Engineering Journal issn: 0125-8281

Review

Speculation of Nano-Gap Sensor for DNA Sequencing Technology: A Review on Synthetic Nanopores

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Abstract. Nano-gap sensor is the next generation of single molecule analysis that consumes less resources, costs, times and spaces. The ultimate goal of this technology is rendering the ability to determine any living genetic code in several seconds using this portable device. In principle, DNA decryption was determined by tracking electrical signal change when DNA is passed through either natural or synthetic nanometer size gap. This review focuses on the synthetic nanopore, which is more robust, reliable and manageable with ease. Biological nanopore has been researched in parallel; however, the device reproducibility is a controversial issue. The history and development toward the future of this prospect technology will be elaborated attentively. The main limitation of nanopore sensor device is the controlling of DNA translocation dynamic through tiny pore. Many attempts had been tried and the synopsis will be contemplated in the review. Nonetheless, the present results of DNA sequencing have not been satisfied, the development of nanogap sensor is promising for genomic sequencing and molecular biology.

Keywords: Nanopore, DNA sequencing, sensor, nanochannel, molecular biology, nanofabrication.

ENGINEERING JOURNAL Volume 22 Issue 6
Received 4 August 2018
Accepted 3 December 2018
Published 4 December 2018
Online at http://www.engj.org/
DOI:10.4186/ej.2018.22.6.229

DOI:10.4186/ej.2018.22.6.229

1. Introduction

Initially, a nanopore refers to the transmembrane protein, which naturally possesses an ability to pull small charge molecules through its ion channel. The discovery of this property initiated the idea to study the molecular analysis via those protein's pores. Many researches adopted this idea to observe the behaviors and characteristics of small molecules in the range of nanometer scale [1, 2]. A wide range of bioanalytical applications using nanopores based devises were lining up, e.g. DNA/RNA sequencing, protein or peptide analysis, nanoparticles detection, and single molecular sieving. Nanopore technology is expected to be the upcoming platform for biotechnology and medical application since it furnishes the ability to analyze the characteristics of biomolecules or even their sub-units at the molecular-scale resolution [3-6]. Same as other miniaturized systems, it grants following benefits, e.g. minute sample consumption, fast analysis system, low operating and fabricating cost, massive parallelization and compactness, and lastly better sensing resolution and efficiency. The nanopore sensor requires much less experimental steps comparing with standard DNA sequencing methods such as; Sanger technique, Pyrosequencing, Shotgun cloning, etc. The earliest application of nanopore for DNA sequencing was introduced by Kasianowicz *et al.* [7]. Alpha-hemolysin, a transmembrane protein of *Staphylococcus aureus*, was utilized as a nanopore in ionic current measurement at that time. Several researchers measured such current trace of DNA molecules via the biological nanopore [7-13]; however, the velocity of DNA was incredibly high (~0.5 cm/s) when it passed through the pore. At that speed, it is impossible to discriminate base pairs along the strands of DNA. Therefore, trend of researches moved toward controlling of DNA translocation in the protein membrane by incorporating enzyme or genetically engineered protein with positive charges in order to slow down the DNA translocation [14-17]. Early researchers studied and utilized the biological nanopores for DNA sequencing. However, many limitations were revealed; fixed size, unstable morphology, environmental sensitivity, and difficulty in manipulation and integration into the device, for example. The solid-state nanopores were then proposed as the next generation of nanopore DNA sequencing, which has high robustness, better ability to adjust the pore dimension, more potential to integrate in array, and less sensitivity to environmental parameters in 2001 by Harvard group [18]. The first solid-state nanopore was fabricated and conducted the ionic current measurement of DNA. However, the velocity of the DNA passing through synthetic nanopore was even faster than that in biological nanopore (~1cm/s) [19]. The better interaction between DNA and transmembrane protein perhaps slowing down the translocation better than in synthetic pore. Nonetheless, the translocation rate for both synthetic and natural nanopores halted the ability to discriminate the nucleotides along the DNA strands. A number of researches have been conducted to overcome this high speed molecular transportation. Some groups tried to understand and control the translocation dynamic of DNA in the nanopore by adjusting the pore dimension, incorporating other molecules to the pore and manipulating the experimental parameters. Others find ways to utilize the alternative detecting methods such as transverse current measurement, nanopore capacitor, force measurement, and optical detection to solve this issue. This review studies and synthesizes the key development of all possible approaches toward DNA sequencing ability of synthetic nanopore sensor and justifies the prospect of this technology. The main objectives of this work are to summarize the previous attempts and current stage of nanopore DNA sequencing and point the way toward future applications which will be beneficial for researches in nanopore technology, DNA sequencing even the biomedicine as a whole.

