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Fluorescent Structural DNA Nanoballs Functionalized With Phosphate-Linked Nucleotide Triphosphates

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Abstract

Highly labeled DNA nanoballs functionalized with phosphate-linked nucleotide triphosphates (dNTPs) were developed as a source of dNTPs for DNA polymerase. The particles were prepared by strand-displacement polymerization from a self-complementary circular template. Imaged by atomic force microscopy, these functionalized particles appear as condensed fuzzy balls with diameters between 50–150 nm. They emit a bright fluorescent signal, detected in 2 msec exposures with a signal-to-noise of 25 when imaged using a TIR fluorescence microscope.

Keywords

single molecule sequencing; phosphate-labeled nucleotide; DNA nanoball; SYBR; fluorescent

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.^{1–4} Sequencing by ligation, hybridization, denaturation, exonuclease, cyclic synthesis, and through nanopores are some of the emerging technologies that are evolving the way we acquire DNA sequences. Our own efforts towards producing a next generation sequencer focused on using single molecule DNA sequencing to produce a high-throughput, low cost sequencing methodology. As with other single molecule sequencing approaches, detecting and correctly identifying individual bases is a daunting challenge. To help overcome the limitations inherent in single molecule detection schemes, we developed a system that uses a highly labeled DNA nanoball for identifying incorporated nucleotides. The highly labeled DNA nanoballs provide small, highly-charged, yet extremely bright fluorescent sources from which to detect binding events with millisecond exposures and high signal-to-noise ratios. Similarly designed DNA nanostructures are also currently being used in other next-generation sequencing endeavors.⁵ Using these bright DNA nanostructures, we investigated a modified form of sequencing by synthesis, in which it is necessary to detect the addition of a nucleotide as it is incorporated in real-time into the growing DNA strand.

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SUPPORTING INFORMATION PARAGRAPH

Detailed experimental procedures covering the production and labeling of our DNA nanoballs is included in the supporting information section. These procedures include: Primed template for nanoparticle construction, Rolling circle amplification for Nanoparticle construction, Sucrose purification of DNA nanoparticle, dCTP-PEG8-amine, Amino acid bridge attachment to modified triphosphate, SYBR[®]101-SE conjugation with modified nucleotide triphosphate, Derivatization of amino-nanoparticle to thiol reactive nanoparticle, and Covalent attachment of nucleotide-dye conjugates to nanoparticle.

We classify this method of single-molecule sequencing as Field-Switch Sequencing⁶, where a surface bound polymerase containing a primed single-stranded DNA template is provided with labeled nanoballs containing attached dNTP's. In this method the nanoballs would be moved near the polymerase using an electric field, allowing the binding and eventual incorporation of the corresponding nucleotide. Unbound nanoballs would be cleared from the surface by reversing the field charge, and the remaining bound nanoballs then detected. This scheme requires both the synchronized movement of the nanoballs toward the surface bound polymerases, allowing the capture of a specific nucleotide by a polymerase, and the synchronized movement of the uncaptured nanoballs away from the surface and out of the evanescent field before the polymerase forms a phosphodiester bond and releases the captured nanoparticle. Rather than using nucleotide triphosphates labeled directly on the base for sequence identification, terminal phosphate-labeled nucleotides are employed, allowing the formation of natural, unlabeled DNA strands.⁶⁻⁸

Using nucleotides that contain a fluorescent moiety attached to the terminal phosphate offers a distinct advantage over directly labeling the nucleotide base.⁶⁻⁹ 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 γ -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.¹⁰

To efficiently detect our terminal phosphate modified nucleotides, we constructed a novel DNA nanoball that is both highly labeled and is functionalized with the modified nucleotides. The DNA nanoballs maintain several characteristics that are essential to this specific method of single molecule sequencing. 1) The nanoballs 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 smaller in size (<200 nm cross-section area) than the optical resolution limit, in order to maximize the potential surface density of sequenced DNA molecules. 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. 6) They may eliminate the issues of blinking and non-functional fluorophores that can cause problems in single fluorophore configurations.

DNA nanostructures have been used as structural material and templates for bionanofabrication of composite materials and metals, providing angstrom level accuracy.^{11, 12} 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 nanoballs 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 (SYBR101, Invitrogen) that is covalently attached to the nanoball, we were able to create an ultrabright fluorescent particle that could serve as a source of nucleotides for a DNA polymerization reaction.

