Fluorescent Structural DNA Nanoparticles Functionalized With Phosphate-Linked Nucleotide Triphosphates

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ABSTRACT

Structural DNA nanoparticles functionalized with phosphate-linked nucleotide triphosphates (dNTPs) were tested as a source of dNTPs for DNA polymerase. The particles were prepared by strand-displacement polymerization from a self-complementary circular template. An intercalating dye (SYBR-101) was joined to the gamma-phosphate of a dNTP by a PEG linker, and the dye-dNTP conjugate was covalently bonded to the nanoparticles. Imaged by AFM, these functionalized particles appear as condensed fuzzy balls with diameters between 50-150 nm. They emit a bright fluorescent signal, detected in 5 msec exposures with a signal-to-noise of 50 when imaged using a TIR fluorescence microscope. A DNA polymerase, adapted by directed evolution to the efficient utilization of phosphate-modified dNTPs, inefficiently utilizes dNTPs supplied from the nanoparticles.

Keywords: single molecule sequencing, phosphate-labeled nucleotide, DNA nanoparticle, SYBR, fluorescent

1 INTRODUCTION

Next generation sequencing efforts are utilizing a variety of new technologies with the aim of reducing the cost of sequencing a human genome to $1000 [1, 2]. Sequencing by ligation, hybridization, denaturation, cyclic synthesis, and through nanopores are some of the emerging technologies that are evolving the way we acquire DNA sequences. Our efforts towards producing a next generation sequencer involve using single molecule DNA sequencing to produce a high-throughput, low cost sequencing methodology. We are investigating a modified form of sequencing by synthesis, in which we directly detect the addition of a nucleotide as it is incorporated into the growing DNA strand.

We classify one of our methodologies as Field-Switch Sequencing, where a surface bound polymerase containing a primed single-stranded DNA template is provided with labeled nanoparticles containing attached dNTP's. The nanoparticles are moved near the polymerase using an electric field, allowing the binding and eventual incorporation of the corresponding nucleotide. Unbound nanoparticles are cleared from the surface by reversing the field charge, and the remaining bound nanoparticles are then detected. In this scheme, rather than using nucleotide triphosphates labeled directly on the base for sequence identification, we employ terminal phosphate-labeled nucleotides [3, 4].

Using nucleotides that contain a fluorescent moiety attached to the terminal phosphate offers a distinct advantage over directly labeling the nucleotide base. As the terminal phosphate-labeled nucleotide is incorporated into the growing DNA strand, the phosphodiester bond formation between the 3'-hydroxyl terminus of the DNA strand and the α-phosphate of the incoming deoxyribonucleoside triphosphate releases the labeled pyrophosphate, resulting in the formation of an unmodified DNA strand. This DNA extension reaction provides an opportunity to visualize and identify the incoming nucleotide, either directly before or after phosphodiester bond formation and release of the labeled pyrophosphate. The release of the labeling moiety and subsequent formation of an unmodified DNA strand provides an opportunity to generate long DNA strands and thus long sequencing reads. Long sequencing reads from base-labeled DNA, on the other hand, are difficult to generate, with the high label content having the tendency to alter the properties of the DNA strand, resulting in insolubility of the highly labeled strand [5].

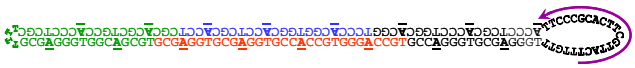
To efficiently detect our terminal phosphate modified nucleotides, we constructed a novel DNA nanoparticle that is both highly labeled and is functionalized with the modified nucleotides. The DNA nanoparticles maintain several characteristics that are essential to this specific method of single molecule sequencing. 1) The nanoparticles produce a sufficiently high signal-to-noise ratio when detected with a 5 msec excitation exposure. 2) They are highly charged and capable of being moved in an ordered fashion within an electric field. 3) They are small (<200 nm cross-section area), providing sufficient resolution for sequencing. 4) They have terminal phosphate-linked dNTP's covalently attached to them that are capable of being incorporated by a DNA polymerase. 5) They are easily produced and stable.

