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Mini Review

Artificial Nanopore Biosensor for Detection of Single Biomolecules

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Abstract

Nano-scale systems have been widely researched in the measurement of ultra-low concentrations of analytes in solution by achieving high SNR (Signal to Noise Ratio). Nanopore systems are outstanding nano-scale biosensors that can detect biomolecules such as DNA and protein through label-free detection and an extremely simple structure. This review will introduce new applications of the nanopore system, as well as the principle behind its use and its approach to DNA sequencing.

Keywords: Nanopore, DNA sequencing single molecule detection

Introduction

The demand for rapid and reliable DNA diagnostic methods has enhanced the establishment of new analytical methods. Outstanding progress has been made in capillary electrophoretic devices[1,2]. But, these devices require a time-consuming separation process and optically detectable reporter molecules for the readout. Although capillary electrophoresis and label-dependent assays achieve good sensitivity and accuracy, they require bulky and complex supporting instruments. Therefore, eliminating these steps can enhance the speed of the assay and simplify the readout. A simple, label-free biosensing method is required to use the nano-scale system to establish new methods with high SNR.

The nanopore system is one of the most promising label-free biosensor technologies for detecting biomolecules, particularly as an alternative to conventional DNA sequencing methods (Sanger sequencing method) and has been studied since α-hemolysin protein was first used by Kasianowicz in 1996[3-5]. α-Hemolysin is a 33-kD protein secreted by *Staphylococcus aureus* that has a nano channel that is about 10 nm in total length. The mouth of the channel is about 2.6 nm in diameter and then widens into a vestibule that abruptly narrows to a limiting aperture of 1.5 nm. This limiting aperture is slightly larger in diameter than an extended single strand of DNA. Several other membrane proteins have been used to measure the ionic current passing through single ion channels in biological membranes or planar lipid bilayers[6-8]. For example, bacterial pore proteins are promising alternatives to α-hemolysin. In artificial phospholipid bilayers, the protein from the *Bacillus subtilis* bacterial membrane measurably reduced ionic current using 4.2 kb double-stranded plasmid DNA[30]. Still, to date, α-hemolysin is one of the best proteins of appropriate size to detect single-stranded DNA or RNA. Many groups, including the Nanopore project group led by Deamer at UCSC and Branton at Harvard, have researched nanopore systems to characterize DNA biopolymers using α-hemolysin[9-13]. Only single -stranded DNA or RNA can be detected by translocation through α-hemolysin. Meller showed that nanopore technology can serve as an alternative DNA sequencing tool by differentiating between the electrical signals of the nucleotides [adenine (A), thymine (T), guanine (G), cytosine (G)][13]. This signal differentiation between the nucleotides was also demonstrated by Akeson using RNA[4]. The signal difference was over 50 pA between poly A and poly C. However, biological membrane that contains α-hemolysin has some disadvantages such as a short lifetime due to the unstable lipid membrane, uncontrollable pore size, and unestablished fabrication methods. In particular, the fixed pore size (1.5 nm) of α-hemolysin is a weak point in terms of broadening its application to double-stranded DNA or protein detection, whereas it is the best characteristic for signal reproducibility.

To overcome the difficulties of using α-hemolysin, artificial solid-state nanopores have been constructed using inorganic material such as silicon and organic material like polymer by the Harvard Nanopore group led by Branton[14,15] and by other groups[16-18] (Table 1). Solid-state nanopores have been used to detect double-stranded DNA, protein, individual particles and even cells by controlling the diameter, structure and surface of the nanopore[19-22]. This single molecule de-

tection approach makes it possible to examine individual molecular details that otherwise cannot be achieved by the ensemble-averaged method. These results suggest that nanopore sensing is a potential future technology for rapid DNA sequencing.

This review will present the principles and characteristics of the nanopore detection system, fabrication of artificial solid-state nanopores, and current applications of nanopore technologies to overcome the difficulties of artificial nanopores.