2. The Principle of DNA Measurement in Nanopore

The earliest principle of nanopore sequencing is the ionic current blockage measurement. This technique was succeeded from the Coulter counter method where the change in electrical signal was detected when the sample is passed through the small aperture [20]. The overview of the principle is depicted in Fig. 1. The current measurement system was filled with ionic solution and applied electrical field on two sides of chamber, which separated by the membrane with aperture. At the beginning, a constant open pore current can be detected by the ionic conduction of free charge species passing through the pore. Whenever the sample comes to block the passage of ion species at the gap, the measured current drops, accordingly. The magnitude of the negative pulse is directly proportional to the size of the blocking molecules. In late nineteen century, nanopore sequencing technology adopted this concept by observing the trace of electrical signal when DNA passed through transmembrane protein, α-hemolysin [7]. The Ag/ AgCl electrode was immersed to drive DNA from one chamber to another. The concept is to stretch the DNA in full length and pass through the

pore in linear configuration in order to read electrical signal of the basepairs sequence along the strand of DNA. When DNA was introduced, a drop in the current was observed. However, DNA travelling speed is too fast and unable to differentiate the basepairs information at that time, the signal was considered for single molecule as a whole. The current drop is known as 'ionic current blockage', which is the parameter to determine the molecular characteristics (see Fig. 1). Another important term is 'translocation time', the time it takes for the analyte to travel through the pore. The translocation time is used to determine the DNA travelling speed.

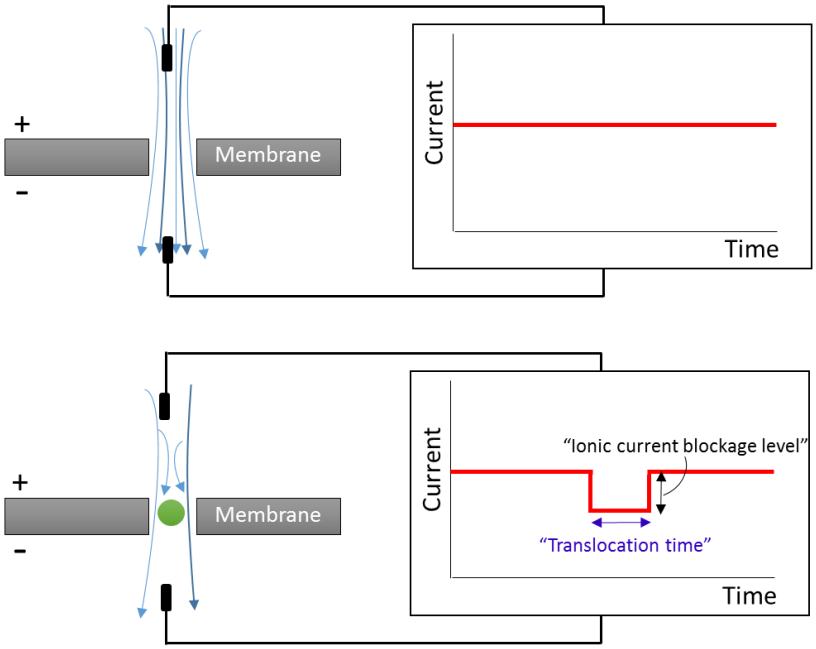

Fig. 1. Schematic of ionic current measurement concept. Without any blockage molecule, ion species can pass through the gap freely, showing a constant current measurement. When the analyte comes and blocks the passage of charge species at the aperture, a drop in the current level known as 'Ionic current blockage level' can be measured. The duration that analyte blocks the current is called the 'Translocation time'.