2 RESULTS AND DISCUSSION

DNA nanostructures have been used as structural material and templates for bionanofabrication of composite materials and metals, providing angstrom level accuracy [6, 7]. The power of using DNA as a building block material rests in its ability to accurately self-assemble into defined structures, based on the sequence chosen. Instead of using the self-assembled DNA nanoparticles as a nanoscale template for materials sciences, we used the DNA nanostructures themselves to serve as nucleotide transporters and bright fluorescent point sources. By functionalizing the DNA nanostructures with phosphate-linked dNTP's and labeling them with an intercalating fluorophore, we were able to create an ultrabright fluorescent particle that could serve as a source of nucleotides for a DNA polymerization reaction.

2.1 DNA Nanoparticle Production

The DNA nanoparticles were produced using the DNA polymerase phi29 (Amersham). A circular template was designed to allow strand-displacement rolling-circle amplification (RCA) of the template DNA [8]. The designed template sequence forms a stable hairpin structure that contains approximately 75% GC content over the paired region of the sequence (Fig. 1). An unpaired region within the template allowed for efficient priming and extension of the template DNA using an extension primer and phi29 polymerase. The template sequence itself consists of four DNA primers that self-assemble into the hairpin structure and are ligated prior to RCA. The nucleotide adenosine (A) was strategically spaced along the DNA template, eventually providing regular positions for which to attach the phosphate-linked dNTP's on the amplified product (Fig. 1).



Primer 1 – 5'-GCCAGGGTGCGAGGGTTTGTTTCATTGCTTCACGCCC
TTACCCTCGCACCCTGGCACGG
Primer 2 – 5'-TCCCACGGTGGCACCTCGCACCT
Primer 3 – 5'-CGCACGCTGCCACCCTCGCTTTTGCGAGGGTGGCAGCGT
Primer 4 – 5'-GCGAGGTGCGAGGTGCCACCGTGGGACCGT
Extension Primer -5'-AAGGGCGTGAAGCAATGA

Figure 1: DNA nanoparticle template design and primer sequences.

The RCA takes place in the presence of a limited amount of the nucleotides, dATP, dCTP, dGTP, and the modified nucleotide aha-dUTP (Invitrogen). The aha-dUTP base-pairs with the adenosines along the template sequence and provides a chemically active attachment group. As the RCA reaction proceeds, the nanoparticle self-assembles into a multi-hairpinned structure containing the multiple reactive groups spaced along its surface. Self-termination of the reaction takes place as the dNTP's are depleted in the reaction. The resulting product is a DNA nanostructure of approximately 1500bp (duplexed hairpin DNA) in length. The extended DNA forms condensed fuzzy balls with diameters from 50-150nm in size, as imaged by AFM (Fig. 2). The double-stranded hairpin structures within the nanoparticle allow the intercalating fluorophore SYBR®101 to effectively bind to the DNA, increasing the fluorophore's intensity.

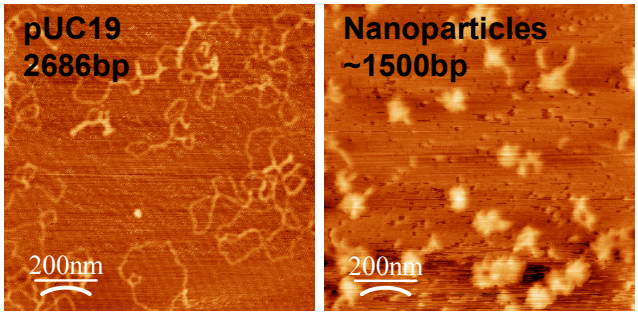


Figure 2: AFM images of DNA nanoparticles and a pUC19 DNA control. The double stranded pUC19 control DNA forms a loose open structure, while the nanoparticles form into compact balls of DNA.

2.2 DNA Nanoparticle Structure

The SYBR®101 terminal phosphate-labeled nucleotides were covalently attached to the DNA nanoparticle through a reactive site on the aha-dUTP (Fig 3). The phosphate-labeled nucleotide itself is first attached to a polyethylene glycol (PEG18) linker purchased from PolyPure (Oslo, Norway). The long linker provides space for the dNTP to move away from the nanoparticle and interact with a DNA polymerase during incorporation. The PEG labeled nucleotide is then reacted with a trifunctional group, allowing both SYBR®101 to be attached, while still providing an additional site for the reaction with the aha-dUTP sites on the nanoparticle. Since SYBR®101 is a DNA intercalating dye, it is possible that the actual covalent attachment of the SYBR®101 terminal phosphate-labeled nucleotides to the nanoparticles is aided by the intercalation of the dye in the DNA nanoparticles.