Characteristics of a Nanopore Detection System

The nanopore is a nano-sized miniaturized coulter counter that measures the size of micro particles and cells as shown in Figure 1. When a voltage bias is applied across an insulating membrane that has a nanopore that separates two chambers filled with ionic solution, a current will flow through the nanopore. Negatively charged biomolecules translocate through the nanopore due to the voltage bias, and the ionic current inside the nanopore is momentarily perturbed by the translocating biomolecule. This phenomenon allows a single translocating biomolecule to be transuced into an electrical signal. In addition, the duration and magnitude of the transient current blockade provide information about the structure of the translocating molecule[23-25]. The conventional coulter counter and nanopore system differ in pore size and in the driving force that moves biomolecules through the membrane.

Table 1. Comparison of bio nanopores and artificial solid-state nanopores.

	Bio-pore (α-Hemolysin)	Solid nanopore (Silicon based)
Membrane (insulator)	Lipid layer	Silicon nitride
Pore size	1.5 nm Dia. (fixed)	Variable diameter
Robustness	· Fragile · 5min-24 hr shelf life · Room temp.	· Robust mechanically · Longevity · Extended temperature · Wide pH and voltage range
Sensitivity	High	Moderate

$AgCl_2$ electrodes are used in the nanopore system. The two chambers are usually filled with the same buffer (KCl, 0.1-0.3 mM). Typically, the average number of events per minute is about 100 when 0.1 nM of lambda-DNA in 0.1 mM KCl solution are used with an applied voltage of 200 mV between transmembranes.

The duration and the magnitude of the signal are proportional to the length and the projected area of the biomolecules translocating through the nanopore, respectively. The magnitude of the ionic current is a function of the KCl concentration, the applied voltage between the two chambers separated by the insulating membrane that contains the nanopore, the length of the nanopore, and the diameter of the biomolecule passing through the nanopore. The dwell time of the ionic current blockade is a function of the interaction between the translocating biomolecule and the inner surface of the nanopore, the size and structure of nanopore, the applied voltage, as well as the length of the biomolecule.

Although the artificial nanopore system was developed to overcome the demerits of a protein nanopore such as α-hemolysin, some difficulties in the nanopore system still remain. The representative problems of artificial nanopores are categorized in Table 2. First, the nanopore can become clogged by proteins, viruses, or other unknown particles even when using

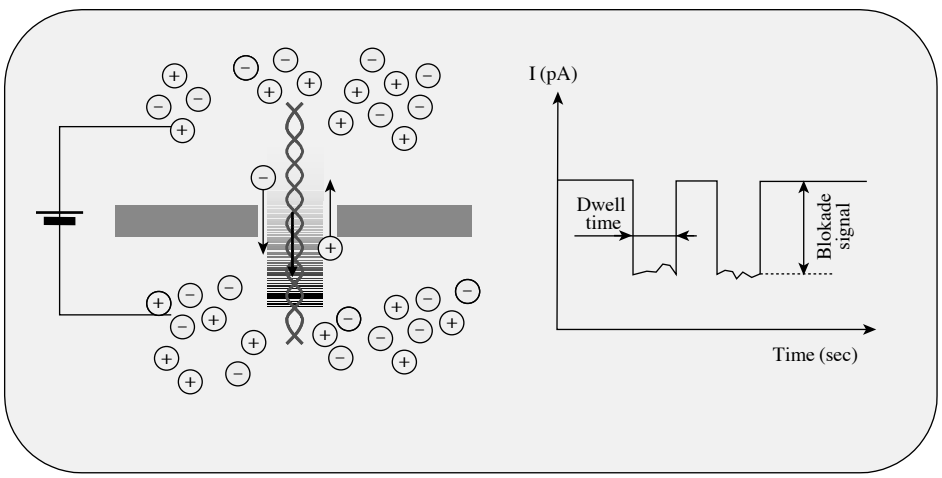

Figure 1. The detection principle of a nanopore system.

Table 2. Common considerations for a nanopore system that characterizes DNA.

Problems	Phenomena	Directions
1. Clogging	Short shelf life	Inherent problem
2. Priming	Long preparation time-wetting problem	Inherent problem
3. Short DNA detection	Minimum measurablee DNA length is 3,000 bps using normal artificial nanopore	Nanopore modification
4. Robustness	Insulating membrane consisting of silicon nitride : 200 nm	Structure modification
5. Specification	Only physical characteristics available	Biological reaction included
6. Non mass production	Nano etching process is not available to mass production	Fabrication methods

ultra-purified water because it is impossible to use a 100%-purified sample. Thus, sample preparation is a key process when using the nanopore detection system to detect DNA or RNA.

