

Current status on single molecular sequencing based on protein nanopores

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Abstract

In the 1960s, Hladky and Haydon have succeeded in detecting ionic flows across thin lipid membrane when adding small lipid-soluble molecules such as surface-active polypeptides to artificial membrane, which has laid the foundation for decoding single strand DNA when it crosses the nanopore under an applied electric field. In this paper, we will review several kinds of protein nanopores used in single molecular sequencing, such as α -hemolysin nanopore, MspA nanopore and membrane-adapted phi29 motor protein nanopore.

Keywords: Single Molecular Sequencing, Nanopore, α -hemolysin, MspA, Phi29 motor

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Introduction

A simple idea for single molecular sequencing is that let nanometer-scale pore (~ 1.3 nm in diameter) provide the sole pathway for single-stranded DNA or RNA. When single-stranded DNA or RNA is driven through the pore under an applied electric field, the ionic current through the pore is reduced and every single nucleotide generates a characteristic residual ionic current which corresponds to its DNA sequence. Theoretically, this method can be used to detect the DNA or RNA sequences rapidly. Based on this idea, several nanopores have been reported to succeed in detecting DNA/RNA sequences. In this paper, we will review some of these nanopores, thus providing readers with a brief framework of the nanopore sequencing.

1. α -hemolysin nanopore

The first used nanopore in single molecular sequencing is the α -hemolysin nanopore. It is a 33kD protein that is isolated from *Staphylococcus aureus*, which undergoes self-assembling in lipid bilayers and finally forms a channel with a relatively large nanopore [1]. It forms a homo-heptameric beta-barrel within biological membranes. This may span the membrane barrier, and a broader segment (called the "mushroom cap") which extends into one of the aqueous compartments [2]. Its total length is about 100Å. The structure of α -Hemolysin is showed in Fig. 1.

Mark Akeson et al. have demonstrated that nanopore

formed by α -Hemolysin can act as a detector that can discriminate between pyrimidine and purine segments rapidly along RNA molecule [4]. The apparatus are shown in Fig. 2. They use a U-shaped Teflon path tube connecting two 70- μ l baths milled into a Teflon support while the baths and the Teflon tube are filled with 1M KCL buffer. These baths are then connected to an Axopatch 200B amplifier by the Ag-AgCl electrodes. One end of the Teflon path tube has a conical tip which narrows abruptly to a 25- μ m conical aperture. The diphytanoyl phosphatidylcholine/hexadecane bilayers are formed across this aperture. one or more α -hemolysins are inserted into the bilayer. Nucleic acids are driven through the α -hemolysin channel under an applied voltage of about +120 mV at the *trans* side [4].

The results are shown in Fig. 3. As predicted by the crystallographic model, the translocation of the ssDNA has caused an 85% blockade which corresponds to sequence of the ssDNA. These blockades could be amplified by PCR from the *trans* compartment. The polynucleotide blockade durations mainly depend on strand-length of the DNA, which shows that DNA traverses the pores with strand extended. That is, the requirement of single strand transport across a nanopore has been fulfilled. However, it still remains unknown that whether the DNA translocation through α -hemolysin is unidirectional, and whether thermal fluctuations could cause the strands to diffuse backward randomly against

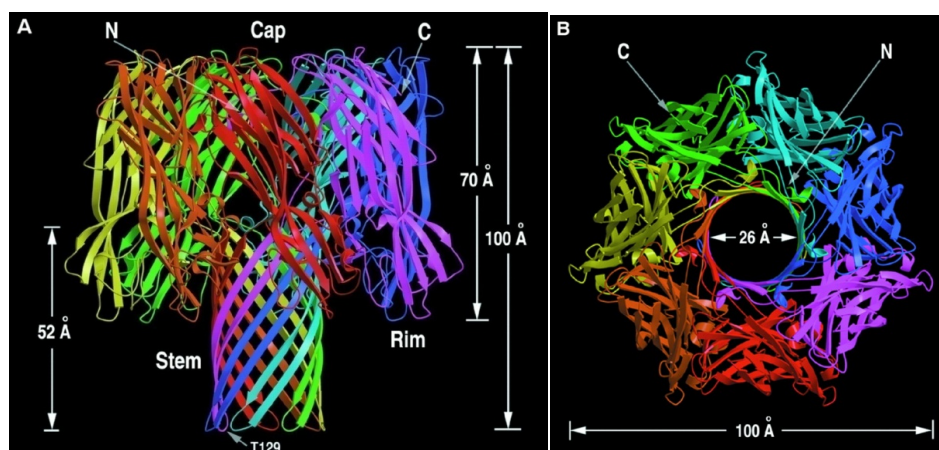


Fig. 1 Ribbon representations of the α -HL heptamer with each protomer in a different color[3]

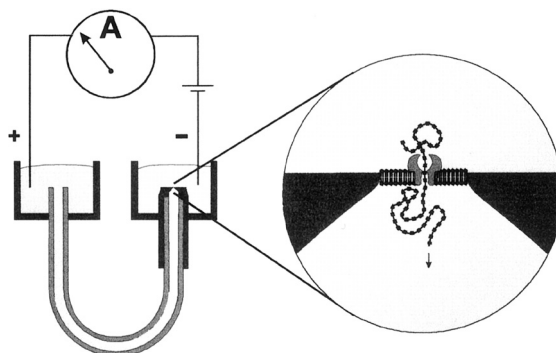


Fig. 2 Horizontal bilayer apparatus[4].

the electric field[2].

