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Solid-state Nanopore for Detecting Individual Biopolymers

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Abstract

Solid-state nanopores have been fabricated and used to characterize single DNA and protein molecules. Here we describe the details on how these nanopores were fabricated and characterized, the nanopore sensing system setup, and the protocols of using these nanopores to characterize DNA and protein molecules.

Keywords

Solid-state nanopore; ionic current blockage; DNA; protein

1. Introduction

1.1. Background

Detecting the change in electrical conductance or in resistance of a small aperture in ionic solution containing particles have been used to count and size particles [1,2] for decades. Starting in the 1990s, protein pores or ion channels suspended in lipid membrane have been used to characterize single DNA and RNA molecules [3-5]. Remarkable results have been published from studying these biopolymers translocation through protein pores [6-10]. Recently, solid-state nanometer size pores have been successfully fabricated from materials such as silicon nitride and silicon dioxide, and these synthetic nanopores have been used to study biopolymers such as single DNA [11-17] and protein molecules [18,19].

1.2. Ionic Current Measurement

As illustrated in Figure 1, the main component of a solid-state nanopore sensing system is a single 2-20 nm diameter pore in a silicon nitride membrane that separates two chambers connected electrically only by the electrolyte solution inside the nanopore. The electrical behavior of a nanopore in an electrolyte solution approximately obeys Ohms law. When a voltage is applied and no biopolymers are presented in the chambers, a stable ionic current, open pore current I_o , will be observed (Fig. 1A&C):

$$I_o = \frac{VA_p}{\rho H_{eff}} \quad (1)$$

Here ρ is the resistivity of the electrolyte solution, V is the applied voltage across the membrane, H_{eff} the effective membrane thickness, and A_p is the mean cross section area of a nanopore. When charged biopolymers are added to the *cis* chamber near the nanopore, the molecules will be captured by an electric field of the right polarity, and forced to pass through the nanopore to the other side (*trans* chamber) of the membrane. In the simple case of high ionic strength, a biopolymer inside the nanopore (Fig. 1B) simply blocks part of the ionic flow in the nanopore causing a detectable ionic current blockade (Fig. 1C). This transient signal depends on the properties of the nanopore, electrolyte solution, and the passing molecule.

1.3. Properties of a biopolymer can be measured from a Current Blockage signal

Three basic parameters can be extracted from a current blockage event like that shown in Fig. 1c: the average blockade current ΔI_b , the translocation time t_d , and the integrated area of an event A_{ecd} . There is no universal equation to describe the amplitude change of this current blockage as a function of time $\Delta I_b(t)$. Approximate analytical solutions can be obtained in the limit of a globular shape molecule much shorter than the nanopore thickness, H_{eff} , or chain-like polymer much longer than H_{eff} .

In the first case when the length of a biopolymer $l_m < H_{eff}$, by neglecting the interaction between the biopolymer and the nanopore (free translocation), Ohm's law and the work by DeBlois et al.[2], estimates the parameters ΔI_b , t_d , and A_{ecd} in our study of protein [18]:

$$\Delta I_b = \frac{VA_m l_m}{\rho H_{eff}^2} \left[1 + f(A_m, A_p, l_m, H_{eff}) \right] \quad (2)$$

$$\Delta I_b = \frac{VA_m}{\rho H_{eff}} \quad \text{for } l_m \gg H_{eff} \quad (3)$$

$$t_d \propto \frac{\eta H_{eff}}{QV} M \quad (4)$$

$$A_{ecd} = \int_{event} \Delta I_b(t) dt \propto M \Delta I_b / Q \quad (5)$$

Here A_m is the mean cross section area of the biopolymer, M is the molecular weight of a biopolymer, Q is its total effective electrical charge, and η is the solution viscosity. $f(A_m, A_p, l_m, H_{eff})$ is a correction factor that depends on the relative values associated with the dimensions of a biopolymer and a nanopore. The estimated value of H_{eff} is about 10 nm in the nanopores we fabricated. According to Eq. 2, in the same nanopore measuring system which H_{eff} , V , and ρ are kept constants, ΔI_b is directly proportional to the product of $A_m l_m$ (the volume) of a biopolymer, thus a change of the size of biopolymer could be measured by the change of ΔI_b . According to Eq. 4, a change in the charge Q can be measured by the change in t_d . A_{ecd} (*ecd*: event charge deficit, in the unit of ke: kilo electrons of charge) represents the net excluded charges caused by an ionic current blockade event. A_{ecd} is determined primarily by the identity of the molecule, its charge, geometry, applied voltage, and ionic solution parameters [16,20].

