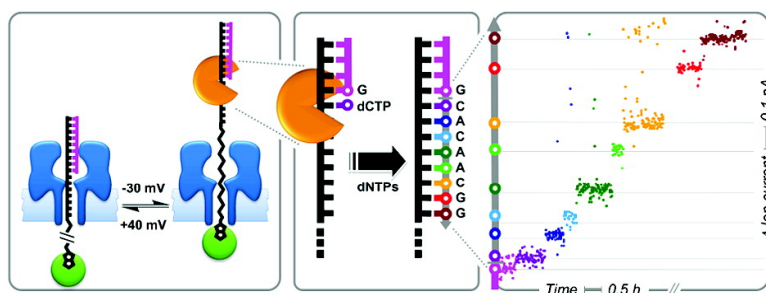


A Single-Molecule Nanopore Device Detects DNA Polymerase Activity with Single-Nucleotide Resolution

Scott L. Cockroft, John Chu, Manuel Amarin, and M. Reza Ghadiri

J. Am. Chem. Soc., 2008, 130 (3), 818-820 • DOI: 10.1021/ja077082c

Downloaded from <http://pubs.acs.org> on February 8, 2009



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 9 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

[View the Full Text HTML](#)



ACS Publications
High quality. High impact.

A Single-Molecule Nanopore Device Detects DNA Polymerase Activity with Single-Nucleotide Resolution

Scott L. Cockcroft, John Chu, Manuel Amarin, and M. Reza Ghadiri*

*Departments of Chemistry and Molecular Biology and The Skaggs Institute for Chemical Biology,
The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037*

Received September 13, 2007; E-mail: ghadiri@scripps.edu

The ability to monitor DNA polymerase activity with single-nucleotide resolution is the cornerstone of a number of advanced single-molecule DNA sequencing concepts.¹ While state-of-the-art single-molecule studies of oligonucleotide–protein complexes have achieved astonishing subnanometer resolutions, the base-by-base activity of DNA polymerase has not been previously resolved.^{2,3} Here we describe a supramolecular nanopore device capable of detecting up to nine consecutive DNA polymerase-catalyzed single-nucleotide primer extensions with high sensitivity and spatial resolution (≤ 2.4 Å). The device is assembled in a suspended lipid membrane by threading and mechanically capturing a single strand of DNA–PEG copolymer inside an α -hemolysin protein pore.⁴ Single-nucleotide primer extensions result in successive displacements of the template DNA strand within the protein pore, which can be monitored by the corresponding stepped changes in the ion current flowing through the pore under an applied transmembrane potential. The ability to resolve DNA polymerase-catalyzed DNA primer extensions with single-base resolution at the single-molecule level represents a promising advance toward nanopore-mediated single-molecule DNA sequencing.⁵ In addition, the system described is expected to have broader utility for studying biopolymer–protein interactions and dynamics.⁶

Single-molecule methods have provided unique views of biomolecules in action.^{2,3,7} Among the emerging single-molecule techniques, *Staphylococcus aureus* α -hemolysin (α -HL) has been employed as a sensor element to probe a range of molecular systems and processes.^{4–6} α -HL forms a heptameric protein pore in lipid bilayers with a central channel approximately 10 nm in length and 1.5 nm in diameter at its narrowest point.⁸ The pore can accommodate the entry and passage of a variety of small molecules and linear polymers including single-stranded DNA (ssDNA) and polyethylene glycols (PEG).^{4–6} A single α -HL protein can be readily inserted into an artificial planar lipid membrane. Due to its permanent open pore structure, a steady and characteristic ionic current results under an applied transmembrane potential. The entry of molecules into the pore gives rise to transient current modulations or blockades that can be used to characterize the molecular species involved.

