Single-Molecule Electronic Detection of Nucleic Acids and Proteins: A New Paradigm for Genetic Analyses and Biomedical Applications

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Abstract - *We explored the use of polymer tags and nanopore for single-molecule electronic detection of nucleic acids and proteins. In this paper, we first report our progress of the research and development of a single molecule real time nanopore-based DNA sequencing by synthesis strategy that accurately distinguishes the 4 bases (A, C, G, T) by electronically detecting 4 different polymer tags attached to the 5'-phosphate-modified nucleotides during polymerase reaction. Then, we present the development of a strategy for an electronic multiplex single nucleotide polymorphism assay using a library of oligonucleotide primers labeled with polymer tags of different lengths by nanopore detection with single molecule sensitivity. The single molecule electronic detection and analyses of target nucleic acids with polymer tagged oligonucleotides are also described. Finally, an approach for the single molecule electronic detection of protein-protein interactions using nanopore and polymer tags is presented.*

Keywords: nanopore, polymer tags, DNA sequencing by synthesis, single-molecule electronic detection.

Introduction

Detection of biological molecules with single-molecule (SM) sensitivity in an efficient cost-effective manner will have wide applications in biomedical science and precision medicine. Currently, the major platform to achieve SM sensitivity uses fluorescence detection that is complex and requires bulky instrumentation. The characterization of polymer transport through a nanometer-sized pore (nanopore) using electrophoresis allows SM electronic detection of polymers in a miniaturized device[1,2]. We have explored the use of polymer tags and nanopore for SM electronic detection of nucleic acids and proteins. The progress of our research efforts is reported in this paper.

1 Single Molecule Electronic DNA Sequencing by Synthesis Using Polymer-Tagged Nucleotides and Nanopore Detection

Single-molecule nucleic acid sequencing platforms will facilitate deciphering complete genome sequences, determining haplotypes, and identifying alternatively spliced mRNAs for biomedical research and precision medicine. DNA sequencing by synthesis (SBS) offers a robust platform to decipher nucleic acid sequences. We have developed a single molecule nanopore-based SBS strategy (Nanopore SBS) that accurately distinguishes the four bases (A, C, G, T) by electronically detecting 4 different polymer tags attached to the 5'-phosphate-modified nucleotides during polymerase reaction. The basic principle of the Nanopore SBS is outlined as follows. As each of the 4 polymer tagged nucleotide analogues complementary to the corresponding base in the template is engaged in the process for incorporation into the growing DNA strand during the polymerase reaction on a complex formed by a polymerase covalently attached to a nanopore and a primed template, its particular tag enters the nanopore and is then released, producing a unique electrical current blockade signature due to the tag's distinct chemical structure, thereby determining DNA sequence electronically at the single molecule level with single-base resolution in real time. As proof of principle, we attached 4 different length polyethylene glycol (PEG)-coumarin polymer tags to the terminal phosphate of the 2'-deoxyguanosine-5'-tetraphosphates. We demonstrated efficient, accurate incorporation of the tagged nucleotide analogues during the polymerase reaction and excellent discrimination among the four tags based on nanopore electrical currents[3]. In a further effort to design additional tagged nucleotide analogues to optimize the Nanopore SBS platform, we explored the potential to use nucleotides tagged on the terminal phosphate with modified oligonucleotide polymers that can be captured and detected by α-hemolysin nanopores. We designed and synthesized several polymer-tagged nucleotide analogues using tags that produce different electrical current blockade levels and verified that they are active substrates for DNA polymerase. A highly processive DNA polymerase was

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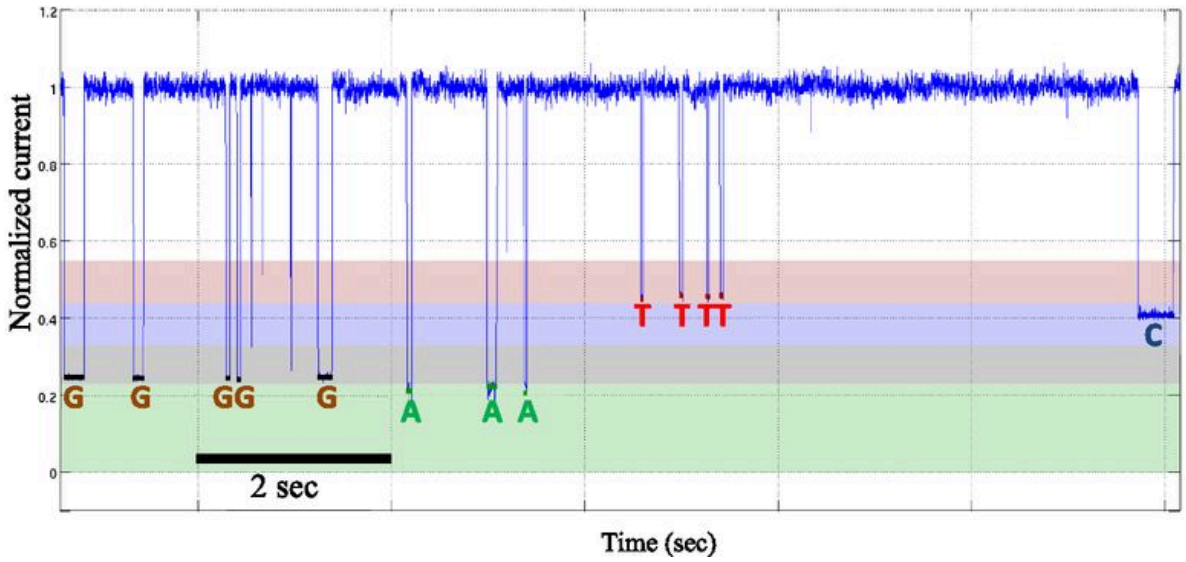