For two decades, many research groups developed the synthetic nanopores for ionic blockage measurement of DNA. The translocation of DNA through the voltage driving ionic system was influenced by several forces and interactions, such as bias voltage, viscous drag, DNA-nanopore interaction, etc. At equilibrium, DNA has a random coil conformation in homogeneous fluid. Under an applied electric field DNA can stretch and penetrate through the pore in a linear manner. The bias voltage, sometimes, cannot fully stretch a very long DNA. Those folded or coiled DNA, at the vicinity of the pore, present a high blockage level. An increase in the voltage bias showed the higher percentage of low level blockage, indicating that a single file (stretched) DNA mainly passed through the pore [21]. The result demonstrated a strong correlation between current blockage level and the cross-sectional area of the analytes. This idea was supported by the observation of a step change in current signal, which considered to be a DNA partial folding event [19]. Two types of the folding events were observed as full-folding and part-folding, which conducted in 15 nm pore [22]. Full-folding showed a blunt blockage peak, while part-folding showed a step peak (see Fig. 2a, 2b, 2c). A comparison between single file and full folding shows that the level of a current blockage was duplicated according to the number of folded times. Other researches only observed DNA folding event in large nanopore about 5-20 nm in diameter [19, 21, 23],whereas, small nanopore (2-5nm diameter) demonstrated a single file (linear DNA) conformation where hydrodynamic diameter of DNA is about 2 nm in an aqueous solution [24-26]. If a certain experiment showed a constant current blockage level, it can be assumed that linear DNA passed through the pore without folding. In the same way, ssDNA measurements reported an ionic blockage level decrease by two folds from those of dsDNA [27]. All the results supported that ionic blockage is directly proportional to the hydrodynamic cross-section of the analytes.

Considering the DNA lengths, an early study observed a small influence of DNA length on translocation time between 3 and 10 kilobasepairs (kbps) in 10 nm pore diameter [19]. The experiment in 15 nm pore showed indifferent electrophoretic velocity to the DNA length variation as most DNA folded [22]. Later, the translocation time scale dependence on the DNA length was reported to follow a power law of 1.27 from a study conducted in 8 nm pore [23]. Smaller pore research (2.7-5nm) exhibited even stronger length dependent and a distinct characteristic between long DNA (1.2-20 kbps) and short DNA (0.4-6 kbps) [26]. The translocation time of long DNA and short DNA grow exponentially with their length; $\tau \approx L^{\alpha}$ with α = 1.4, 2.28 respectively, where τ is translocation time, L is the DNA length, and α is an arbitrary exponential factor. From those results, it was abundantly clear that the small pore showed a stronger effect of DNA length onto translocation speed than the larger pore. This is probably due to the fact that there is more chance of DNA to interact with the pore surface in narrow gap that could retard the translocation speed.

Nevertheless, it was difficult to determine the effect of DNA length under folded conditions. In 2005, Fologea *et al.* proposed the determination of the overall charges on DNA called the 'Event charge deficit (Ecd)' [27]. DNA unit has a unique specific charge regardless of its conformation. Ecd can be calculated by integration of ionic blockage over translocation time;

$$Ecd = \int \Delta I(t)dt = \Delta I_t \tau_t \tag{1}$$

where ΔI_t is current blockage, τ_t is translocation time of DNA molecules. Further study supported this assumption where same DNA molecules with three different conformations; linear, circular relaxed, and supercoiled DNA, exhibited indifferent Ecd value (see Fig. 2d, 2e) [28]. Moreover, ecd showed a strong relationship with DNA length (L) as Ecd = CL^{β} where C=26.4±0.7, β=1.4±0.1 in their report. Thus, ecd could be used as an indicator to define the length of unknown DNA samples. Recently, this value had been applied as a universal factor to normalize molecular size effect for many biomolecule analysis via nanopore sensor [29-31].