Dye that is not covalently bound to the nanoparticles is removed by purifying the DNA nanoparticles in the presence of a chaotropic salt [9], such as using a MiniEluit™ PCR clean-up kit from Quagen. Following the removal of the free dye from the nanoparticles, the labeling efficiency was quantitated based on the extinction coefficients of the fluorophore and the DNA nanoparticle. Approximately 30% of the potential binding sites on the DNA nanoparticle were functionalized with the SYBR®101-nucleotide triphosphate moiety, corresponding to a little over 100 fluorophores and free nucleotide triphosphates per nanoparticle.

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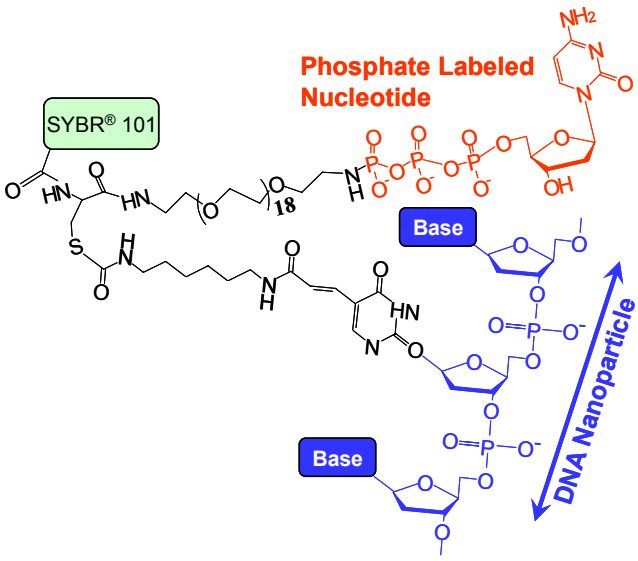

Figure 3: Chemical structure of a SYBR®101 terminal phosphate-labeled nucleotide (Red) attached to one dUTP base on the DNA nanoparticle (Blue). The SYBR®101 can intercalate into the double stranded portions of the DNA nanoparticle. The DNA nanoparticle contains approximately 100 of such labeled nucleotides.

2.3 Fluorescence Detection

The ability to detect single nanoparticles using only 2-5 msec exposure times is paramount in our ability to sequence using our Field-Switch methodology. Since a single nanoparticle bound to a polymerase is detected only after clearing of all unbound nanoparticles by the electric field, but prior to the completed nucleotide incorporation, the detection times are limited to only a few milliseconds. Total internal reflection (TIR) optics are used to detect either single fluorophore molecules or our labeled nanoparticles. Signal-to-noise (SNR) ratios were determined using various exposure times, with the nanoparticles providing a SNR of 27 with only a 5 msec exposure, compared to a maximum SNR of 6 with the single free dye (Table 1). The enhanced SNR of the nanoparticle corresponds to the multiple fluorophores attached to a single nanoparticle within a compact molecular structure of the DNA nanoparticle. The use of only 2 msec exposures with single fluorescent dye molecules gave SNR's that were similar to the background of the signal, and thus not detectable. To further increase the SNR of our nanoparticles, a new 100X TIR objective containing a 1.65 numerical aperture (Olympus) was used on our system. The 100X objective provided a SNR of 50 with a 5 msec exposure and 25 with only a 2 msec exposure (Table 1).

	60X Objective			100X Obj	
	80 msec	5 msec	2 msec	5 msec	2 msec
Labeled DNA Nanoparticles	——	27	19.8	48.6	24.7
AlexaFluor 633	24.4	6	ND	——	——
TAMRA	11.4	3	ND	——	——

Table 1: SNR measurements obtained from either free dye or SYBR®101 labeled DNA nanoparticles for a given exposure time and microscope objective. ND = not detected.

2.4 Nanoparticle Motion

Beyond detecting the labeled nanoparticles at a high SNR given short exposure times, our methodology requires that the dNTP-functionalized nanoparticles effectively move within an electric field. The negative charges associated with DNA provide a structure that is easily influenced by an electric field. Other synthetic nanostructures, such as polystyrene beads and metal nanoparticles produce sufficiently high SNR's, but lack a highly charged surface capable of efficient movement within a field gradient.