We recently described a single-molecule method for threading and mechanically capturing ssDNA–PEG hybrid molecules inside the α -HL protein pore.^{4a,d} In the present study, we have employed a similar strategy to configure a supramolecular nanopore device capable of resolving the small spatial changes associated with DNA polymerase-catalyzed single-nucleotide DNA primer extensions. We reasoned that, with an appropriately configured nanopore device, each single-nucleotide primer extension should successively change the position of the threading strand and alter the proportion of the DNA to PEG residing within the pore. Since we have previously shown that the transmembrane ion conductance depends on whether ssDNA or PEG is held inside the pore,^{4a} primer extensions were

expected to give rise to stepped changes in the ion conductance that could be used to ascertain the polymerized state of the primer strand. To test this hypothesis, we employed the following thread design and nanopore device configuration.

The threading component of the nanopore device consists of a 31 nucleotide ssDNA segment bearing the primer binding sites and the template sequence, an 11 nucleotide poly d(A) section, a PEG portion made from eight units of hexaethylene glycol phosphate, and a terminal biotinyl group (Figure 1). The design provides DNA and PEG regions of appropriate length to allow full threading of the α -HL pore as well as the means for locking the threading strand at both ends after its insertion into the pore. Binding of streptavidin to the terminal biotin moiety creates a cap structure that is wider than the α -HL pore entrance and provides a mechanism to direct the formation of the desired α -HL•DNA–PEG configuration.⁴ Under the influence of an electric field, the capped DNA–PEG strand can only thread through the pore from its free 3'-ssDNA terminus and can be held in that configuration as long as the applied electric field is maintained (Figure 1a). Threading of the DNA–PEG strand is signalled by the characteristic reduction in ion conductance caused by the presence of the PEG segment inside the pore. Furthermore, since double-stranded DNA (dsDNA) is also too large to pass through the narrowest part of the α -HL pore, a stable mechanically interlocked complex can be formed by the hybridization of a DNA primer to the 3'-ssDNA end of the threading strand protruding from the pore (Figure 1b). Once the fully interlocked complex has been formed, the position of the threading strand inside the pore can be flipped back and forth by changing the sign of the applied transmembrane potential (Figure 1b and 1c).

Upon application of a positive transmembrane potential, the primer-template DNA duplex region is forced into the pore and held up against the narrowest part of the channel (Figure 1c). Therefore, according to our hypothesis, changes in length of the primer were expected to afford different conductance states at positive potentials by altering the ratio of DNA to PEG occupying the channel. We tested this hypothesis by preparing a series of interlocked complexes **a–d** (Figure 2a–d) using the DNA–PEG threading strand (Figure 1d) and a set of fully complementary DNA primers ranging from 23 to 31 nucleotides in length (primers **+0** to **+8**). In agreement with our hypothesis, each complex displayed a characteristic current–voltage (I – V) trace that depended on the length of the primer (Figure 2e). To further establish that a change in the primer length could be monitored by changes in the ion conductance, we performed the following experiment. Under negative transmembrane potentials, an 8 nucleotide stretch of ssDNA in complex **a** resides outside the pore and is thus free to serve as an initial binding site (toe-hold) for the longer primer (**+8**). Once bound to the toe-hold, primer **+8** should rapidly invade the adjacent duplex region and replace the shorter primer (**+0**) via a

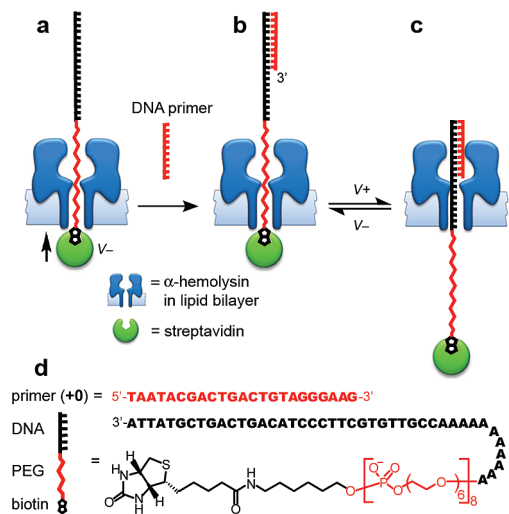


Figure 1. Fabrication and configuration of the interlocked α -HL-DNA-PEG transmembrane complex. (a) A single streptavidin-capped DNA-PEG strand on one side of the membrane is driven into and held within an α -HL pore under an applied negative transmembrane potential. (b) Hybridization of a DNA primer to the protruding 5'-ssDNA region of the threading strand on the opposite side of the membrane furnishes the fully interlocked α -HL-DNA-PEG complex. (c) The position of the threading strand held inside the pore can be flipped back and forth by changing the sign of the applied potential. (d) The sequence of primer (+0) and the composition of the threading DNA-PEG strand.