1.4. An example of a nanopore measurement and its application on determining DNA conformation and size

Our results showed that a nanopore assay can simultaneously evaluate a DNA molecule's conformation and its number of bases [21]. The conformation of a DNA molecule can be evaluated by the blockage amplitude ΔI_b . The number of bases or the size of a DNA molecule can be estimated from the integrated area A_{ecd} of an event. In this example, a linear dsDNA ladder (contains 2027, 2322, 4361, 6557, 9416, and 23130 bp dsDNA) was used as a marker (New England Biolabs). A known size circular dsDNA pBR322 (4.4 kbp)

was to be evaluated the distribution of its supercoiling. An “unknown” DNA sample “X” was to be evaluated about its conformation and size.

First, the DNA ladder was added to the negatively biased cis chamber and ~7000 events were recorded. The chambers were washed, the pBR322 was added, and ~7000 events were recorded. The chamber was again flushed, the unknown sample “X” was added, ~9000 events were recorded. The plot of number density versus ΔI_b (vertical) and t_d (horizontal) in Figure 2a shows the linear ~2.17 kbp (a mixture of 2027 and 2322 kbp), 4.36k, 6.56k, and 9.42k events from the ladder DNA, the 4.4 kbp circular DNA, and the unknown sample “X”. From the ΔI_b (doubled comparing with linear dsDNA) and the shape of each DNA shown in the density plot, the 4.4 kbp PBR322 DNA contains circular relaxed (the main peak, ~40% events), and supercoiled (the tail, 60% events) form of DNA. The unknown “X” contains circular relaxed DNA only.

In order to estimate the length of the unknown “X”, we extracted the integrated areas of events or A_{ecd} as shown in Figure 2 b. Fitting the A_{ecd} data calculated from the DNA ladder measurement to $A_{ecd} = CL^\beta$, $C=26.4\pm0.7$ and $\beta=1.40\pm0.01$ were obtained. The corresponding A_{ecd} peak value for the unknown sample “X” is $A_{ecd}^X=392\pm47$ ke, the calculated DNA size from this relation, $L^X=(A_{ecd}^X/26.4)^{1/1.40}$ is $L^X=6.9\pm0.6$ kbp (It was a circular relaxed 7.2 kbp dsDNA).

This example has demonstrated that a nanopore measurement can determine the conformation of a DNA accurately. The measurement can also determine estimate the conformation distribution, and the size of a biopolymer at the single molecule level.

2. Nanopore Fabrication and Characterization

2.1. Fabrication of solid-state nanopore chips

There are several methods to fabricate solid-state nanopores, here we only introduce two of them: by feedback controlled low energy (0.5-5.0 keV) noble gas ion beam sculpting, and by high-energy (200-300 keV) electron beam illumination.

2.1.1. Feedback Controlled Ion beam Sculpting (IBS) Method—Three major steps are involved in this method: **A1.** Fabrication of a freestanding silicon nitride membrane supported by a silicon substrate as illustrated in Fig. 3a. **A2.** Fabrication of a ~100 nm diameter hole or cavity in the freestanding silicon nitride membrane as illustrated in Fig. 3b. **A3.** Low energy ion beam sculpting the ~100 nm hole or cavity to a desired size nanopore as illustrated in Fig. 3(c) and (d).

