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Detection of RNAP-DNA complexes using solid state nanopores

C. Raillon, P. Granjon, M. Graf and A. Radenovic

Abstract—**Transcription is the first step in gene expression where DNA is copied into RNA. It is extensively studied at the bulk level especially the regulation mechanism, which in cancerous cells is impaired. We were interested in studying E. coli RNAP enzyme at the single-molecule level for its functional as well as molecular motor properties. With nanopore sensing, we were able to observe RNA polymerase-DNA complexes translocate through nanopores and able to distinguish between individual complexes and bare RNA polymerase. We were also able to observe orientation of RNA polymerase in the nanopore whether flow or electric field predominates. The complexity of the signals from the protein-DNA complexes experiment motivated us to develop level detection software. This software is based on a change detection method called the CUSUM algorithm. OpenNanpore software was designed to analyze in details current blockages in nanopore signals with very little prior knowledge on the signal. With this work one can separate events according to their number of levels and study those sub-populations separately.**

I. Introduction

In this paper, we summarize recent findings on the power of nanopore-based sensing to detect and identify specific proteins or DNA-protein complexes based on the shape of its corresponding ionic current translocation traces [1, 2]. Nanopore-based analysis is an innovative technique used for a wide range of applications such as molecule length measurement, single-molecule dynamics or sequencing [4-6].

In the past decade, many single-molecule techniques have been used for a wide variety of biological systems: proteins, nucleic acids or complexes of the two. Most of what we know today about the transcription reaction was learnt from bulk studies, in which one measures the average signal issued from a very large number of molecules. In recent year, however, researchers have started measuring fundamental properties using single-molecule experiments. For example, by attaching a single molecular motor such as RNAP to a bead, researchers have been able to probe properties like step size, speed and force using optical tweezers [7-10]. Here, we show how nanopore sensing and nanopore based force spectroscopy can be used to study RNAP-DNA interaction at the single-molecule level.

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A. Solid state nanopore fabrication

For single-molecule applications such as nanopore sensing, the nanopore chip should satisfy several requirements. The membrane should contain one pore only, and the pore size should be comparable to the DNA diameter (2 nm for dsDNA) so that DNA passes through the pore in a linear fashion and the ion flux is significantly reduced when DNA molecule translocates.

The pore geometry needs to be well defined and reproducible. In addition, the pore surface should be hydrophilic to facilitate the microfluidic cell filling with solution and it should be neutral or positively charged to avoid DNA repulsion. The membrane has to be strong enough to withstand experiments for several hours but thin enough to make a nanopore in it. The chip should also tolerate salt concentrations ranging from 0.1 - 2 M KCl and different pH conditions (2-9 pH). The starting substrate, for nanopore fabrication, is a double-sided polished silicon wafer with a triple stack of insulating layers, 20 nm of low-stress silicon nitride (Si_xN_y), 100 nm of silicon dioxide (SiO_2) and 100 nm of Si_xN_y. The supporting silicon and insulating layers on the backside are dry and wet etched to form a 50x50 μm^2 large, 220 nm thick membrane. Then electron beam lithography (EBL) is used to define a small square (500 x 500 nm^2) in the center of the Si_xN_y membrane. This region is then thinned to less than 20 nm using dry and wet etching.

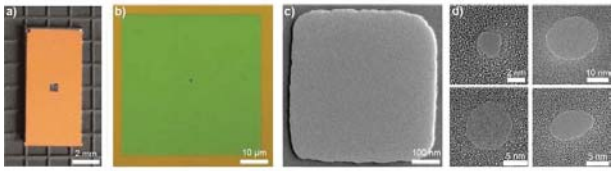

Figure 1. a) 4 x 8 mm^2 silicon chip, KOH groove is visible in the center of the chip **b)** Optical image of a 50 x 50 μm^2 Si_xN_y. membrane (in green) in the center of the chip shown in a). This 220 nm thick membrane is composed of three stacked layers (20 nm low-stress Si_xN_y., 100 nm SiO_2 and 100 nm low-stress Si_xN_y.) and is created by standard processes of photolithography, dry and wet etching. The small black dot in the center of the membrane corresponds to the thinned region **c)** TEM image of the thinned region; fabricated by electron beam lithography and etched down to a thickness of 20 nm. d) TEM images of several nanopore sizes. Taken from [3].

