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Detection of Local Protein Structures along DNA Using Solid-State Nanopores

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ABSTRACT Nanopores have been successfully employed as a new tool to rapidly detect single biopolymers, in particular DNA. When a molecule is driven through a nanopore by an externally applied electric field, it causes a characteristic temporary change in the trans-pore current. Here, we examine the translocation of DNA with discrete patches of the DNA-repair protein RecA attached along its length. Using the fact that RecA-coated DNA and bare DNA yield very different current-blockade signatures, we demonstrate that it is possible to map the locations of the proteins along the length of a single molecule using a solid-state nanopore. This is achieved at high speed and without any staining. We currently obtain a spatial resolution of about 8 nm, or 5 RecA proteins binding to 15 base pairs of DNA, and we discuss possible extensions to single protein resolution. The results are a crucial first step toward genomic screening, as they demonstrate the feasibility of reading off information along DNA at high resolution with a solid-state nanopore.

KEYWORDS Nanopores, translocation, local structure, DNA-protein, genetic screening

In a complex cascade of events, proteins constantly bind and process DNA in order to maintain, transcribe, and replicate this carrier of genetic information. Examples of DNA-binding proteins include transcription factors that modulate the process of transcription, nucleases that cleave DNA, and histones that are involved in tight chromosome packaging in the cell nucleus. The need for the sensitive detection of proteins bound to DNA is manifest. Solid-state nanopores are a good candidate for such a DNA−protein sensor. Both biological pores[1−5] and artificial solid-state nanopores[6−12] have proven their ability to detect single biopolymers. Solid-state nanopores can be fabricated with great control of pore size,[7] allowing molecules of large and variable diameter to translocate. Here, we demonstrate for the first time that it is possible to obtain length-wise "topographic reading" of proteins along a single, unstained DNA molecule at high speed and high resolution using a solid-state nanopore.

Figure 1a shows the experimental layout, depicting a DNA molecule that is locally coated with proteins at a number of sites along its length. Upon threading the molecule through a nanopore, one can monitor the changes in the ionic current and thus potentially distinguish protein-coated patches along the DNA. The experimental setup has been described in detail previously.[13] Briefly, a single nanopore is fabricated in a 20 nm thick, low-stress silicon nitride (SiN) membrane using the focused electron beam of a transmission electron microscope (TEM). The inset of Figure 1a depicts an example of such a nanopore. The membrane is then placed between two compartments filled with a monovalent salt solution (1 M KCl). Application of an electric field across the membrane results in a measurable ionic current through the pore, which is temporarily reduced upon passage of a molecule.

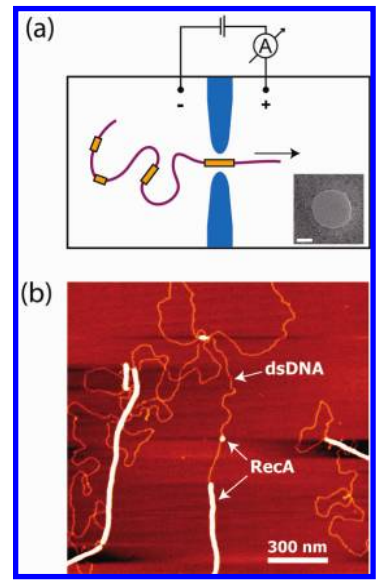

FIGURE 1. Schematic layout and AFM image of partly RecA-coated DNA molecules. (a) Schematic layout of the experiment, showing a nanopore between two liquid compartments. DNA molecules (purple) that are locally coated with RecA proteins (orange) are added to the left liquid compartment and are then pulled through the nanopore electrophoretically. The inset shows a TEM of a 30 nm diameter nanopore. The scale bar is 10 nm. (b) AFM image of partly RecA-coated dsDNA molecules on mica. Thick regions are RecA-coated dsDNA; thinner, more flexible lines are bare dsDNA.