Second, wetting the inside wall of the nanopore is also a key preparation process when using an artificial nanopore. When somewhat hydrophilic materials such as silicon or silicon oxide are used to fabricate the nanopore, the wetting problem might not be as critical because water molecules have strong binding energy with the surface. However, an air bubble can be trapped inside the nanopore because both ends of the nanopore meet the water interface. To overcome this problem, the nanopore is kept in an ethanol solution that contains water to reduce the surface tension of the water.

Third, the translocation time of DNA through a solid-state nanopore is too fast to identify individual DNA nucleotides. The velocity of DNA translocating through a 10-nm pore at 120 mV is ~27 nucleotides/μs[26]. A full λ-phage DNA (48.5 kbp, 16.5-μm long dsDNA) takes only about 1.8 ms to traverse a 10-nm pore. Although nanopore sensors provide simple, rapid detection of DNA without any labeling, the fast translocation of DNA limits the widespread use of nanopore sensors for more practical diagnostic applications, such as detecting, sizing or quantifying short DNAs typically generated by PCR. Due to the high translocation speed (~27 bases/μs), detection of short double-stranded DNA requires an electronic system with extremely high bandwidth. But microsecond resolution is currently not achievable when designing a bench-top or LOC type diagnostic system. It should also be noted that high bandwidth is not the only requirement for detecting short ds-DNA. The concomitant electronic noise makes it even more difficult to resolve the signals transduced from the fast translocation of short ds-DNAs. Slowing the DNA translocation speed is by far the most practical solution for obtaining a clear and deterministic signal[27].

Fourth, the current artificial nanopore membranes are still too fragile to be used as commercial biosensors to detect DNA or RNA, particularly for integration in micro-fluidic devices such as lab on a chip. The insulating membrane developed by the Branton group at Harvard University is usually 200-250 nm of silicon nitride, which is stiff and brittle. The insulating membrane is disrupted by the hydraulic force used to continuously input sample from the purification chamber in lab on a chip. The fabrication of robust nanopores should be considered to enlarge the fields of application. Some nanopores consisting of polymer such as PETP (polyethyleneterepthalate) might be useful for integration of the whole process[28]. Several polymer nanopores are introduced in the following section, which covers nanopore preparation.

Fifth, the nanopore system that is mainly based on the principles of a coulter counter does not characterize the biological properties of DNA, RNA or proteins, but rather the physical properties such as length and sample size. Detecting only the physical properties of DNA is useful when the nanopore system is combined with another process such as PCR because specificity can be achieved by the PCR process. However, most biosensing requires direct measurement of the extent of biological reaction of biomolecules against chemicals, other biomolecules, or specific biological phenomena. To endow a nanopore system with sensing specificity, the probes can be immobilized on beads[21] or on the aperture[29] of the nanopore to capture the biomolecules through biological reactions.

Finally, the nanopore system is generated by unestablished methods such as ion track etching, ionic sculpting method, focused ion beam, or TEM. By any of these methods, each chip is individually manufactured so that it is so difficult to sustain nanopore uniformity. Several technologies have been developed to fabricate uniform and reproducible nanopores. The next section will discuss several tools used in nanopore fabrication.