The DNA nanoballs were produced using the DNA polymerase phi29 (GE Healthcare). A circular template was designed to allow strand-displacement rolling-circle amplification

(RCA) of the template DNA.¹³ 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 oligonucleotides 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).

The RCA takes place in the presence of a limited amount of the nucleotides, dATP, dCTP, dGTP, and the modified nucleotide aha-dUTP (Invitrogen) (see supplementary material). 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 nanoball 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 nanoball allow the intercalating fluorophore SYBR[®]101 to effectively bind to the DNA, increasing the fluorophore's intensity.

The SYBR[®]101 terminal phosphate-labeled nucleotides were covalently attached to the DNA nanoball 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) (supplementary material). The long linker provides space for the dNTP to move away from the nanoball 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 nanoball. 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 nanoballs is aided by the intercalation of the dye in the DNA nanoballs.

Dye that is not covalently bound to the nanoballs is removed by purifying the DNA nanoballs in the presence of a chaotropic salt¹⁴, such as using a MiniElut[™] PCR clean-up kit from Quagen. Following the removal of the free dye from the nanoballs, the labeling efficiency was quantitated based on the extinction coefficients of the fluorophore and the DNA nanoball. Approximately 30% of the potential binding sites on the DNA nanoball were functionalized with the SYBR[®]101-nucleotide triphosphate moiety, corresponding to a little over 100 fluorophores and free nucleotide triphosphates per nanoball.

The ability to detect single nanoballs using only 2–5 msec exposure times is paramount in a successful implementation of the Field-Switch methodology. Since a single nanoball bound to a polymerase is detected only after clearing of all unbound nanoballs by the electric field, but prior to the completed nucleotide incorporation, the detection times are limited to only a few milliseconds. An Olympus IX70 inverted microscope (Olympus) fitted with total internal reflection (TIR) optics and a Roper Cascade 512B back-illuminated EMCCD camera (Roper Scientific) were used to detect either single fluorophore molecules or our labeled nanoballs. Signal-to-noise (SNR) ratios were determined using various exposure times, with the nanoballs 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 nanoball corresponds to the multiple fluorophores attached to a single nanoball within a compact molecular structure of the DNA nanoball. 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 nanoballs, a 100X TIR objective, 1.65 numerical aperture (Olympus), was used in our system. The 100X objective provided a SNR of 49 with a 5 msec exposure and 25 with only a 2 msec exposure (Table 1).

Beyond detecting the labeled nanoballs at a high SNR given short exposure times, our methodology requires that the dNTP-functionalized nanoballs 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 nanoballs also produce sufficiently high SNR's, but may not maintain a charge-to-mass ratio capable of efficient movement within a field gradient.

Experiments on our functionalized DNA nanoballs show that the nanoballs can be moved near the surface of a slide and then quickly cleared from the TIR excitation field using an electric field (Fig 4.). For these experiments, a sample chamber was constructed to both observe the nanoballs and to move them in an electric field (Fig 5). Corning No. 1 1/2 coverglass slides were custom coated with index-matched indium-tin oxide (ITO) and used as the bottom electrode for the chamber. An insulating layer consisting of Q-Tape (Grace Bio-Labs) with 1.6mm diameter through-holes and a 2mm thick silicone isolators (part # JTR12R-2.0, Grace Bio-labs) served as the chamber wall. The top electrode consisted of a gold voltammetry electrode (part # MF-2014, BAS Inc.). An electrochemistry potentiostat/galvanostat (BAS Inc.) was used to control the potential and measure current flows of the system. As current flows were changed, the signal intensity of our labeled nanoballs was recorded with our inverted TIR microscope using a 60x/NA 1.45 objective. To effectively move the nanoballs, voltage potential on the chamber was varied between zero and 1.5V, resulting in a current of approximately 350 μ A (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 nanoballs need only to move about 200 nanometers away from the slide surface to be out of the intense excitation

A gel extension assay was used to verify that our dNTP-functionalized nanoballs 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 (unpublished results). The results of the gel extension assay indicate that the mutant polymerase is capable of utilizing the nanoball bound nucleotides, however, the ability to incorporate these bound nucleotides is severely reduced compared to γ -labeled dNTP's in solution (Fig. 6). 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 cannot be responsible for the gel extension results.