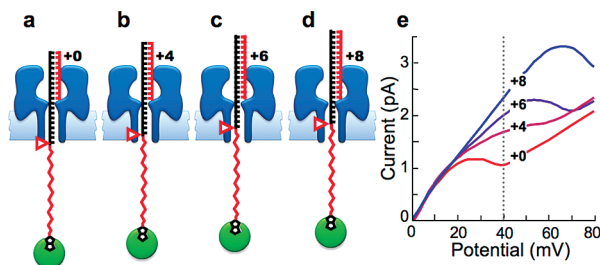


Figure 2. Establishing the device response to changes in primer length. The interlocked α -HL-DNA-PEG transmembrane complexes containing primers of different lengths: (a) 23 nucleotides (+0), (b) 27 nucleotides (+4), (c) 29 nucleotides (+6), and (d) 31 nucleotides (+8). All primer sequences are fully complementary to the 3'-end of the template sequence (Figure 1). As the length of the primer increases, the ratio of PEG to DNA in the pore is increased (red pointers mark the PEG-DNA transition in each complex). (e) Primer length differences manifest themselves in the characteristic current-voltage (I - V) trace of each complex. Although the factors that influence the shape of the I - V traces are not clearly understood, the largest and most reproducible current difference between the four complexes was observed at +40 mV (vertical dotted line). Currents were averaged from the final 0.2 s of the 2 s ion current recording at each potential. The I - V traces were averaged from 10 or more recordings. Experiments were performed under conditions appropriate for DNA polymerase activity (150 mM KCl, 25 mM Tris, 4.5 mM $MgCl_2$, at pH 8.0 and 22 ± 2 °C, see Figure S1).

three-way branch migration mechanism (Figure 3g experiment D, and Figure S3).⁹ Indeed, addition of primer +8 to complex **a** led to an almost immediate and irreversible change in the ion conductance to a state corresponding to that of complex **d** (Figure S16). These initial experiments established that the ion current readout provides a direct measure of the primer length and thus give credence to the application of the nanopore device for monitoring DNA polymerase-catalyzed primer extensions.

Using the nanopore device, we opted to monitor the progress of DNA polymerase-catalyzed primer elongation at +40 mV (*monitoring mode* Figure 3a) since the largest and most consistent differences between the ion currents of the primer-template complexes were observed at this potential (Figure 2e). Elongation