This allows the creation of a very thin membrane, mechanically stable because made of Si_xN_y. That region is thin enough to drill a nanometer-sized hole with a highly focused electron beam. In this microfabrication process, we obtain 72 chips per wafer one such chip is shown in Figure 1. The tool used to drill nanoscale holes is typically a Transmission Electron Microscope (TEM). We use a Philips/FEI CM300 to make the nanopores. Nanopores form due to localized heating that fluidizes matter, and surface tension that creates geometry-dependent contraction or expansion [12]. The optimal parameters for nanopore drilling are following: an accelerating voltage of 200 kV and an extraction voltage of 4.2 kV using condenser lens 3 and spot size 5 (~10 nm) at a magnification of 220 000 X. In TEM mode, the beam is focused with the intensity knob for about 1 min. Sometimes it can take up to several minutes to obtain a nanopore; this mostly depends on the chip cleanliness. There are several ways to clean a nanopore chip from hydrocarbon contamination, we often use O_2 plasma.

The problem with this contamination is that it can migrate into the electron beam and be immobilized [13]. We were originally drilling nanopores at an accelerating voltage of 300 kV but, in order to limit hydrocarbon contamination we have lowered it to 200 kV and we have used the cold trap; which condenses all vapors and so limits contamination at the nanopore drilling site. It is also best to wait that drift stabilizes before starting the drilling process, and 30 min is usually enough. The use of TEM as a nanopore fabrication tool allows for the variation of nanopore sizes. Figure 1 d) shows four nanopores ranging from 2 to 20 nm. This is extremely important since it allows tuning of the pore size, one of the prerequisites for recognition of protein-DNA complexes [3].

B. Measurement conditions and setup

Once the pore is fabricated, it is put into a microfluidic cell that is filled with an ionic solution Figure 2 a). To optimize translocation signal, the size of the pore is matched to the size of the protein, DNA or Protein DNA complex. In our case pores slightly bigger than RNAP-DNA (around 20 nm) complex gave best results.

To get an ionic flow, the pore needs to be hydrophilic, to do so we store the chips in degassed and filtered 1:1 ddH_2O:EtOH solution until use. This straightforward step yields highly reproducible wettability of nanopores. Two other solutions are to use O_2 plasma or to deposit aluminum hydroxide (Al_2O_3) on the chip after pore drilling [13]. ALD (Atomic Layer Deposition) can also be used to precisely control the nanopore diameter. In our hands, the first simple solution of storing nanopores in a mixture of water and ethanol worked better than the Al_2O_3 deposition.

Transparent microfluidic cells allow for fast detection, hence removal, of large air bubbles. An Axopatch 200B (Molecular Devices, Inc. Sunnyvale, CA) is used to amplify the ionic current through the nanopore. The Axopatch is a low noise patch clamp amplifier widely used in nanopore setups. The Ag/AgCl electrodes are immersed in the buffer solution immersed in the reservoirs on each side of the nanopore and connected to the Axopatch preamplifier.

The microfluidic cell and the Axopatch preamplifier are mounted on a damping breadboard (Thorlabs, NJ) and closed in a Faraday cage.

The sample is introduced into the cis side of the nanopore, and a voltage is applied across the membrane In the absence of a flow, the DNA will face two different regimes, one regime that is diffusion limited (far away from the nanopore) and one regime that depends on the pore diameter, its length and the applied voltage (closer to the nanopore. Signals are filtered at 10 kHz using the low-pass Bessel filter built-in the Axopatch and sampled at 100 kHz using a National Instrument PXI-4461 DAQ card. This allows for low-noise measurement.