We were able to show for the first time that a DNA nanoball, functionalized with covalently linked dNTP's, could be used to feed a DNA polymerization reaction. The Field-switch single molecule sequencing is predicated on the ability to use these nanoballs. Not only do the functionalized DNA nanoballs 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 nanoballs may also be useful in a variety of other applications that require increased SNR's, much like DNA nanotags.¹⁵ It should be possible to functionalize the nanoballs with a variety of structures and chemical moieties. This flexibility in the ability to construct the nanoballs should allow for the detection of specific DNA and peptide sequences, by attaching the relevant DNA or antibody to the labeled nanoball. The use

of a DNA nanoball also allows for the efficient clearing of any unbound background signal by using a simple electric field.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Service RF. Gene sequencing. The race for the \$1000 genome. *Science* 2006;311(5767):1544–6. [PubMed: 16543431]
2. Huang X, Barbee K, Chen YJ, Roller E. Towards a \$1000 human genome. *Nanomedicine* 2006;2(4):271–2.
3. Schloss JA. How to get genomes at one ten-thousandth the cost. *Nat Biotechnol* 2008;26(10):1113–5. [PubMed: 18846084]
4. Shendure JA, Porreca GJ, Church GM. Overview of DNA sequencing strategies. *Curr Protoc Mol Biol* 2008;Chapter 7(Unit 7):1. [PubMed: 18231983]
5. Drmanac R, Sparks AB, Callow MJ, Halpern AL, Burns NL, Kermani BG, Carnevali P, Nazarenko I, Nilsen GB, Yeung G, Dahl F, Fernandez A, Staker B, Pant KP, Baccash J, Borcharding AP, Brownley A, Cedeno R, Chen L, Chernikoff D, Cheung A, Chirita R, Curson B, Ebert JC, Hacker CR, Hartlage R, Hauser B, Huang S, Jiang Y, Karpinchyk V, Koenig M, Kong C, Landers T, Le C, Liu J, McBride CE, Morenzoni M, Morey RE, Mutch K, Perazich H, Perry K, Peters BA, Peterson J, Pethiyagoda CL, Pothuraju K, Richter C, Rosenbaum AM, Roy S, Shafto J, Sharanhovich U, Shannon KW, Sheppy CG, Sun M, Thakuria JV, Tran A, Vu D, Zaranek AW, Wu X, Drmanac S, Oliphant AR, Banyai WC, Martin B, Ballinger DG, Church GM, Reid CA. Human genome sequencing using unchained base reads on self-assembling DNA nanoarrays. *Science* 2010;327(5961):78–81. [PubMed: 19892942]
6. Williams JG, Steffens DL, Anderson JP, Urlacher TM, Lamb DT, Grone DL, Egelhoff JC. An artificial processivity clamp made with streptavidin facilitates oriented attachment of polymerase-DNA complexes to surfaces. *Nucleic Acids Res* 2008;36(18):e121. [PubMed: 18723573]
7. Reynolds B, Miller R, Williams JG, Anderson JP. Synthesis and Stability of Novel Terminal Phosphate Labeled Nucleotides. *Nucleosides Nucleotides Nucleic Acids* 2008;27:18–30. [PubMed: 18188766]
8. Williams, JG. Composition and method for nucleic acid sequencing. US Patent Application. US 20050042633A1. 2004.
9. Eid J, Fehr A, Gray J, Luong K, Lyle J, Otto G, Peluso P, Rank D, Baybayan P, Bettman B, Bibillo A, Bjornson K, Chaudhuri B, Christians F, Cicero R, Clark S, Dalal R, Dewinter A, Dixon J, Foquet M, Gaertner A, Hardenbol P, Heiner C, Hester K, Holden D, Kearns G, Kong X, Kuse R, Lacroix Y, Lin S, Lundquist P, Ma C, Marks P, Maxham M, Murphy D, Park I, Pham T, Phillips M, Roy J, Sebra R, Shen G, Sorenson J, Tomaney A, Travers K, Trulson M, Vieceli J, Wegener J, Wu D, Yang A, Zaccarin D, Zhao P, Zhong F, Korlach J, Turner S. Real-Time DNA Sequencing from Single Polymerase Molecules. *Science*. 2008
10. Tasara T, Angerer B, Damond M, Winter H, Dorhofer S, Hubscher U, Amacker M. Incorporation of reporter molecule-labeled nucleotides by DNA polymerases. II. High-density labeling of natural DNA. *Nucleic Acids Res* 2003;31(10):2636–46. [PubMed: 12736314]
11. Yan H, Park SH, Finkelstein G, Reif JH, LaBean TH. DNA-templated self-assembly of protein arrays and highly conductive nanowires. *Science* 2003;301(5641):1882–4. [PubMed: 14512621]
12. Chow DC, Lee WK, Zauscher S, Chilkoti A. Enzymatic fabrication of DNA nanostructures: extension of a self-assembled oligonucleotide monolayer on gold arrays. *J Am Chem Soc* 2005;127(41):14122–3. [PubMed: 16218572]
13. Fire A, Xu SQ. Rolling replication of short DNA circles. *Proc Natl Acad Sci U S A* 1995;92(10):4641–5. [PubMed: 7753856]