Experiments on our functionalized DNA nanoparticles show that the nanoparticles can be moved near the surface of a slide and then quickly cleared from the TIR excitation field using an electric field (Fig 4.). The use of TIR optics produces an evanescent field that decays exponentially in intensity as you move away from the surface of the slide. Therefore, the nanoparticles need only to move slightly away from the slide surface to be out of the excitation field.

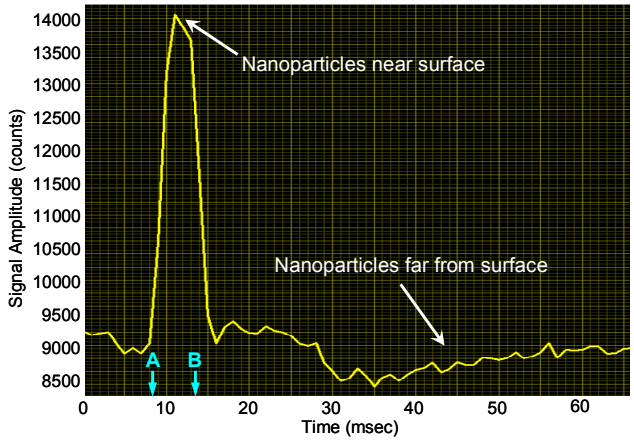

Figure 4: Plot of average signal counts over time detected by TIR microscopy and produced by SYBR®101 labeled DNA nanoparticles. The field charge is switched at time A to move the particles toward the slide surface, producing higher signal amplitude. The field charge is then reversed 5 msec later, at time B, moving the nanoparticles away from the slide surface and reducing the signal amplitude.

2.5 Nucleotide Incorporation

A gel extension assay was used to verify that our dNTP-functionalized nanoparticles were capable of supplying nucleotides for primer extension by a mutant DNA polymerase. The polymerase tested is a mutant form of Therminator (New England Biolabs) and was developed to efficiently incorporate terminal-phosphate labeled nucleotides [10]. The results of the gel extension assay indicate that the mutant polymerase is capable of utilizing the nanoparticle bound nucleotides, however, the ability to incorporate these bound nucleotides is severely reduced (Fig. 5). The wild type Therminator polymerase was shown to be incapable of incorporating these nucleotides over the times tested. The reactions all contained phosphatase, ensuring that any natural dNTP's that could be present in the solutions are deactivated and can not be responsible for the gel extension results.

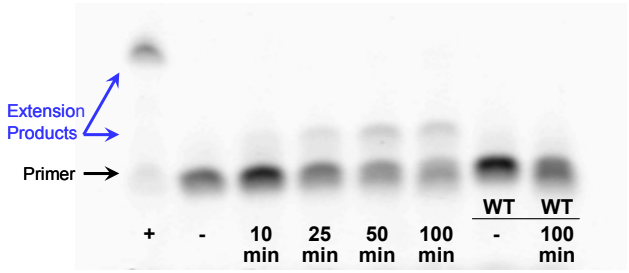

Figure 5: Gel extension assay results showing the successful incorporation of nucleotide triphosphates that were attached to DNA nanoparticles by a mutant DNA polymerase. A positive control (+) containing natural dNTP's, and negative controls (-) containing no dNTP's are shown, along with reactions performed exclusively with nucleotide triphosphate functionalized nanoparticles, using either a mutant polymerase or the wild-type (WT) version of Therminator DNA polymerase (Invitrogen). The mutant polymerase shows an extension product for all time-points, while the wild-type polymerase shows no extension product.

3 CONCLUSIONS

We were able to show for the first time that a DNA nanoparticle, functionalized with covalently linked dNTP's, could be used to feed a DNA polymerization reaction. The ability to use these nanoparticles advances our efforts to use Field-Switch single molecule sequencing. Not only do the functionalized DNA nanoparticles allow for an increased SNR by using the highly labeled balls of DNA, but they also allow for the controlled movement of the nucleotides toward and away from the surface bound polymerases. This combination of bright fluorescent signals and controlled movements is prerequisite for detecting single incorporation events.

The development of these ultra-bright DNA nanoparticles may also be useful in a variety of other applications that require increased SNR's, much like DNA nanotags [11]. It should be possible to functionalize the nanoparticles with a variety of structures and chemical moieties. This flexibility in the ability to construct the nanoparticles should allow for the detection of specific DNA and peptide sequences, by attaching the relevant DNA or antibody to the labeled nanoparticle. The use of a DNA nanoparticle also allows for the efficient clearing of any unbound background signal by using a simple electric field.

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