2.1.1.1. Major steps A1 to A3

A1. Fabrication of a freestanding silicon nitride membrane supported by a silicon substrate: Double side polished silicon wafers coated with low stress (~200 MPa tensile) silicon nitride membranes of 50-500 nm in thicknesses can be custom made at the Cornell Nanofabrication Center (CNF). Fig. 3a shows the fabrication steps to create the freestanding membrane. Photolithography and wet chemical (KOH, 30% by volume) etching of silicon are used. The dimension of the freestanding silicon nitride membranes is ~25 $\mu\text{m} \times 25 \mu\text{m}$ supported by 3 mm $\times$ 3 mm (or 4 mm $\times$ 6 mm) silicon substrate (100).

A2. Fabrication of a ~100 nm diameter hole or cavity in the freestanding silicon nitride membrane: The initial 0.1 μm diameter holes or ball shaped cavities (Fig. 3, bottom) in the membrane's center can be milled using a focused ion beam (FIB) machine. In our work, a focused 50 KeV Ga^+ ion beam (Micrion 9500) was used. The initial 0.1 μm diameter hole or cavity has also been made by electron beam lithography at the Cornell Nanofabrication

Center. TEM images are taken at the end of this step to verify and measure the size of the FIB holes or cavities.

A3. Feedback controlled Low energy Ion Beam Sculpting: Here, exposure of silicon nitride films to a feedback-controlled low energy ion beam provides an approach to fabricating and fine-tuning the size of nanopores by controlling the lateral transport of matter across the surface. This “Ion Beam Sculpting or IBS” technique has demonstrated that tunable single nanometer-sized pores can be fabricated in silicon nitride and silicon dioxide membranes [11,22,23].

There are two ways to make a nanopore by feedback controlled IBS as illustrated in Fig. 3c and 3d [11]. The first is to shrink a larger pore by ion beam induced lateral mass transport on the membrane surface (Fig. 3d). The second way is to remove membrane material by ion beam sputtering (Fig. 3c) layer by layer from the flat side of the membrane containing a cavity on its opposite side. The flat surface will ultimately intercept the bottom of the bowl shaped cavity, forming a sharp edge nanopore. In both cases the ion beam exposure is extinguished when the ion current transmitted through the pore is appropriate for the desired pore size.

2.1.1.2. The Apparatus of Feedback Controlled Low Energy Ion Beam Sculpting: Fig. 4a is a schematic drawing of the feedback controlled IBS System. The main components of this system are housed in a high vacuum chamber pumped to 10^{-9} mbar by a turbo molecular pump. The system includes: a differentially pumped ion gun which generates an ion beam of energy 0.5 to 5.0 keV; a focusing einzel lens and 60° electrostatic deflection system; an electron gun to neutralize insulating surface (SiN) charging effects; a “Channeltron” electron multiplier style single ion detector to measure the ions transmitted through the forming nanopore. The temperature of the sample holder is monitored with a resistor temperature detector, and adjusted with liquid or gas nitrogen and a resistance heater.

This system not only uses keV ions as a nanopore sculpting tool, the ions transmitted through the pore is directly proportional to the area of the nanopore, thus it also measures the area (the size) of the forming nanopore. The transmitted ions are refocused, energy analyzed, and measured by a single ion detector, which converts every detected ion into an electronic pulse. The electronic pulses are then counted by a counter and read by a PC Labview program that also controls a voltage that can deflect the ion beam away from the sample. When the desired pore size is reached, the labview program will terminate the ion beam sculpting process. Fig. 4d illustrates the ion beam sculpting process of shrinking a larger ~ 100 nm FIB hole (4b) to a ~ 4 nm diameter pore (4c).

If we adjust ion beam sculpting parameters so that ion sputtering removing material process dominates, we can fabricate nanopores from a bowl shaped cavity as illustrated in Fig. 3c. Fig. 5 shows a real example of a 10 nm pore fabricated by this method.

In summary, feedback controlled ion beam sculpting technique is capable to fabricate silicon nitride nanopores with desired diameters ($D_p > \sim 1$ nm). The estimated length of the nanopores (H_{eff}) is between 5 to 15 nm. However, the length or thickness of nanopores fabricated by this technique is not well controlled.