To investigate the ability of the solid-state nanopore system to detect local molecular structure, we use the RecA protein as a model DNA-binding protein. RecA plays a

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central role in DNA repair in prokaryotes, where it catalyzes the pairing of broken DNA with complementary regions of undamaged DNA.[14−16] Related to its in vivo role, this protein is able to polymerize long filaments on both double-stranded DNA (dsDNA) molecules and single-stranded DNA (ssDNA) molecules, as well as form sequence-defined filament stretches involved with triple-strand DNA constructs.[17,18] RecA binding to dsDNA is highly cooperative and RecA-dsDNA complexes formed in the presence of ATPγS (a poorly hydrolyzable ATP analogue) are known to be very stable.[15] The DNA is bound within this filament at a ratio of one RecA monomer per three base pairs and the resultant filament is a factor of 1.5 longer than bare DNA.[17,19] From the crystal structure,[17] we estimate a 7.0 ± 0.5 nm diameter of the local cross section[20] of the RecA-dsDNA complex.

Translocation of such nucleoprotein filaments was recently demonstrated,[21] showing that the electrical conductance blockade associated with RecA-dsDNA molecules is about 12 times larger than that of bare dsDNA at high ionic strength (1 M KCl). This indicates that coated and uncoated molecules can be easily distinguished from each other and, by extension, that coated regions along an individual molecule could also be easily distinguished from bare regions. In the present work, we therefore explore this idea by forming discrete RecA patches on dsDNA and investigating nanopore detection of local structure along a single molecule.

Patchy RecA coatings are formed on dsDNA (λ-phage, 48.8 kbp) by using low protein concentrations and a mechanical filtering method (details in Methods section). Figure 1b shows an atomic force microscopy (AFM) image of the resultant molecules (see Supporting Information Figure 1 for a height profile along the length of this molecule). Discrete RecA patches (thick, white regions in Figure 1b) of variable length, ranging from a few monomers to filaments several micrometers long, can be observed on the dsDNA (thinner, more flexible regions). We obtain a solution with a mixture of DNA molecules with a partial coverage of RecA, ranging from bare DNA to fully RecA-coated DNA. The fraction of RecA-coated DNA is found to be around 20% (see Supporting Information Figure 2). This is in good agreement with the stoichiometric ratio of 1:15 (RecA monomers: bp of DNA) that was used for the formation of the molecules, considering that one RecA protein binds to 3 DNA base pairs.

Upon addition of these molecules to the negatively biased reservoir, we observe short temporal current blockades (see Figure 2a). Each downward spike ΔI in the current represents the translocation of an individual molecule through the pore. We distinguish a number of discrete current levels in this trace. The origin of these levels becomes manifest in examining a histogram of conductance blockades ($\Delta G = \Delta I/V$) of the recorded events ($N = 2799$; Figure 2b). The first peak (0 nS, corresponding to a current of 15.9 nA at 60 mV) is the open pore conductance, which is recorded before and after each event. The peaks at 0.8 and 1.6 nS indicate the

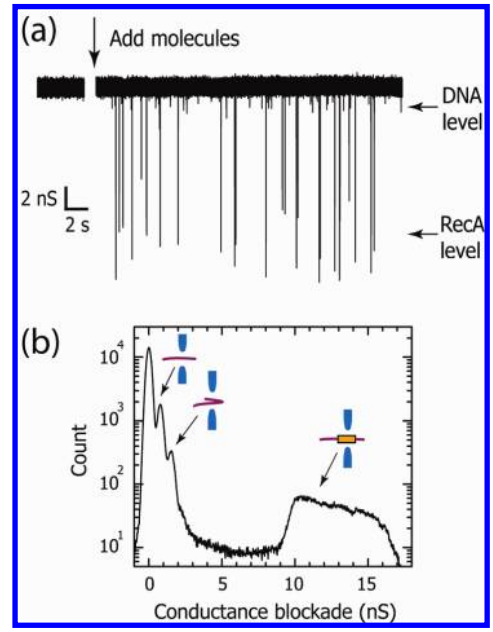

FIGURE 2. Nanopore data. (a) Example current trace before and after addition of the molecules. (b) Conductance histogram of all events recorded at 60 mV (10 μs current samples obtained from 2799 events, including 4 ms of baseline before and after each event). The peaks corresponding to unfolded bare DNA, folded bare DNA,and RecA-coated DNA are indicated in the insets.