Nanopore Preparation

Artificial solid-state nanopores have been suggested

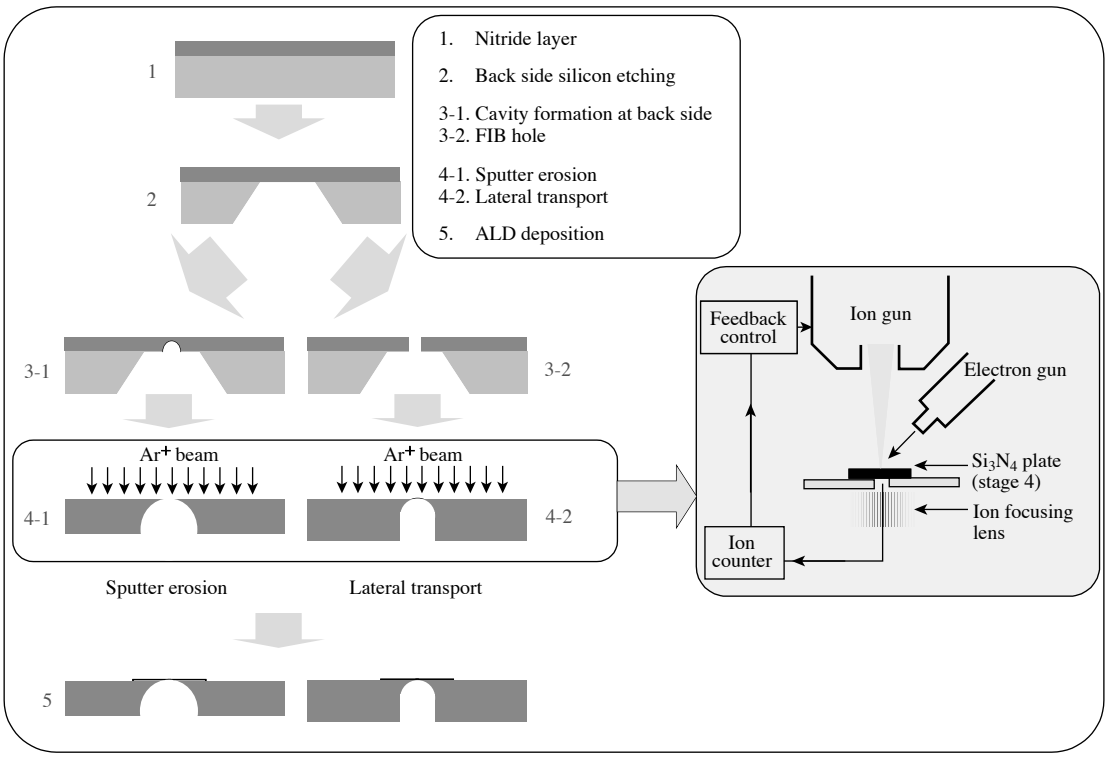

Figure 2. Schematic diagram of the ion-beam sculpting method for nanopore fabrication.

to overcome the demerits of protein nanopores. Various fabrication methods have been introduced to control the nano-scaled pore size since there is no well-established photo-lithographical method for fabricating nanopores a few nanometers in diameter, even in semiconductor development protocols.

The first artificial nanopore was introduced by the Nanopore group at Harvard University led by Branton[15]. Their method, called "ion beam sculpting" contains a feedback-loop etching technique as shown in Figure 2. Dimensional control of the nanopore is achieved by this feedback control of the Argon ion beam. As shown in Figure 2, a silicon nitride layer 200-500 nm thick is formed by LPCVD, before silicon on the back side is wet-etched to make a 20×20 μm^2 silicon nitride window. The few hundred-nanometer-sized cavity or pore in the silicon nitride membrane can be made by normal etching or with a focused ion beam. A surface with a cavity in the silicon nitride window is exposed to Argon ion beams. Material is removed from the silicon nitride surface through the bottom of cavity, causing a pore to open. A slightly larger pore fabricated by focused ion beam can also be closed by Argon ion beam exposure due to the lateral transport phenomena. For fine control of nanopore size with angstrom resolution, atomic layer deposition of Aluminum oxide (Al_2O_3) can proceed after the erosion process by Argon ion beam. The main reason to use Al_2O_3 as the outermost surface material is its iso-electric point. Silicon oxide and silicon nitride have relatively low iso-electric points (pH 2-4); therefore, it is difficult for negatively charged biomolecules such as DNA or RNA to pass through the nanopore by electrostatic repulsion due to the debye length effect of the nanopore. In contrast, charged biomolecules such as DNA or RNA can be translocated through the Al_2O_3-coated nanopore because the isoelectric point of Al_2O_3 is slightly higher than pH 7. Finally, controlled pore size by ion beam sculpting method is tuned to 1.8 nm.