However, the speed of ssDNA transporting the nanopore is so high that this method can only identify between pyrimidine and purine rather than a single base of the ssDNA. Moreover, the nanopore can accommodate ~ 15 bases at the same time, which may cause signal confusion. Last but not least, the secondary structure of the single-stranded DNA and the nanopore is easy to aging and the experiment environment plays an important role, which can easily interfere the experimental condition.

2. MspA nanopore

α -hemolysin's 5 nm-long cylindrical beta barrel may dilute the ion current which is specific to the single nucleotide and will generate a small current difference between nucleotides. This structural drawback is believed to be overcome by the channel protein *Mycobacterium smegmatis* porinA (MspA)[5]. MspA is a channel-forming protein which constitutes the major diffusion pathway for hydrophilic solutes in *M. smegmatis*. MspA has remarkable stability when under strong environmental stresses. It can retain channel-forming activity even after exposure to any pH ranging from 0 to 14, extraction at 100°C for about 30 minutes and further incubation at 80°C in the presence of 2% SDS for about 15 minutes. It can also be rationally modified based on its crystal structure. Furthermore, MspA has a narrow and short channel constriction that is very suitable for DNA sequencing [6]. The constriction is about 1-nm in total

length and 1-nm in width. The geometry of MspA may provide improved spatial resolution. The characteristics of the ionic current blockades are mainly determined by the polynucleotide segment passing through the narrow region of the nanopore.

The wide-type MspA is initially negatively-charged, thus, preventing ssDNA translocation. Butler, T. Z., Pavlenok, M. et al[6] have used the site-directed mutagenesis to remove the three negatively-charged aspartic acids and replaced them with neutral asparagines. And the resulting mutant of MspA is called M1-NNN-MspA which allows ssDNA translocation.

In order to overcome the problem that DNA translocation is so rapid, with the speed of $>10\text{nt}/\mu\text{s}$ in solid state nanopores and $>1\text{nt}/\mu\text{s}$ in α -hemolysin and MspA nanopores, Derrington, I. M., Butler, T. Z. et al. [5] have proposed a method. That is, adding a section of double stranded DNA (called hairpin duplex) at the head of single stranded DNA (called hairpin tail which we are interested in can halt the translocation of the DNA and hold the single-stranded section of interests in the nanopore's constriction. At a driving voltage of about 180mV, the hairpin duplex dissociates after $\sim 10\text{ms}$. While the hairpin tail is held in the constriction of MspA, the measured ionic current primarily depends on the composition of the single-stranded DNA residing in the confining constriction. After the dissociation of the hairpin duplex, the DNA completes translocation into the lower potential chamber at the speed of faster than $1\text{nt}/\mu\text{s}$. Thus, the ssDNA section can be sequenced in this way.

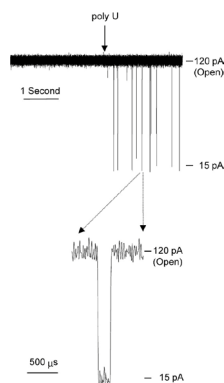


Fig. 3 Blockade of ionic current through the α -hemolysin pore during the translocation of poly(U) RNA homopolymers. The poly(U) strands is 150 nt in length on average. The applied potential was about 120 mV at room temperature. The buffer was 10 mM HEPES/KOH and 1 M KCl at pH 8.0 [2].

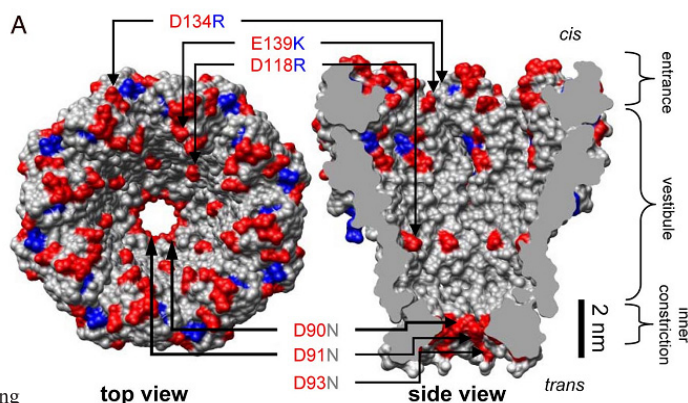


Fig. 4 The structure of MspA[6]