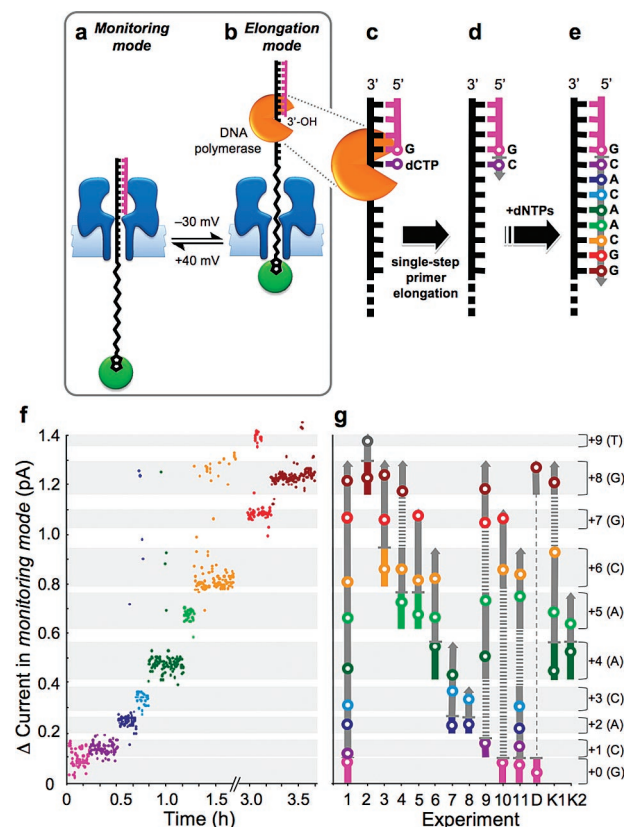


Figure 3. Monitoring DNA polymerase-catalyzed single-nucleotide primer extensions. (a) The length of the DNA primer in a fully interlocked α -HL-DNA-PEG complex is reported by ion current measurements in the monitoring mode (+40 mV). Since in the *monitoring mode* the dsDNA segment is pulled into the pore under the applied positive potential, the DNA polymerases are typically knocked off the DNA template but are able to rebind in the *elongation mode* (-30 mV), the 3'-OH of the primer is accessible to the DNA polymerase. (b) DNA polymerase bound to the starting primer-template complex, with the correct incoming deoxynucleotide triphosphate. (c) DNA polymerase catalyzes the elongation of the primer by incorporating a single dNTP against the DNA template threaded through the pore. (d) Templated extension of the primer sequence after multiple base incorporation steps. (e) Aligned primer sequence after multiple base incorporation steps. (f) A plot of cumulative change in the ion channel current recorded in the monitoring mode versus time. The data contain nine distinct current levels corresponding to the starting primer (+0, Figure 2a), the seven intermediate polymerized states, and the extended primer (+8, Figure 2d). Primer extension was paused for over 1 h after the incorporation of six bases (+6) until the required dGTP was added (note the broken time axis). (g) Alignment of multiple experiments initiated with primers of different lengths (vertical colored bars). The dark gray arrows indicate the direction of primer extension. The white dots in colored circles represent the average cumulative ion current for each length of primer (sem ± 0.05 pA). The horizontal light gray stripes indicate the average current maxima and minima for each primer length. The numbers to the right indicate the base extension relative to the shortest primer (+0), and the letters in parentheses identify the base at the 3'-end of each primer. Dashed lines represent nucleotide incorporation steps that were not sampled by current recordings. Experiment 1 corresponds directly to the current recordings shown in Figure 3f. Experiment D shows the displacement of a short primer (+0) by a longer primer (+8) as described in the main text. TopoTaq DNA polymerase was used in experiments 1–11. The Klenow fragment (exo-) of *E. coli* DNA polymerase-I was used in experiments K1 and K2. Primer extension was terminated with the appropriate dideoxynucleotide triphosphate (ddNTP) in experiments 2, 7, and K2. Experimental conditions were as described in Figure 2, but with the addition of 2 nM DNA polymerase and 0.01–0.2 mM dNTPs. See Figures S5–S18 for complete current versus time recordings for all experiments.

of the primer is inhibited in the *monitoring mode* since the terminal 3'-OH of the primer, which serves as the site for DNA polymerase-catalyzed incorporation of deoxynucleotide triphosphates (dNTPs),

is concealed within the pore vestibule. Conversely, primer elongation is allowed in the *elongation mode* when the 3'-OH of the primer strand is expelled from the pore vestibule under the applied negative transmembrane potential (-30 mV, Figure 3b). No current change was observed in the *monitoring mode* readings when *TopoTaq* DNA polymerase was added to a preformed complex containing the starting primer (+0). However, after the further addition of dCTP, a small increase in the average current at $+40$ mV was observed (Figure 3f, 0–0.5 h). We interpreted this change as arising from the incorporation of a single deoxycytosine opposite the first base (deoxyguanosine) in the threaded template strand (Figure 3c–d). After the addition of dATP, five further current steps were observed, corresponding to the incorporation of five bases against the remaining deoxythymidines and deoxyguanosines in the template sequence (Figure 3d–f, 0.5–1.5 h). Since the next two bases in the template sequence were deoxycytosines, primer extension could be paused at this primer length (Figure 3f, +6) by deliberately delaying the addition of dGTP. Primer extension was allowed to resume by the addition of dGTP, with two further current steps, indicating that the fully extended primer (+8) had been synthesized by the DNA polymerase (Figure 3f, 1.5–3.5 h). A total of nine distinct current levels were observed during this experiment, corresponding to the starting primer (+0), the seven intermediate single nucleotide incorporations (+1 to +7), and the fully extended primer (+8) (Figure 3f and 3g, Experiment 1).