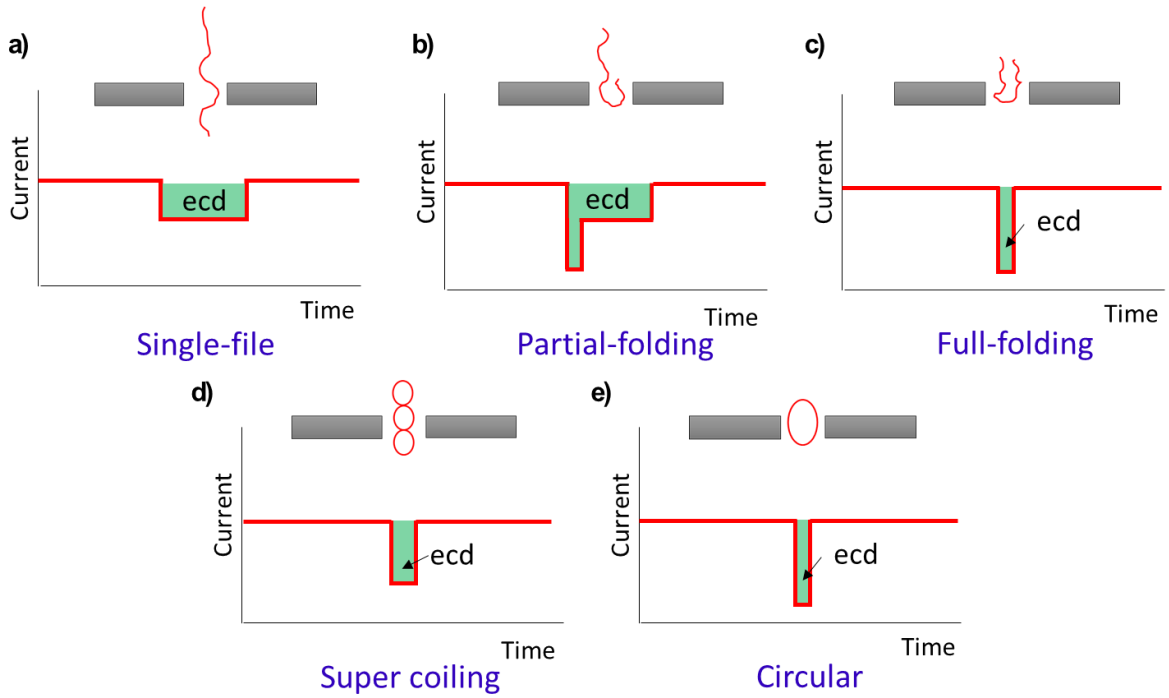

Fig. 2. The conceptual ionic current blockage results for different DNA conformation when travel through nanopore; (a) 'Single file', (b) 'Partial-folding', (c) 'Full-folding', (d) 'Supercoiling', and (e) 'Circular'. The ionic current measurement shows different patterns; however, the ecd are same for all cases with same DNA length.

Each research studied the current blockage measurement of DNA using distinct pore sizes. Studies conducted on larger pores (5-20 nm in diameter) observed that the high ionic current blockage events were slightly faster than those conducted on low blockage one [19, 23, 32, 33]. The high current block event usually corresponded to folded DNA, and the translocation speed were in the range 3-4 cm/s. Low current block event represented single file (linear) DNA which slowly travels through the pore (0.5-1cm/s). Those folding DNAs became shorter than single file DNA, hence they tend to travel faster, independence of their lengths. In contrast, small pore research (2.7-5 nm in diameter) observed an invariance current blockage level since only linear DNA were allowed to pass [26]. The translocation speed largely depends on the length of DNA from 2.7 mm/s of 400 bp down to 0.11 mm/s of 10kbp DNA. However, their travelling speed is much slower compared to that of large pore. The faster events (> 2 cm/s) of small pore observed were found to be DNA collision that rarely happened in large pore. The collision duration in small pore was comparable to the translocation time in large pore, while the actual DNA translocation can be prolong up to 100 millisecond (0.11 mm/s). The interaction between DNA and nanopore leads to a much longer translocation time in all cases. By allowing only single file DNA pass through, it would promote the determination of basepairs along the strand, unlike the folded DNA in large pore. At this point, small nanopore renders a better chance to achieve DNA sequencing. The most proper diameter of nanopore should be slightly larger than the diameter of the analytes; ssDNA (~1 nm), dsDNA (~2 nm), dsRNA (~2.6 nm).