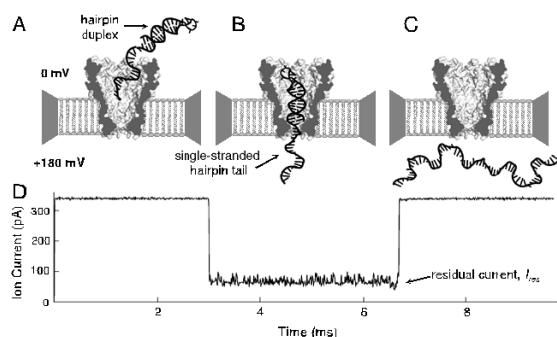


Fig. 5 The process of DNA passing through the nanopore of MspA. (A) The positive voltage attracts the negatively-charged hairpin DNA into the nanopore. (B) The DNA threads through the nanopore until the wilder hairpin duplex section of the DNA prevents further translocation. (C) After a couple of milliseconds, the hairpin duplex dissociation takes place and allows for complete translocation (D) the resulting current shows that the hairpin duplex of the DNA present in the nanopore allows a residual current until the hairpin duplex dissociates [5].

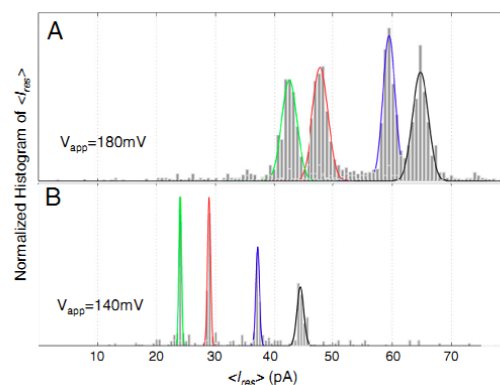


Fig. 6 Example histograms of the measured average residual ion current. The current is shown for different homopolymer single-stranded tails of a 14 base-pair hairpin. (A) under the voltage of +180mV (B) under the voltage of +140mV. The reduction in widths at +140mV is due to increased time averaging because the dissociation times are nearly 30 times longer than the dissociation times at 180mV [5].

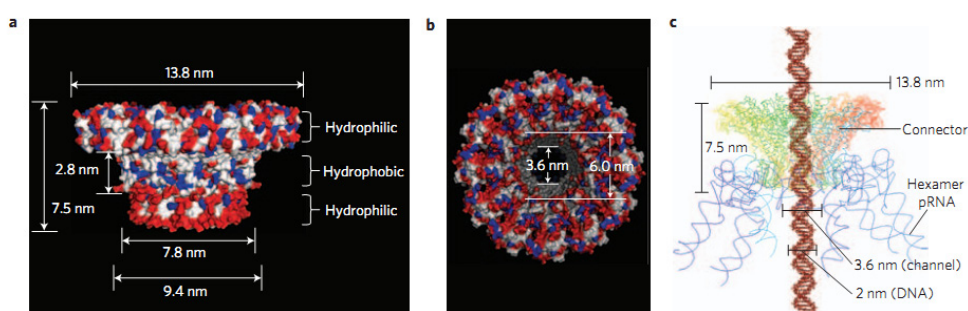


Fig. 7 The structure of the phi29. (a) Side view of the phi29 connector. The acidic is shown in red while the basic is shown in blue, and amino acids are shown in white. (b) Top view of the phi29 connector. (c) Illustration of the entire phi29 DNA packaging motor showing DNA translocation through the connector[7].

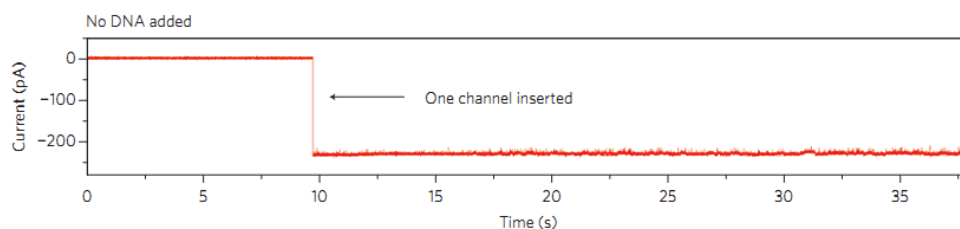


Fig. 8 (a) Translocation of double-stranded DNA through connector channels. (a) The current trace when no DNA is presented (control).