We established the reproducibility and generality of this method for the observation of DNA elongation by performing multiple single-molecule primer extension experiments using primers of different starting lengths and two different types of DNA polymerase. Nucleotide incorporation steps were assigned from the cumulative ion current changes and knowledge of dNTPs added to each experiment. An excellent agreement between the current changes corresponding to each polymerized state was obtained after alignment using a least-squares analysis (Figure 3g). Primer extension could be halted in the absence of a required base or alternatively by the incorporation of a terminating dideoxynucleotide triphosphate (ddNTP), which lacks the 3'-OH group required for the addition of further nucleotides (Figure 3g, Experiments 2, 7, and K2).

The spatial resolution of the nanopore device was assessed by assuming that the incorporation of a single nucleotide displaces the DNA held inside the pore by 3.4 Å (the distance between stacked bases in B-form DNA). From the alignment of the primer extension data in Figure 3g, we observed a total ion current change of 1.3 pA arising from the incorporation of nine bases (equivalent to a translocation of the DNA–PEG strand by $9 \times 3.4 = 30.6$ Å). In this window, the average ion current noise is ± 0.05 pA (Figure 3g). Thus the 30.6 Å displacement can be effectively resolved in thirteen 0.1 pA segments, each representing a 2.4 Å step (30.6 Å/ $13 = 2.4$ Å). The nanopore device achieves its highest spatial resolution (1.4 Å) in the region where single-nucleotide incorporations result in the largest current change, (i.e., +6 to +7, Figure 3g). The high spatial resolution (≤ 2.4 Å) of the nanopore device places it alongside the most spatially sensitive single molecule techniques available for studying biomolecular systems.^{2,3,10}

In conclusion, we have developed a supramolecular assembly capable of monitoring the activity of DNA polymerase as it operates on a single strand of DNA threaded through a protein pore. To our

knowledge, this work represents the first observation of the base-by-base DNA polymerase activity at the single-molecule level. Furthermore, the ability to detect single-nucleotide, angstrom-scale changes in an oligonucleotide template-primer complex using a nanopore opens a new avenue for the study of biopolymers and their interactions with proteins.⁶ The single-base resolution of our approach and the ability to control the passage of DNA in single-base steps satisfy the two minimal requirements of a nanopore-based sequencing device.¹ Nevertheless, further significant advances are required before nanopore-based approaches can be employed in *de novo* DNA sequencing.^{1,11}

Acknowledgment. We thank Professor Hagan Bayley for valuable discussions and are grateful to NIH (Grant HG003709) for financial support. M.A. is a MEC/Fulbright Postdoctoral Fellow.

Supporting Information Available: Materials and method for the nanopore experimental setup, procedure for data recording and analysis, ion current recordings, and complete author list for ref 11b. This material is available free of charge via the Internet <http://pubs.acs.org>.