This image shows single-molecule nanopore DNA sequencing by synthesis data from a template with homopolymer sequences[4].

conjugated to the nanopore, and the conjugates were complexed with primer/template DNA and inserted into lipid bilayers over individually addressable electrodes of the nanopore chip. When an incoming complementary-tagged nucleotide analogue forms a ternary complex with the primed template and polymerase, the polymer tag enters the pore, and the current blockade level is measured. The levels displayed by the four nucleotide analogues tagged with four different polymers captured in the nanopore in such ternary complexes were clearly distinguishable and sequence-specific, enabling continuous sequence determination during the polymerase reaction. Thus, real time single-molecule electronic DNA sequencing data with single-base resolution were obtained[4,5]. The use of these polymer-tagged nucleotide analogues, combined with polymerase tethered to nanopores and multiplexed nanopore sensors, should yield a high-throughput single-molecule electronic SBS platform for a wide range of applications in biological research and precision medicine.

2 Single Molecule Electronic Multiplex Single Nucleotide Polymorphism Assay and PCR Analysis Using Polymer Tags and Nanopore Detection

Single nucleotide polymorphism (SNP) refers to a single base variation in the genome of a living organism. SNPs may occur in both coding and non-coding sequences of genes, including regulatory regions. SNPs are the most common genetic differences between human beings. Over 3 million SNPs have been characterized from the human genome. Thus, SNPs are important biomarkers for investigating the molecular basis underlying the mechanism for disease development, thereby laying a foundation for precision medicine.

Using polymer tags and nanopore detection, we have developed a strategy for an electronic multiplex SNP assay using a library of oligonucleotide primers labeled with PEG polymers of different lengths by nanopore detection with single molecule sensitivity[6]. PEG polymers of different sizes have been shown to be electronically detected and differentiated at single molecule level by a protein nanopore[3,7]. The principle of the single molecule electronic multiplex SNP assay is described as follows. A library of primers corresponding to different SNP sites of a target gene is labeled with PEG polymers of different sizes through an azido-based linker that can be efficiently cleaved by tris-(2-carboxyethyl)phosphine (TCEP). The nucleotide at the 3'-end of each PEG-labeled primer is designed to be complementary to a particular SNP in the DNA template. These PEG-labeled primers are used with biotinylated dideoxynucleotides (biotin-ddNTPs) to perform single base extension (SBE). Only the PEG-labeled primer that is fully complementary to the DNA template is extended with a biotin-ddNTP by DNA polymerase. The PEG-labeled DNA extension products that carry a biotin at the 3'-end are captured with streptavidin-coated magnetic beads; the unextended PEG-labeled primers and other components of the SBE reaction are removed by washing. Treatment of the captured DNA products with TCEP cleaves the PEG polymers, which are analyzed by the nanopore. Each different-sized PEG polymer produces a unique nanopore electrical current blockade signature at the single molecule level, which leads to the SNP identification.

Polymerase chain reaction (PCR) that uses primer extension to amplify nucleic acids is a fundamental tool for genetic analysis and clinical diagnosis. Using polymer tags and nanopore detection, we have formulated a method for simultaneously detecting the presence of one or more different target nucleic acids in a sample. This method consists of the following steps: (a) contacting the target

nucleic acids with a plurality of nucleic acid primers simultaneously for primer extension to occur, wherein (i) for each target nucleic acid at least one predetermined primer that corresponds to the target nucleic acid is used, and (ii) each primer has a removably attached polymer tag that generates a unique nanopore electrical current blockade signature; (b) separating the unextended primers from the primer extension products; (c) simultaneously removing the polymer tags from any primer extension products; and (d) detecting the presence of any released polymer tags by the nanopore to characterize the presence of the target nucleic acid in the sample, which is specifically recognized by that predetermined primer.

We have also constructed a PCR method for identifying and quantifying a target nucleic acid using polymer tags and nanopore detection. This approach consists of the following the steps: (i) incubating the target nucleic acid with a complementary oligonucleotide probe, which contains a polymer tag that generates a unique nanopore electrical current blockade signature, to allow the probe to hybridize with the target nucleic acid; (ii) performing PCR that leads to the release of the polymer tag from the hybridized oligonucleotide probe; (iii) detecting by nanopore an electrical current change caused by the released polymer tag; and (iv) correlating the amplitude of the electrical current change determined in step (iii) with the quantity of the polymer tag, thereby identifying and quantifying the target nucleic acid.

3 Single Molecule Electronic Detection of Protein-Protein Interactions Using Nanopore and Polymer Tags

Protein–protein interactions are essential for initiating and regulating biological functions. By attaching one protein to a nanopore and labeling the partner protein with a polymer tag, the occurrence of protein–protein interaction can be detected at single molecule level electronically when the tag is captured by the nanopore[8]. Using such a principle, single molecule electronic characterization of antibody-antigen interaction can be developed for clinical diagnoses.

Conclusions

In summary, using polymer tags and nanopore detection, single-molecule electronic characterization of nucleic acids and proteins can be achieved, offering a new paradigm for genetic analyses and biomedical applications.

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