PDMS [poly (dimethylsiloxane)] can be used to easily fabricate nano-sized structures. The pore (3 μm in length and 200 nm in diameter) can be fabricated by the micromolding technique[22]. A well-established conventional lithographic technique is used to fabricate a negative master template. The nano-sized part (200 nm sized pore) of the system is created by electron-beam (e-beam) lithography. The master is then reproducibly cast into a slab of PDMS. This slightly larger pore can be used to detect somewhat bigger biomolecules or assisted particle-bound biomolecules. Saleh and Sohn used the nanoparticle-bound biomol-

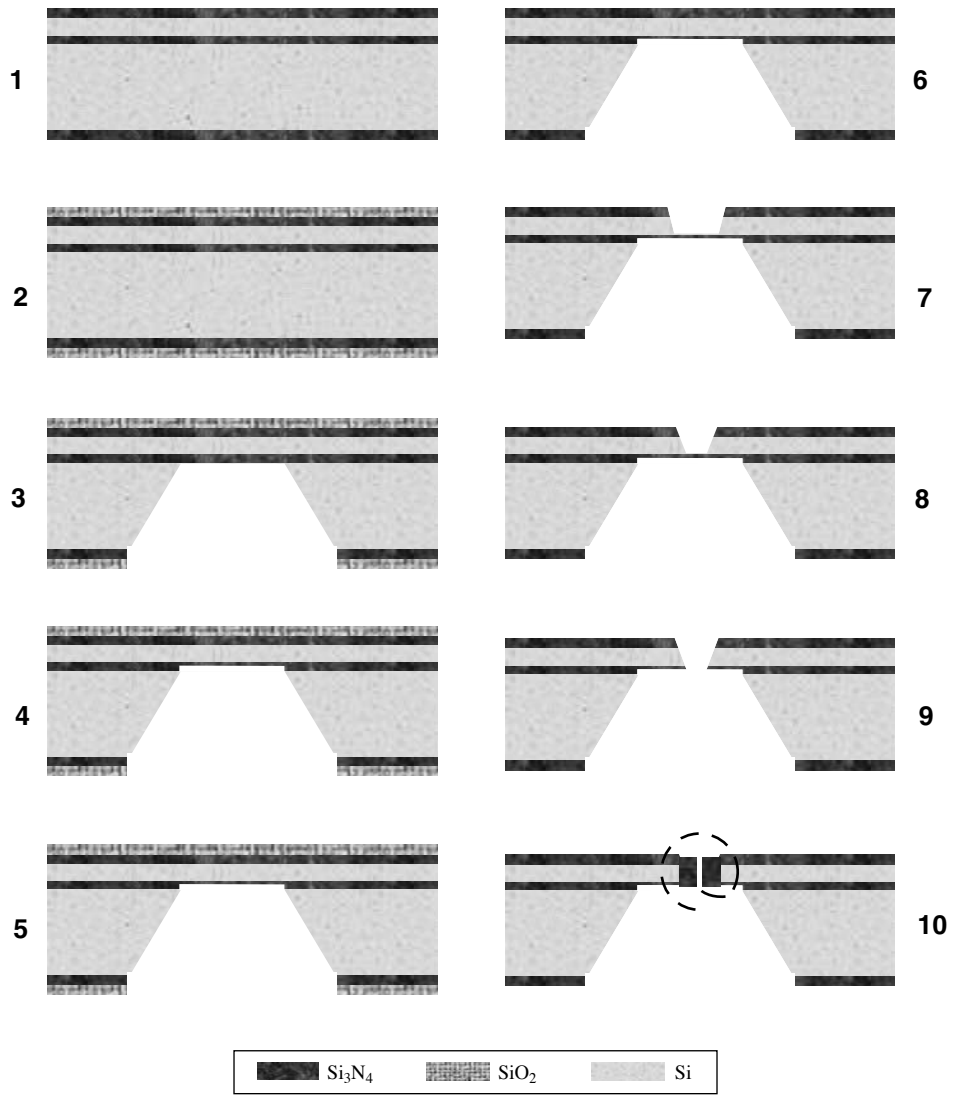

Figure 3. Schematic diagram of nanopore fabrication using SOI wafer.

ecules as capture probes. The size of the nanoparticle after a biological reaction between the capture probe and sample increased the ionic current blockade. Although this nanopore system is based on coulter counter principles, this system uses hydraulic pressure as a driving force to move the sample, whereas other nanopore systems use an electric field as the driving force to make the biomolecule pass through the nanopore.