Another dimension that would likewise be concerned with is the thickness of nanopore. The influence of biological nanopore thickness showed an inverse effect on the current amplitude, but it had no effect on translocation time [12]. The solid-state nanopore thickness was constrained by fabrication methods and materials properties. It is usually proportional to the pore diameter; thus effect of thickness would be similar to pore width consideration. No actual determination of membrane thickness was studied, although thinner membrane expected to give a better resolution on nucleotide discrimination. Graphene was then proposed as a prospective thin membrane material for high-resolution nanopore DNA sequencing. Graphene is a one-atom thick planar sheet. Its thickness is comparable to the single nucleotide on the strand of DNA (0.34nm). In addition, distinct properties such as high electron mobility, excellent mechanical behavior characteristics, and remarkable electrical sensitivity of graphene, made it an outstanding candidate in nanopore technology [34-36]. Those points made graphene a superior choice for future electronic applications, so that many researchers exploited graphene membrane for nanopore DNA current measurement [37-41]. Nonetheless, the major limitation was pore clogging due to the strong hydrophobic attraction between DNA and graphene molecule. Other researchers proposed the hydrophilic molecules to functionalize on graphene in order to redeem this issue [42, 43]. They reported a fairly slow speed of about 0.13 mm/s of DNA passing through graphene pore. Strong interaction between sp^2 carbon and nucleotide strands could prolong the translocation time, despite remaining unable to discriminate the nucleotide. In addition, there are some unrevealed concerns such as electrical noise in graphene membrane and significance of multilayer graphene about the DNA translocation dynamic through graphene nanopore. Elaborated simulation studies on DNA translocation mechanism demonstrated the complicated DNA transport through graphene material [44, 45]. Even though this incredibly thin material still has the prospective future toward this field.

3. Parameters Affecting the Measurement Performance

The overview of the measurement system was explained above in previous section. It is well established that the main limitation of this promising technology is the controlling of DNA translocation. Other researches focused on tuning the experimental parameters in the measurement system (i.e. bias voltage, temperature, viscosity and electrolyte concentration) to overcome the translocation dynamic of DNA, rather than on pore characteristic and DNA length consideration. The endeavor on the development of those factors will be discussed in the following section.

3.1. Bias Voltage

Bias voltage is one of the factors that affects both DNA capturing and translocation. In principle, a bias voltage of 200 mV, is typically required to drive and stretch DNA through the pore via electrolysis force. Many research groups have studied the relationship between bias voltage and translocation dynamics [19, 21, 26, 33, 46]. One interesting result is that the ionic current is proportional to the bias voltage, as simply defined by Ohmic law. However, the voltage dependence on translocation speed required elaborated studies. In large pore (>10 nm), the bias voltage demonstrated a weak influence on the DNA's velocity [19, 21]. Whereas an

inverse linear relationship between bias voltage and translocation time was observed in 6 nm pore [33]. In order to slow down the translocation speed to about 0.34 cm/s, they decreased the voltage bias down to 40 mV, which was about three times less than the original driving voltage. Although decreasing voltage diminishes the ionic blockage signal, manipulating the bias voltage to control the speed seems impractical. Later, research on small nanopore (3.5 nm) reported an exponential decay behavior of bias voltage on translocation time [26]. Their work showed that much slower translocation speed (0.45 mm/s) can be achieved at a bias voltage of 200 mV. In this case, decreasing the bias voltage had less disruption in the interaction between DNA and nanopore, so the DNA translocation time was prolonged due to strong attraction. In larger pores, the translocation dynamic was governed by viscous effects where larger bias voltage suppression required to slow down the translocation speed. Small nanopores (< 5 nm) provided a better chance to manage the translocation dynamic while maintaining the current signal level due to strong interaction. Certain research demonstrated an ability to control DNA translocation speed by tuning voltage in very large nanopore (30-60 nm) [47]. Applying 200mV could translocate DNA at a speed of 0.15 mm/s; however, it could be realized for merely large DNA, i.e. lambda DNA (48.5 kbp). Apart from the study on voltage dependence, recent research reported the ability to control the movement of a short (3.27 kbps) DNA back and forth at the entrance of 10 nm pore [46]. They adjusted the bias voltage until it balances with the viscous dynamic of DNA molecules. This result is very promising to trap DNA at the nanopore for sequencing purposes.

3.2. Viscosity

Increasing viscosity is possibly the straightest way to retard the translocation speed. The change in current blockage and translocation time was investigated by adding glycerol from 0 to 50% solution concentration to the electrolyte [33]. As predicted, the translocation speed was retarded to about 0.17 cm/s by increasing the percentage of glycerol up to 50% in the electrolyte. The result complied with the theoretical consideration in previous research explained by the linear relationship between translocation time and viscosity [19];

$$t_d = K\frac{\eta L}{\lambda V_b} \tag{2}$$

where t_d is the translocation time, η is the viscosity of fluid, K is an arbitrary constant, λ is a linear charge density, L is the length of DNA, and V_b is the bias voltage. The above relationship is established by a balance between the electrical force and the viscous drag. Despite slowing down the speed, the current was dropped in accordance with glycerol adding. Effect of polyelectrolytes on DNA electronic property was explained where the conductivity (κ) is inversely proportional to the viscosity of fluids (η). The ionic current of DNA solution is defined as;