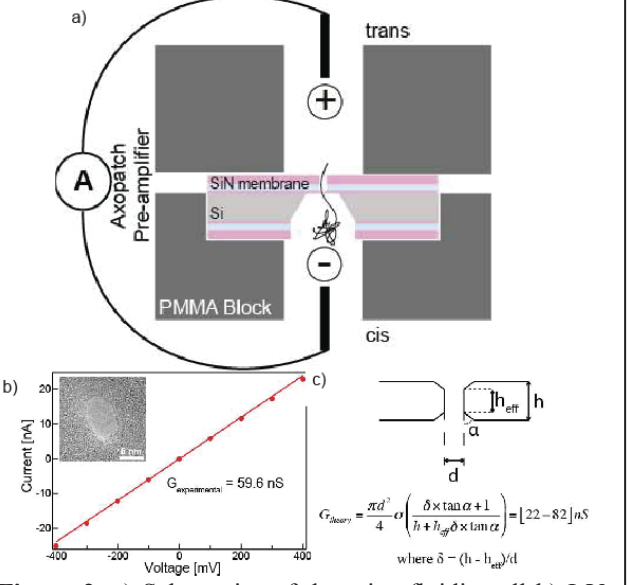

Figure 2. a) Schematics of the microfluidic cell b) I-V characteristic of nanopore in inset (diameter of 5.4 x 9.6 nm2) taken at 1 M KCl at room temperature, resulting experimental conductance is written in the graph c) Formula taken from Kim et al. [7] to calculate the theoretical conductance range for this nanopore with α = [0° - 45°]. Illustration taken from [3].

To allow for discrimination of events above the noise level, a custom made LabVIEW program only records data when the current signal peak depth is above 4 x IRMS and the event longer than 50 μs, which makes files lighter and easier to analyze. The applied voltage can be controlled directly on this interface. This same voltage can be swept to establish the I-V characteristic of the nanopore before starting the experiment Figure 2 b) and c) [2, 3].

II. Results

The higher the voltage, the bigger the capture radius [14]. In the experiments on bare RNAP and RNAP-DNA

complexes, we tried to keep the applied voltage as low as possible (50 mV) in order to minimize the electrophoretic force and maximize the dwell times for each event. This proved to be successful in most experiments and allowed us to visualize orientation of bare RNA whether force or flow predominates as well as detect individual protein-DNA complexes [2, 3]. Typical ionic current events from experiment with RNAP-DNA complexes with RNAP halted at one end of the DNA template are shown in Figure 3.

In our recent publications [2, 3] we described the appropriate volume-based approach using previous knowledge on RNAP, its dipole, its charge and its atomic structure. For the first time, we have shown that at low bias voltage:

1) RNAP translocates in different measureable orientations whether it is the RNAP dipole or the drag force that predominates.

2) using a volume-based approach we have shown that we could distinguish between bare RNAP and single RNAP-DNA complexes using nanopore sensing.at higher voltages:

3) RNAP subunits could break off the core enzyme in measurable subunits

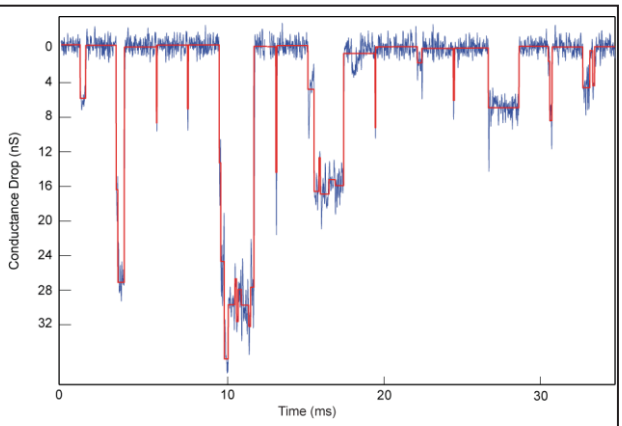

Figure 3. Concatenated events from experiment with RNAP-DNA complexes with RNAP halted at one end of the DNA template. Example of a current trace (in blue) after addition of RNAP-DNA complexes to the 16 x 19 nm pore in 1 M KCl solution with 50 mV applied. In red the fit obtained using the *cumulative sum* (CUSUM) algorithm. Illustration taken from [1].