presence of one and two strands of bare dsDNA in the nanopore, respectively.[8,22] Finally, the broad peak at 10−16 nS represents the passage of RecA-coated DNA. The peak value agrees well with the earlier measurements of 11.4 ± 0.7 nS for fully RecA-coated DNA.[21] The distribution of RecA blockade levels found is slightly broader than reported by Smeets et al.[21] This is because the molecules in the current work are only locally coated with RecA proteins in contrast to this previous work. In addition to the RecA-coated DNA, bare DNA is thus present. This bare DNA is relatively flexible and can sometimes enter the pore simultaneously with the RecA-coated part, something which is not possible for fully coated molecules. This can result in somewhat larger conductance blockades and thus contributes to a broader distribution in the RecA blockade level.

Interestingly, we observe that individual events can demonstrate over their entire time duration a low-blockade level (<2 nS, 65% of the recorded data) (Figure 3a), a high-blockade level (>10 nS, 6%) (Figure 3b) or can interchange between the two (Figure 3c). We identify this behavior with the passage through the pore of bare dsDNA, fully coated RecA-dsDNA, and partially coated RecA-dsDNA, respectively. Of the partially coated events, we occasionally detect events in which the current level changes frequently between the DNA and RecA levels (Figure 3d,e), in agreement with the random patchiness of the protein coating observed in AFM imaging. For example, Figure 3d shows an event with two sharp spikes; at the left is a downward spike,

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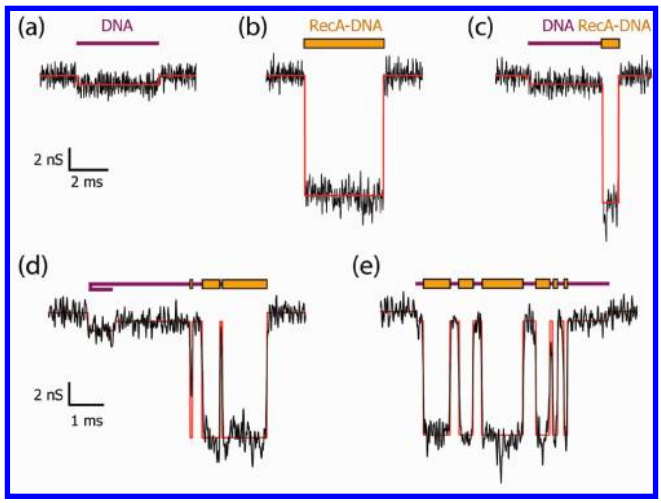

FIGURE 3. Typical nanopore translocation events recorded at 60 mV. Example of current recording of translocations of (a) bare DNA, (b) fully RecA-coated DNA, and (c) partially RecA-coated DNA. (d,e) Current recordings of more complex events in which the current level changes frequently between the DNA and RecA level. The cartoon molecules above the traces indicate the type of molecule that went through the nanopore, where purple stands for DNA and orange for RecA protein.