Recently, the e-beam-assisted fine-tuning technique has been widely used to fabricate artificial nanopores. This technique, developed by the Dekker group at Delft University, uses SOI (silicon on insulator) wafers as starting materials as shown in Figure 3[31]. First, a 100-nm LPCVD Si_3N_4 layer is deposited on both sides of the SOI wafer (Figure 3.1). Then, e-beam lithography is performed, with subsequent plasma etching using CHF_3/O_2 on the backside of the wafer to generate the exposed pattern (Figure 3.3). A wet KOH etch using the remaining silicon as a mask is performed to obtain pyramid-shaped holes through the handle wafer. The buried oxide layer can be used as an etch-stop, due to the slow etch rate of silicon oxide in KOH solution. At this stage, V-shaped grooves are etched next to the membranes so that the wafer can be broken into smaller pieces at a later stage. After normal processing to make the pattern using silicon oxide and silicon nitride as shown in Figure 3, free-standing silicon membrane can be achieved. Finally, a thermal oxidation process is used to obtain a 40- to 50-nm-sized nanopore (Figure 3.10). Then, TEM (Transmission electron microscopy) was used to tune the pore size. The pore shape is deformed when the nanopore is exposed to high electron intensity (300 eV), resulting in reduced pore size. This

method also uses a feedback mechanism, in that the change in pore diameter is monitored in real-time using the microscopic tools.

Some research groups have used a polymer to fabricate a single conical nanopore[16,28,32]. To make nanopore on polymer membrane, a heavy ion shower is required to make tracks in the polymer membrane along which the chemicals etch. Ions penetrate and etch the polymer along the tracks to form a one-pore membrane. This technique requires precisely optimized etchant concentration, temperature, and etching time to control pore size and shape. Polymer nanopore is very useful because it is less expensive and therefore commercially available. However, polymer is restricted when surface modification is required to control the surface tension or other surface properties to adjust the translocation speed of biomolecules or to immobilize capture probes.

Other materials are used to fabricate artificial nanopores as alternatives to the bio-nanopore. MWNT (multiwall carbon nano tubes) are also used as nanopores[33,34]. Although this fabricated nanopore has several merits, other than silicon-based technique, such as uniform size, well-defined surface properties and structures, and controllable pore size, this fabrication technique is too difficult to be established.

Although various other techniques have been developed to replace α-Hemolysin, the current fabrication techniques still have problems in controlling pore size, fabrication reproducibility, and unknown surface characteristics. The next-generation nanopore is expected to be fully integrated into a micro-fluidic chip for a complete analytical system.

Current Nanopore Technologies

Cost-effective DNA sequencing methods that provide reasonable coverage and accuracy have been required since the Human Genome Project. Indeed, the Human Genome project motivated a 100-fold reduction in sequencing costs, from US $10 per finished base to US $1. Several academic and commercial efforts led by NIH are developing new ultra-low cost sequencing technologies to further reduce sequencing costs. One of the suggested new sequencing techniques is continuous DNA sequencing technology using the nanopore. Biomedical research could be improved dramatically with whole information from individual human beings or animals if ultra-low cost continuous DNA sequencing tools are developed.

The nanopore is an ideal method if it could discriminate each nucleotide as the DNA translocated the nanopore. However, it is impossible to characterize DNA at single-nucleotide resolution by the blockade of ionic current signal detection through a nanopore. There are two major reasons[35]: First, the difference between nucleotides is only a change in the movement of ~100 ions. Second, there are 12 nucleotides in the nanopore (α-hemolysin), even if the nanopore is 5 nm in length. That means the ionic current blockage by DNA translocation is the signal averaged over at least 12 nucleotides. Marziali did a simple computer simulation of the signal expected from a hypothetical infinitely thin membrane with a 1.5-nm aperture[35]. The results are as follows: the change by the periodic structure of ssDNA for an open channel current of ~100 pA is only 1 pA. To detect this tiny difference, the noise level, including thermal motion of nucleotides and 1/f noise, should be lower than 1 pA rms. Can we use the nanopore to characterize DNA at nucleotide resolution if we develop new technologies to fabricate a nanopore with 0.1-nm thickness and 1.5-nm diameter that can detect less than 1pA resolution current with ultra-low noise? Furthermore, there is one more problem with nanopore use. DNA translocates a 10-nm pore at 120 mV with a velocity of ~27 nucleotides/μs[26]. Thus, the current measuring unit must have 30 ns time resolution and 1 pA current resolution to characterize DNA with nucleotide resolution.