References

- (1) (a) Shendure, J.; Mitra, R. D.; Varma, C.; Church, G. M. *Nat. Rev. Genet.* **2004**, *5*, 335–344. (b) Bayley, H. *Curr. Opin. Chem. Biol.* **2006**, *10*, 628–637.
- (2) For an optical tweezers-based monitoring of RNA polymerase activity with single-nucleotide resolution, see: Greenleaf, W. J.; Block, S. M. *Science* **2006**, *313*, 801.
- (3) (a) Maier, B.; Bensimon, D.; Croquette, V. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 12002–12007. (b) Braslavsky, I.; Hebert, B.; Kartalov, E.; Quake, S. R. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 3960–3964. (c) Bustamante, C.; Bryant, Z.; Smith, S. B. *Nature* **2003**, *421*, 423–427. (d) Abbondanzieri, E. A.; Greenleaf, W. J.; Shaevitz, J. W.; Landick, R.; Block, S. M. *Nature* **2005**, *438*, 460–465.
- (4) (a) Sanchez-Quesada, J.; Saghatelian, A.; Cheley, S.; Bayley, H.; Ghadiri, M. R. *Angew. Chem., Int. Ed.* **2004**, *43*, 3063–3067. (b) Mathe, J.; Visram, H.; Viasnoff, V.; Rabin, Y.; Meller, A. *Biophys. J.* **2004**, *87*, 3205–3212. (c) Nakane, J.; Wiggin, M.; Marziali, A. *Biophys. J.* **2004**, *87*, 615–621. (d) Ashkenasy, N.; Sanchez-Quesada, J.; Bayley, H.; Ghadiri, M. R. *Angew. Chem., Int. Ed.* **2005**, *44*, 1401–1404.
- (5) (a) Kasianowicz, J. J.; Brandin, E.; Branton, D.; Deamer, D. W. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 13770–13773. (b) Vercoutere, W.; Winters-Hilt, S.; Olsen, H.; Deamer, D. W.; Haussler, D.; Akeson, M. *Nat. Biotechnol.* **2001**, *19*, 248–252. (c) Rhee, M.; Burns, M. A. *Trends Biotechnol.* **2006**, *24*, 580–586. (d) Astier, Y.; Braha, O.; Bayley, H. *J. Am. Chem. Soc.* **2006**, *128*, 1705–1710.
- (6) (a) Movileanu, L.; Howorka, S.; Braha, O.; Bayley, H. *Nat. Biotechnol.* **2000**, *18*, 1091–1095. (b) Howorka, S.; Cheley, S.; Bayley, H. *Nat. Biotechnol.* **2001**, *19*, 636–639. (c) Cheley, S.; Xie, H.; Bayley, H. *ChemBioChem* **2006**, *7*, 1923–1927. (d) Hornblower, B.; Coombs, A.; Whitaker, R. D.; Kolomeisky, A.; Picone, S. J.; Meller, A.; Akeson, M. *Nat. Methods* **2007**, *4*, 315–317. (e) Benner, S.; Chen, R. J. A.; Wilson, N. A.; Abu-Shumays, R.; Hurt, N.; Lieberman, K. R.; Deamer, D. W.; Dunbar, W. B.; Akeson, M. *Nature Nanotechnology* **2007**, *2*, 718–724.
- (7) (a) Wuite, G. J.; Smith, S. B.; Young, M.; Keller, D.; Bustamante, C. *Nature* **2000**, *404*, 103–106. (b) Shaevitz, J. W.; Abbondanzieri, E. A.; Landick, R.; Block, S. M. *Nature* **2003**, *426*, 684–687. (c) Revyakin, A.; Ebright, R. H.; Strick, T. R. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 4776–4780. (d) Revyakin, A.; Liu, C.; Ebright, R. H.; Strick, T. R. *Science* **2006**, *314*, 1139–1143. (e) Kapanidis, A. N.; Margeat, E.; Ho, S. O.; Korkhonia, E.; Weiss, S.; Ebright, R. H. *Science* **2006**, *314*, 1144–1147. (f) Zlatanova, J.; van Holde, K. *Mol. Cell* **2006**, *24*, 317–329. (g) Luo, G.; Wang, M.; Konigsberg, W. H.; Xie, X. S. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 12610–12615.
- (8) Song, L.; Hobaugh, M. R.; Shustak, C.; Cheley, S.; Bayley, H.; Gouaux, J. E. *Science* **1996**, *274*, 1859–1866.
- (9) Li, Q.; Luan, G.; Guo, Q.; Liang, J. *Nucleic Acids Res.* **2002**, *30*, e5.
- (10) Moffitt, J. R.; Chemla, Y. R.; Izhaky, D.; Bustamante, C. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 9006–9011.
- (11) (a) Shendure, J.; Porreca, G. J.; Reppas, N. B.; Lin, X.; McCutcheon, J. P.; Rosenbaum, A. M.; Wang, M. D.; Zhang, K.; Mitra, R. D.; Church, G. M. *Science* **2005**, *309*, 1728–1732. (b) Margulies, M.; et al. *Nature* **2005**, *437*, 376–380.

JA077082C