2.1.1.3. Major steps of making nanopores by IBS

1. Align the nanopore chip (with a FIB hole in it, for example) to the sample holder
2. Load the nanopore chip to the main chamber

3. Calculate the Ion beam Flux (Ions $\text{nm}^{-2}\text{s}^{-1}$) by using the initial ion beam counts divided by the area of the FIB hole, and then estimate the final ion beam count threshold for a targeted size nanopore.
4. Starting the Ion Beam Sculpting, the process will be automatically terminated when the ion beam counting rate passing the threshold set.

2.1.2. High energy electron beam illumination—Dekker and co-workers at the Delft University of Technology have developed another method for producing SiO_2 and SiN pores by high energy (200-300 keV) electron beam illumination in a TEM [15,16]. Using semiconductor processing techniques, e-beam lithography, reactive-ion etching of SiO_2 mask layers, and anisotropic KOH etching of Si, they produced pyramidal 20×20 nm and larger pores in a 40 nm thick membrane. They used the electron beam in a TEM to shrink the larger 20 nm pores to small ones. Since in the TEM approach the electron beam is used to image the pore normally, thus the shrinking process can be observed in real-time. Using a high energy focused electron beam in a TEM, nanopores could also be drilled in free-standing < 10 nm thick SiO_2 membranes [13].

2.2. Characterization of solid-state nanopores –TEM and AFM

Several nanopore properties are important for biopolymer analysis. These properties are the geometry of the nanopore (diameter, length, and the profiles of these parameters), chemical, and charging state of the nanopore surface. Characterization of these properties is challenging due to the small scale involved. A TEM can measure the smallest diameter of a nanopore accurately, an AFM can be used to characterize the surface properties of a nanopore, and an I-V measurement can be used to probe the nanopore electrical properties. The details of these characterization tools are described below.

2.2.1. Transmission Electron Microscope (TEM)—TEM can measure the size and shape of a nanopore with sub-nanometer resolution. TEM has been the best tool for nanopore size characterization. Standard TEM images can characterize two-dimensional nanopore profile (Fig. 4c and 5c). Recently, tomographic techniques have been applied to extract three-dimensional information [24].

2.2.2. Atomic force microscopy (AFM)—AFM has been used to characterize nanopore surface profiles [24-27]. Figure 6 shows AFM images for a FIB made ~ 70 nm hole (Fig. 6a), a 10 nm nanopore made by IBS, and the cross-section profiles of the two images (Fig. 6c). AFM images provide surface roughness and shape, however, limited by the lateral resolution, this technique is not widely used for measuring the size of small nanopores.

2.3. Materials sciences of nanopore formations

2.3.1. IBS—The IBS method has demonstrated ability to continuously monitor changing dimensions in the nanometer scale while varying experimental parameters provides an unusual opportunity to test microscopic models to account for the observed materials phenomena. Competing processes are probably at work. One is responsible for opening the pore, probably driven by ion sputter erosion of the pore edge, this process dominates at low temperatures and high fluxes. The other one is responsible for closing the pore, probably driven by ion beam induced mass transport on surface.

When an ion beam is directed to a material surface, a number of processes are understood to occur. In one such process, a sputtering process, atomic-scale erosion occurs at the material surface, removing atoms from the surface for incident ions. Due to this phenomenon, as material is removed from a solid-state surface, e.g., a Si_3N_4 surface, which has been

processed to contain a bowl-shaped cavity on its opposite surface, as shown in Fig. 3c, the flat surface will ultimately intercept the bottom of the bowl shaped cavity, forming a nanopore. Production of a nanopore requires knowledge of precisely when to stop the ion sputtering erosion process. Since we do not have a built in reference point on the size of the nanopore being made, the absolute size of the nanopores could be difficult to anticipate by this method.