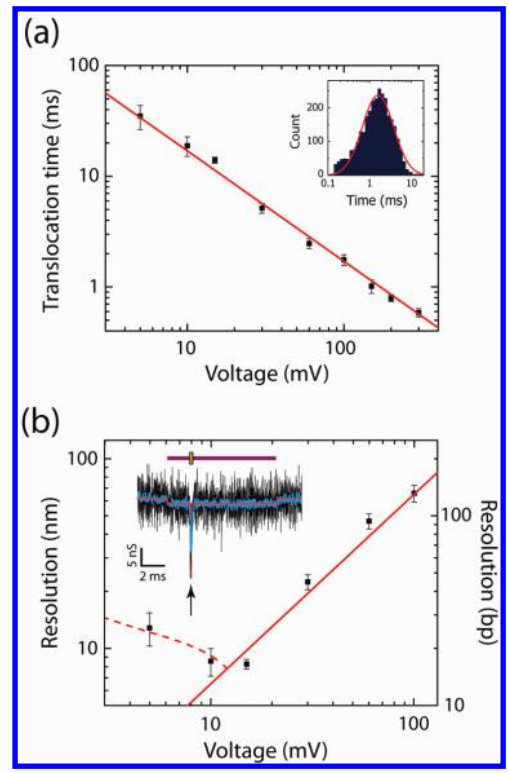

FIGURE 4. Voltage-dependence of translocation time and resolution. (a) Average translocation time versus voltage. The red line is a fit to $\tau \sim 1/V$. The error bars denote the errors from the fit on the positions of the peaks. Only unfolded events are considered. The inset shows a log-normal-distribution fit to a histogram of translocation times at 60 mV. (b) Resolution versus voltage. We find a minimum resolvable distance of about 8 nm (5 RecA proteins binding to 15 base pairs of DNA) at voltages around 10−15 mV. The solid red line is the interpolated resolution at full bandwidth (70 kHz), assuming $\tau \sim 1/V$, as fitted in (a). At low voltages, additional low-pass filtering is required for sufficient signal-to-noise (see text), resulting in a deviation described by the dashed red line. The inset shows the current recording for a DNA molecule with a tiny spot of RecA protein, recorded at 15 mV.

presumably due to a tiny spot of RecA on a bare section of dsDNA, and at the right is an upward spike, presumably due to a small piece of bare dsDNA between two filaments of protein-covered DNA. These example traces show that for each single-molecule translocation event, we can identify the absence or presence and position of proteins along a DNA molecule.

We observe that partially RecA-coated dsDNA molecules show a preference to enter the nanopore with the more flexible bare DNA part first (for example, at 60 mV, bare DNA enters the pore first in 89% of the cases). Presumably, this is due to the lower persistence length of bare DNA (50 nm for bare dsDNA compared to 800 nm for RecA-coated dsDNA[17,18]). We also note that the translocation times are quite similar (see also the scatter plot, Supporting Information Figure 3) for both bare and fully RecA-coated DNA molecules (with log-normal average values of 1.44 ± 0.07 and 1.45 ± 0.18 ms at 60 mV for example), consistent with previous observations.[21] Since the speed at which bare DNA and coated DNA translocates is approximately the same, the time trace is a direct representation of the spatial distribution of RecA protein along the linear DNA molecule, if we assume a uniform translocation speed.

Having established the ability to discriminate local structures along a single molecule, we comment on the resolution capabilities of the technique. The ultimate goal would be to measure a single RecA protein on bare DNA. The (spatial) resolution Δl for the shortest distance that can be resolved along a DNA molecule of length L is defined as $\Delta l = L(\Delta t/\tau)$, where Δt is the minimum resolvable time set by the measurement bandwidth, and τ is the total translocation time of the molecule. A slower translocation speed (with consequent increase in event duration) thus yields a higher resolution, provided that the transitory spikes caused by locally bound proteins can be discriminated above the noise. In Figure 4a, we plot the average translocation time for a range of voltages from 5 to 300 mV. We find that the average translocation time τ increases markedly at lower voltages. A clear $\tau \sim 1/V$ dependence is observed. This dependence is expected intuitively; lower voltage yields a lower electrophoretic force on the molecule, which leads to proportionally lower velocity and thus a longer translocation time. There is, however, a trade-off; to effectively slow down translocation, a lower driving voltage must be applied, which in turn decreases the signal-to-noise ratio.