14. Singer, V. Personal Communication - Molecular Probes. 2006.
15. Benveniste AL, Creeger Y, Fisher GW, Ballou B, Waggoner AS, Armitage BA. Fluorescent DNA nanotags: supramolecular fluorescent labels based on intercalating dye arrays assembled on nanostructured DNA templates. *J Am Chem Soc* 2007;129(7):2025–34. [PubMed: 17256855]

**Primer 1: 5'- GCCAGGGTGCGAGGGTTTGT
TTCATTGCTTCACGCCCTTAC
CCTCGCACCCTGGCACGG**

Primer 2: 5'-TCCCACGGTGGCACCTCGCACCT

**Primer 3: 5'- CGCACGCTGCCACCCTCGCT
TTTGCAGGGTGGCAGCGT**

**Primer 4: 5'- GCGAGGTGCGAGGTGCCACC
GTGGGACCGT**

Extension Primer: 5'- AAGGGCGTGAAGCAATGA

Figure 1.
DNA nanoball template design and primer sequences. Underlined A's represent potential positions for covalent attachment of terminal phosphate-labeled nucleotides.

Figure 2.

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

Figure 3.

DNA nanoball construction. A) Chemical structure of a SYBR[®] 101 terminal phosphate-labeled nucleotide attached to one dUTP base on the DNA nanoball. B) The SYBR[®] 101 can intercalate into the double stranded portions of the DNA nanoball. C) Rolling circle amplification generates multiple stem loop structures that serve as attachment points for the SYBR[®] 101 terminal phosphate-labeled nucleotides, and D) produces a compact DNA nanoball that contains approximately 100 such labeled nucleotides.

Figure 4.

Moving labeled nanoballs. A) Plot of voltage potential and associated current used to move the DNA nanoballs as measured using an electrochemistry potentiostat/galvanostat. B) Plot of average signal counts over time detected by TIR microscopy and produced by SYBR®101 labeled DNA nanoballs. 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 nanoballs away from the slide surface and reducing the signal amplitude.

Figure 5. Schematic representation of sample chamber constructed to both observe the nanoballs and to move them in an electric. A) The chamber is constructed of an ITO-coated coverglass base with layers of Q-tape and a silicone isolator serving as the sample chamber. B) A gold voltammetry electrode serves as the top of the sample chamber.

Figure 6.

Gel extension assay results showing the successful incorporation of nucleotide triphosphates that were attached to DNA nanoballs by a mutant DNA polymerase.⁶ 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 nanoballs, using either a mutant polymerase or the wild-type (WT) version of Terminator DNA polymerase (Invitrogen). The mutant polymerase shows an extension product for all time-points, while the wild-type polymerase shows no extension product.

SNR measurements obtained from either free dye or SYBR®101 labeled DNA nanoballs for a given exposure time and microscope objective. ND = signal not detectable. Empty cells in the table represent experimental setups that were not performed. Experiments using the 60X objective (Olympus TIRF Plan APO 60x/NA 1.45) were done with standard (n = 1.52) cover glass and index matched immersion oil, while experiments using the 100X objective (Olympus TIRF APO 100x/1.65 NA) were performed with specific (n = 1.788) cover glass (Olympus) and index matched immersion oil (Cargill Laboratories).

Table 1

	60X Objective			100X Obj	
	80 msec	5 msec	2 msec	5 msec	2 msec
Labeled DNA Nanoballs		27	20	49	25
AlexaFluor 633	24	6	ND		
TAMRA	11	3	ND		