It is preferred that the apparatus be operated to control a number of processing parameters those can make the flow of matter to a developing or closing nanopore dominate. These parameters are sample temperature, ion beam duty cycle that is defined as the time the beam is on divided by the sum of the times the beam is on and off, for a pulsed beam, and the instantaneous ion beam flux, F , in ions $\text{nm}^{-2}\text{s}^{-1}$ when the beam is directed to the eroding material. Since the initial FIB hole can serve as a reference point for determining the size of a nanopore, the shrinking hole method (3d) has better size control for making nanopores compared with the opening a cavity method (3c).

Two different views can explain the motion of matter necessary to account for the pore closing phenomenon. In the first, a very thin (~ 5 nm) stressed viscous surface layer may be created by the energy and matter deposited by the ion beam. An enhanced collective motion, driven by a reduced viscosity and/or enhanced stress due to implantation effects or surface tension, causes the layer to relax. Alternatively, we can account for our observations with a model in which incident ions both create and annihilate excess, independent and mobile surface “adatoms” (e.g., atoms or molecular clusters) that can diffuse to the pore [11,28].

2.3.2. High energy electron beam illumination—The underlying mechanism for the shrinking process by electron beam illumination appears to differ from the ion-beam sculpting. The authors believe that the electron beam effectively melts the SiO_2 , and surface tension causes the molten SiO_2 to flow to minimize its free energy. A model was also proposed by Storm *et al* describing the contraction and expansion kinetics based on the competition of surface-tension driven contraction with electron beam induced sputtering.

3. Nanopore biopolymer sensing apparatus

3.1. Sample holders, electrodes, and fluidic system

An example of the nanopore sensing system is shown in Fig. 7. In our design, the nanopore chip is sandwiched between two PDMS (polydimethylsiloxane) chambers. Using the solid-state nanopore chip as the main sensing component, we have designed and constructed a nanopore sensing system as illustrated in Fig. 7. This sensing system has the capability of changing sample solution with a microfluidic system (manually or computer controlled), and varying the solution temperature from 0-100 °C by a thermoelectric cooling-heating system. The PDMS chambers are made by casting PDMS into metal moulds. Both chambers are held in place separately. The measuring system shown in Fig. 7 can be assembled under an optical microscope. Each chamber is equipped with a Ag/AgCl electrode to which a voltage bias is applied during experiments. The bottom of the *cis* chamber has a 50-100 μm funnel aperture that sits atop the freestanding membrane window with a nanopore in it. It is aligned with the help of the optical microscope. The whole apparatus is placed in a Faraday cage on top of a vibration isolation table to reduce noise.

3.2. Electronics and data recording

Ionic current through a nanopore is generated and recorded by an Axopatch 200B patch clamp system (Molecular Devices) with the amplifier set in resistive feedback mode. The 10 kHz or 100 kHz low pass Bessel filter in the Axopatch 200B was selected for measurements.

Current blockage events are recorded in event driven mode with pre-trigger length set for 1 ms.

4. Recommended Procedures for a Nanopore Measurement

4.1. Nanopore Sensing System Preparations

1. Clean the nanopore chip by soaking the whole chip in acetone, isopropanol, and DI water for 10 minutes each. Clean the PDMS chambers with isopropanol, followed by DI water, and blow dry the chambers with clean air or nitrogen. Prepare the Ag/AgCl electrodes by bleaching them in regular bleach solution (Clorox, for example) about 10 minutes.
2. Align the nanopore chip with the *cis* and *trans* chambers as shown in Fig. 7b. New PDMS chambers are used for each set of experiments.
3. Fill the *cis* and *trans* chambers with a standard 1 M KCl TE (10 mM tris and 1mM EDTA at pH 7) buffer solution for initial nanopore conductivity testing. The solution should be degassed before use.
4. Apply a biased voltage V (120 mV usually) to the electrodes. If the open pore current is stable, is close to the value estimated from Equation 1, and the ionic current noise RMS is below 10 pA, then the system is ready for measurements.