To allow for discrimination of sharp spikes above the noise, we impose the condition that the signal conductance peak depth ΔG is appreciably larger than the noise level. That is, $\Delta G > p(\delta G)_{\mathrm{rms}}$, where $(\delta G)_{\mathrm{rms}}$ indicates the rms conduc-

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tance fluctuations around the average conductance. We adopt a conservative estimate of $p = 3$ (i.e., the signal exceeds 3 times the rms noise level). Thus, we plot the obtained resolution Δl for detection of a RecA protein patch on the DNA versus applied voltage in Figure 4b. In the lowest voltage range ($V < 12$ mV), additional low-pass filtering is required to meet the criterion of $p = 3$, increasing the value of Δt and thus of Δl. The best resolution is observed in the 10−15 mV range, where we obtain a spatial resolution of about 8 nm. This corresponds to a protein patch of about 5 RecA proteins, binding to 15 base pairs of DNA.

It may be possible to improve the resolution even further. Fologea et al.[23] reported that DNA translocation can be slowed down by increasing the viscosity of the fluid through the addition of glycerol, and additionally by lowering the temperature. This projects a potential resolution approaching single RecA monomers, close to a few base pairs. Alternatively, an integrated optical tweezer approach,[24,25] which allows for the controllable insertion of a DNA molecule into the nanopore, could provide arbitrary control over molecular position relative to the nanopore.

In conclusion, we have demonstrated the detection of proteins locally along DNA using solid-state nanopores for the first time. We show a voltage-dependent resolution of these structures, reaching dimensions as small as 8 nm, or 5 RecA monomers binding to 15 base pairs of DNA. This new capability of measuring local structures along DNA at high resolution is promising for future high-speed, direct-read genetic screening.

Methods ***Solid-State Nanopores.*** Solid-state nanopore fabrication starts with the production of 20 nm thin, free-standing SiN membranes through the use of electron-beam lithography and wet etching. In each such membrane, we drill a nanopore of the desired size through the use of a highly focused electron beam in a TEM. Details of the fabrication process are described elsewhere.[13] In this study, we used nanopores of 30 nm diameter. Nanopores are treated in an oxygen plasma for 30 s on both sides prior to use. Subsequently, the nanopores are mounted in a polyether ether ketone (PEEK) microfluidic flow cell and sealed to liquid compartments on either side of the sample. Measurements are generally performed in 1 M KCl salt solution containing 100 mM Tris-HCl at pH 8.0 at room temperature. The measurements presented in Figure 4 were performed at 1.5 M KCl, 10 mM Tris-HCl. Ag/AgCl electrodes are used to detect ionic currents and to apply electric fields. Current traces are measured at 100 kHz using a resistive feedback amplifier (Axopatch 200B, Axon Instruments). When necessary, further low-pass filtering is performed before digitization at 500 kHz. Only pores with minimal low-frequency current noise (<20 pA rms) are used.[26]

Formation of Dna Molecules with Random RecA Coating. A mixture of 2.4 nM Lambda-DNA (Promega, WI), 7.6 μM RecA (New England Biolabs, Ipswich, MA), 8.6 mM ATPγS (Roche, Switzerland) in 570 mM KCl and 8 mM $MgCl_2$ was incubated for 1 h at 37 °C. ATPγS, a poorly hydrolyzable ATP analogue, was used to prevent ATP hydrolyzation-induced disassembly of RecA from the DNA.[14,15] Subsequent centrifuge filtration (100 kDa filter) serves to both remove unbound protein from the solution and detach loosely bound protein from the DNA itself. The resultant material is left with randomly sized patches of protein-coated DNA. Filtering conditions were 10 000 rpm for 5 min in formation buffer, then rinsing with deionized H_2O under the same spin conditions. Finally, the filtered material was resuspended in 30 μL of measurement buffer (described above). Tapping-mode atomic force microscopy images (Nanoscope IIIa, Digital Instruments, Santa Barbara, CA) were made in air after depositing the DNA molecules from measurement buffer containing an additional 20 mM $MgCl_2$ on freshly cleaved mica.

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Supporting Information Available. Height profile analysis of AFM measurement, comparison of AFM height data to translocation data and to scatter plots, demonstrating statistically equivalent dwell times for bare- and RecA-coated dsDNA. This material is available free of charge via the Internet at http://pubs.acs.org.

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