III. OpenNanopore software

The complexity of our nanopore signals tailored the needs for a level-detection software that we developed [2]. This software is based on the *cumulative sum* (CUSUM) algorithm that does fast and automated detection of events and their levels even when the signal to noise ratio is close to 1. A major advantage of this software is that events can be classified according to their number of levels and so it is easy to make statistics on sub-populations. This new method has demonstrated promising results on complex signals such as protein-DNA complexes. This new software was critical to demonstrate the small difference of levels depending on the orientation of the translocating molecule [2, 3].

As mentioned above, in a typical nanopore experiment a constant voltage is applied between the two sides of the pore and the current passing through the pore is measured. Thanks to the electrical potential, charged molecules are driven through the pore (if their size allows it). During this translocation process, the molecules provoke a current blockade in the pore, which gives a characteristic current drop motif. This current-drop motif, which is generated during molecule transition through the pore, is referred to as *event.*

The software uses two key concepts the CUSUM algorithm and a recursive lo pass filtering. With this software we can compute event dwell times and conductance drops for each level inside each event. All events are classified as one, two or multi-level event. Statistics can also be performed directly with the OpenNanopore Statistics. The GUI (Graphical User Interface) is extremely intuitive each point in the scatter plot can be selected, and the event is then displayed at the bottom. Dwell time and current blockage histograms are displayed on each side of the scatter plot. Both histograms are level histograms; this means each level corresponds to one count in the histogram. Such tools allow the user to select a sub-population of events based on the number of levels in each event and do statistical studies on each population separately. We have first validated OpenNanopore software on prototypical data of λ DNA molecules translocating a nanopore.

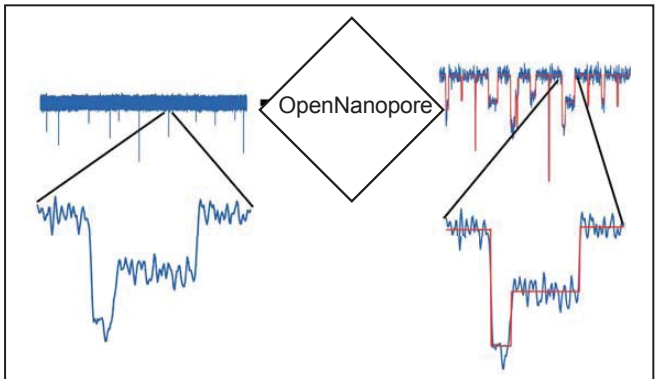

Figure 4. Depiction of the OpenNanopore's tasks : Level fitting and creating database of fitted events.

In the current blockage histogram displayed in Figure 5 from our recent publication [2], it is easy to distinguish two sub-populations of events, the one-level events (in green) and the two-level events (in blue and red). What stems out is that one-level events are centered around two different current blockages: 0.2 and 0.4 nA but the majority is around the 0.2 nA peak whereas, for two-level events, the quantity of 0.2 nA levels is equal to the quantity of 0.4 nA levels. This shows the statistical repartition of λ DNA molecules translocating a nanopore one-level events are mostly events where the DNA molecule translocates in a linear and unfolded fashion and two-level events are events where the DNA molecule translocates partially unfolded (0.2 nA) and partially folded

(0.4 nA). Such a statistical study can be used further in experiments with proteins and biocomplexes where the aim is to distinguish between different sizes of molecules and their orientation while translocating the nanopore [2, 3].

OpenNanopore software can be downloaded from http://lben.epfl.ch/page-79460.html.

IV. Conclusion

Nanopore sensing is a versatile technique; which can also be integrated, in more complex platforms such as optical tweezers, to do force measurement on molecular motors for example. In 2006 *Keyser et al.* [15] published the first experiment of optical trapping force measurement of DNA translocating through a nanopore giving a direct measurement of the electrical force exerted on DNA during translocation. This recent concept of coupling nanopores to optical tweezers opened up a variety of new single molecule studies for biomolecules [16, 17]. This technique could be used to further explore molecular mechanics of transcription, to study forces applied by RNAP to DNA during transcription and give a more detailed view of the kinetic of transcription.

Compared to current single-molecule experiments done on RNAP [8-10] nanopore unfolding can remove structure by shearing rather than pulling. It can also open structures strictly sequentially as they pass through the nanopore. Such an experimental configuration more accurately simulates "natural" biological processes such as transcription [3].

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