4.2. Sample Preparation and Translocation Event Recording

4.2.1. DNA molecules

1. Depending on the size of DNA molecules, about ~10 nM final concentration of a DNA in the *cis* chamber is usually recommended for a translocation experiment. The concentration can be less for long and more for short molecules.
2. For one DNA sample, the recommended number of recording events is about 3000-5,000.
3. For a series of DNA samples, after the measurement of one sample, the chambers should be extensively flushed with fresh electrolyte solution to remove residual DNA. The chambers are considered clean when no translocation events are observed during about 10 minutes.

4.2.2. Protein molecules—Protein molecules could be positively, neutral, or negatively charged depending on the solution pH values. Both positive and negative polarities are applied to test the electrical charge of an unknown protein. Different voltage amplitudes are also applied to find the appropriate voltage for the measurements.

4.3. Data analysis

Here we describe the data analysis routines to extract basic parameters from the recorded translocation data.

1. Baseline correction is usually applied to the raw data. To correct for baseline drift in the open pore current during an experiment, the average current amplitude of the beginning 70% of the 1ms pre-trigger point was used to define the open pore current (I_0), or baseline, for each event.
2. Classification and sorting of translocation events are effected after baseline correction. The start of a translocation event was defined as one that caused the nanopore current to drop monotonically below two thresholds; the end of a translocation event was signaled by the current trace climbing monotonically back

to the open pore current past both of these thresholds. All of these events have to be classified and sorted first to discard the short time spikes and extremely long events. We discard the current blockage pulses that are either very short (less than the time constant of the electrical measuring system, 30 μ s for a 10 kHz low pass filter, for example) or extremely long in time (molecules stuck in the nanopore).

3. Calculate parameters ΔI_b , t_d , and A_{ecc} . The arithmetic mean of the current blockage value was calculated from the current range between the downward and upward crossings of the second threshold. The event time duration, t_d , was defined by the time between the current drop across the first threshold (~ 40 pA below the baseline) and its rise through the first threshold to the open channel current. The first threshold value was set to be beyond the baseline noise level and about the half peak height of the ΔI_b . The second threshold was set to be at 70% of the maximum ΔI_b values. The event integral, A_{ecc} , was defined as the integral of obstructed ionic current over the duration of an event.
4. Further data analysis routines can be applied to detect features such as DNA folding states, supercoiling distribution, hybridization, and protein secondary structures.

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Fig. 1.

The process of generating an ionic current blockage signal. (A) Ions flow driven by the applied voltage generate an open pore current I_{open} . (B) When a nanopore is partially occupied by a long chain shape negatively charged biopolymer. (C) A current blockage signal generated due to the translocation of a DNA molecule through a small nanopore.

Fig. 2.

(a) Event density plot vs. t_d and ΔI_b for a sample contains a mixture of a linear dsDNA ladder (contains 2027, 2322, 4361, 6557, and 9416 bp dsDNA), a supercoiled circular dsDNA pBR322 (4.4 kbp), and an “unknown” sample measured with a 12 nm pore. The experiment was performed in 1.6 M buffered KCl solution containing 20% glycerol at pH 7. The applied voltage across the electrodes was 120 mV. (b) length determination of a DNA sample “X” from the nanopore experiment. *ecd* of the DNA ladder (•) and sample X (X). The solid curve is a fit of $ecd = C L^\beta$ for the DNA ladder.

Fig. 3. Fabrication steps for solid state nanopore chips (a). FIB milling (b). Ion Beam Sculpting nanopores by keV noble gas ions by sputter removing materials from the flat side of a cavity (c), and by closing a larger pore (d).

Fig. 4. Schematic drawing of the feedback controlled IBS system (a). TEM images of a ~100 nm FIB hole (b) and a ~4 nm nanopore (c). Shrinking a FIB hole to a nanopore by IBS (d).

Fig. 5.
A nanopore (c) fabricated from a bowl shaped cavity (a) by Ion beam sculpting (b).

Fig. 6. AFM images of a FIB hole (a) and a nanopore made by IBS. (c) the cross section profiles of (a) and (b).

Fig. 7.
Schematic of a solid-state nanopore detection system for charged biopolymers.