New methods to slow the rate of DNA translocation or to anchor a DNA molecule in the pore to increase the nucleotide dwell time are possible solutions to the resolution limit of the nanopore. Vercoutere used a hairpin DNA structure in order to allow sufficient time to record the signal for the DNA sequence information, essentially hanging the DNA on the aperture of the nanopore (α-hemolysin)[36]. Although the problems with characterizing DNA using the nanopore have not been solved yet, the nanopore system is still one of the most promising technologies for a cheap, new sequencing technique. A few months ago, the National Human Genome Research Institute (NHGRI), part of the National Institutes of Health (NIH), announced the latest round of grant awards totaling more than $13 million to speed the development of innovative sequencing technologies to reduce the cost of DNA sequencing and expand the use of genomics in medical research and health care. Over 50% of these grants from NHGRI were awarded to research on ultra-low cost DNA sequencing technologies using nanopores.

The shortest DNA length detected using a normal artificial nanopore fabricated with silicon was 3,000 bps with 1,000-2,000 bps resolution[19], although stretched DNA and folded DNA could be discriminated by the magnitude and shape of ionic current blockage from the nanopore. This result indicates that nanopores cannot be used as detectors of PCR product

unless some modification is achieved, because PCR products are usually 200-1,000 nt. Slowing the DNA translocation speed is by far the most practical solution for obtaining a clear and deterministic signal. There are three major possible techniques to slow the DNA translocation rate.

First, a reduced pore size can incrementally decrease the DNA translocation by friction force between the DNA and inner surface. However, as the pore size decreases, the event number of DNA translocations into the nanopore decreases. Second, a blocking layer consisting of a porous material such as a gel that directly interferes with DNA movement can be used to slow the DNA movement. This method requires additional structures over the nanopore and reduces the event number. Third, and similarly, a surface modification is introduced on the inner surface to capture and hold the DNA. This method is the simplest of those mentioned above, and does not require additional structures or affect the event number. Of course, electrolyte temperature, salt concentration, viscosity and bias voltage also affect the translocation time[37]. But reducing DNA translocation speed by combining all of these factors also decreases ion mobility, which diminishes the amplitude of current blockade signals[37].

The surface chemistry of the inner wall of a nanopore that can reduce DNA translocation speed improves the detection capability of the nanopore for short ds-DNA[27]. For example, the surface charge of a nanopore was modified by γ-aminopropyltriethoxysilane (pKa=10.6) to replace a negative charge at pH 7 with a positive charge that would hold DNA; the translocation of short ds-DNA molecules in the modified nanopore produced a measurable electrical signal. The positively charged surface played a major role in reducing the translocation speed of short ds-DNA by increasing the energy barrier created by electrostatic interaction between the modified surface and oppositely charged DNA molecules[27]. As a result, the translocation time of a short ds-DNA was exponentially distributed with a time constant of 56.7 ± 2.6 μs and 104.6 ± 28.6 μs for 539-bp DNA and 910-bp DNA (PCR products), respectively[27].

Some researchers have used modified DNA to slow the translocation speed. Kasianowicz quantified analyte in a sample using biotinylated single-strand DNA as a protein-capturing polymer[38]. The other end of this single-stranded DNA had a hairpin structure or other particle that could not translocate through the nanopore. This capturing polymer hung over the aperture of the nanopore, although the biotinylated end had already passed through the nanopore. However, when macromolecules such as protein react with biotin, the capturing polymer became a dumb bell-like molecule that can be trapped within the nanopore. Still, this technique seems ideal because it is difficult for proteins to react with the capturing polymer due to spatial hindrance.

Conclusion

Nanopore technology is considered one of the most promising techniques for characterizing DNA or RNA continuously at single nucleotide resolution, as well as for detecting single molecules without any labels. To achieve these purposes, several techniques have been developed for reproducible nano fabrication to fabricate uniform nanopores and for surface modification to slow DNA translocation speed. However, most investigations of this technique are still in the initial proof-of-principle stage. Although the experimental and theoretical results do not clearly explain all phenomena occurring in the nanopore, the future of nanopores is still promising because the nanopore has so many advantages such as a label-free use, fast detection of single molecule, simplicity, etc., compared to other methods that have been developed to date. So, further investigation is required for better understanding of the factors that control polymer translocation through a pore, as well as for the design of new biosensing concepts.

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