized with 0.5nM of DNA target did not contain capture probes at a density of 78 ± 5 captures/ μm^2 .)