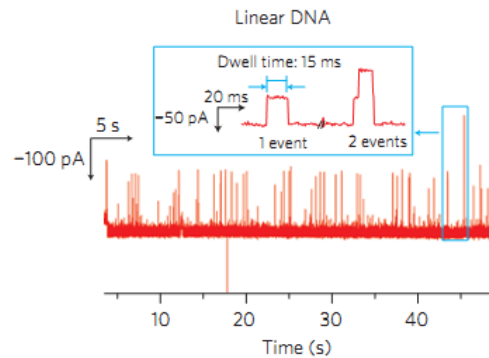


Fig. 8 (b) Blockades caused by 45 pM double-stranded linear plasmid DNA.

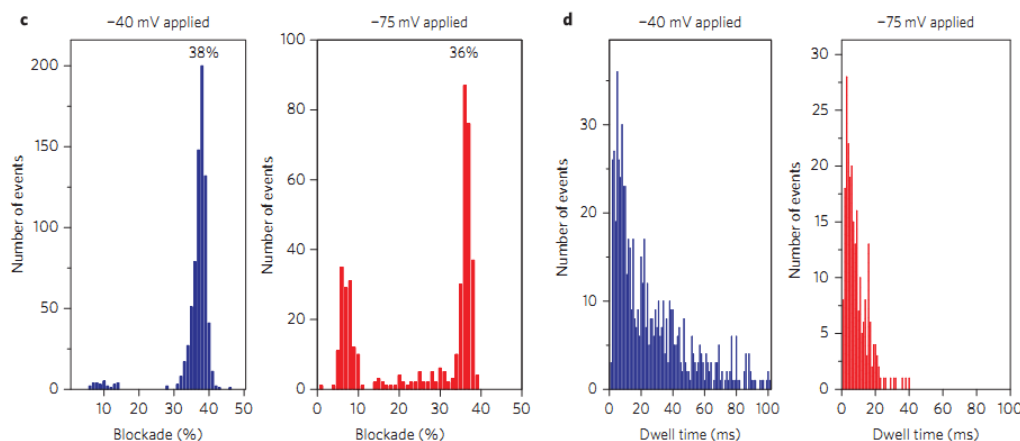


Fig. 8 (c) The percentage of current blockade caused by linear plasmid double stranded DNA under -40 mV and -75 mV.

Fig. 8 (d) The dwell time caused by linear plasmid double stranded DNA under -40mV and -75mV [7].

The process is shown in Fig. 5 and Fig. 6.

3. membrane-adapted phi29 motor protein nanopore

The previous mentioned protein nanopores have channels that only allow the movement of small molecules and single stranded DNA or RNA. In this section, we will introduce the membrane-adapted phi29 motor protein nanopore, which allows double stranded DNA to enter the virus during maturation and to exit during infection. It contains a connector protein with a channel whose width is between 3.6 nm and 6 nm. David Wendell, Peng Jing et al. [7] have modified this connector protein. And when it is reconstituted into liposomes and inserted into planar lipid bilayers, it allows the translocation of double stranded DNA. It uses 6 packaging RNA strands to gear the machine.

The motor of bacteriophage phi29 uses 1 ATP to package 2 or 2.5 base pairs of DNA. The structure of the protein is shown in Fig. 7. It has a truncated cone structure, and termed a connector that allows double stranded DNA to enter during maturation and to exit during infection. The connector has a central channel consisting of 12 GP10 protein subunits. The phi29 connector is ideally suited for undergoing nanopore sequencing because its available crystal structure allows for explicit engineering. Moreover, procedures for large scale production and purification of the phi29 connector have already been developed [7].

After modifying the phi29 connector, reconstituting the connector into liposomes and incorporating the connector into planar lipid membranes, the desired product has been prepared. (detailed procedure in reference [7]) when linear DNA plasmid passes through the nanopore, its translocation induced numerous current blockades which led to the current jump of single connector insertion to be transiently reduced by 25-45 %. At the same time, blockade events in the range of 5-15 % are occasionally detected, which is due to the interactions of connector pores with lipids or lipid micelles rather than DNA translocation because they were also found in the absence of DNA. This kind of blockades are minimized when diluted connector reconstituted liposomes are used or a lower transmembrane voltage is applied. And when DNA was premixed with buffer before connector insertion, the DNA blockades were detected immediately after the first insertion took place. Details are shown in Fig. 8.

The blockade rates were affected by two factors: DNA concentration and transmembrane voltage. And the dwell time of DNA translocation was affected by applied the size of DNA and voltage. Moreover, the DNA translocation rate was affected by the number of inserted connectors [7].

This engineered form of the GP10 connector is incorporated into a lipid bilayer, forming a highly conductive nanopore. The translocation of the linear

plasmid DNA may be confirmed by evaluating the changes of the conductance and performing Q-PCR analysis. This work provides other researchers with a tool or a system for future electrophysiology studies of the phi29 DNA packaging motor. Moreover, this kind of connector is easy to engineer and extremely reproducible because it is a biological nanopore, which makes it suitable for future nanotechnological and biomedical applications.

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