$$I_b = \frac{\kappa A V_b}{d} \tag{3}$$

where I_b is the current blockage, κ is the solution conductivity, V_b is the biased voltage, A is the hydrodynamic cross-sectional area of DNA, and *d* is the effective thickness of nanopore. Therefore, adding glycerol presented a counterbalance effect on translocation speed and current signal level. Adding glycerol to slowdown the DNA transportation would not be a good choice in virtue of decreasing the signal to noise ratio. Other group proposed glutamate as a candidate for ionic solution. They reported that the translocation speed could slow down by 11 times (down to 0.7 mm/s) while the signal was only suppressed by 7 times. [48]. This shows a promising result in manipulating viscosity to slow down DNA speed. Other fluids would be introduced in the future as the research work is still continuing. Ideally, slow motion of analytes could be realized in high viscous media. It showed a potential toward DNA sequencing, though a better signal level sustaining is required.

3.3. Temperature

A strong dependence of temperature on DNA translocation was reported in biological nanopore [12, 49]. Temperature change can alter the transmembrane protein conformation and cause a large effect on translocation dynamic in biological nanopore. The temperature effect also studied in solid state nanopores; however, only weak dependence of temperature on DNA speed and current blockage level was observed in synthetic pore (4-8 nm in diameter) [33]. The decrease of temperature from 22℃ to 4℃ only slowed down the short DNA (3 kbp) from 0.62 to 0.36 cm/s. They concluded that temperature dependence on translocation dynamic in solid-state nanopore was directly related to the effect of viscosity from Arrhenius model. Conversely, another group proposed a strong temperature dependence on the translocation time in 4 nm pore [26]. Decreasing temperature from 30 ℃ to 0 ℃ can prolong the translocation time up to 10 milliseconds (0.26 mm/s). This study contradicted the previous findings that the translocation dynamic was actually governed by DNA-nanopore interaction rather than the viscous effects; exhibited a strong influence on temperature. This finding supported that the interaction is mainly governed by the translocation dynamic in small nanopore. It also agreed with other previous parameter's studies where small nanopore exhibited a strong effect of each parameters on translocation time, while large pore showed less effect on almost all those parameters. Recent report demonstrated a strong effect of temperature on the DNA dynamic in large nanopore (10 nm) [50]. Decreasing temperature to 0 ℃ could prolong the translocation speed down to 7.5 mm/s. Their theoretical study explained that DNA dynamic in large pore was governed by the viscous drag of untranslocated part of lambda DNA sample. In summary, low temperatures could reduce the translocation speed of DNA, but the effect of temperature on the overall measurement system should be considered carefully.

3.4. Ionic concentration

Chang *et al.* observed an unexpected positive pulse current that increased during DNA translocation in the nanopore [51]. This result was contrary to all previous results that showed only drop of current when DNA came and blocked the pore. This phenomenon explained that DNA passed through the pore and attract additional cations into the pore surface. Those additional cations acted as interfacial charge in the pore, hence increasing the measurement current. Smeets *et al.* investigated this uncommon observation of the positive pulse and revealed that it was solely due to low ion concentration in the system [32]. They varied the KCl concentration from 1M to 0.05M and demonstrated that the 0.4 M is the turning point from negative to positive pulse current. This finding helped reveal the contradiction of the ionic pulse between Chang *et al.* and other researches. Chang conducted the experiment in 0.1 M electrolyte and observed the positive pulse, while other researchers commonly used 1M electrolyte where the measurement only showed a negative pulse. When the number of ion species is too low, the inverse effect appeared in which DNA molecules functioned as an ion species instead of blocking the pore. In contrast, DNA current blocking dominated the effect if the ionic concentration was high in the system. Previous researchers have not studied the influence of the ionic concentration on the translocation time. Such a study was revealed by varying the KCl concentration from 0.5-3 M [33]. Increasing ionic concentration can slow down the translocation speed but only in the range of 0.5-1 M. From 1 M to 3 M, translocation speed was saturated to about 0.76 cm/s, so that concentration rarely affected the translocation time after 1 M. Although 1 M KCl concentration is the standard value, indicating that they have already employed the optimum one. Up to this point, we were considering the manipulation of ionic concentration with symmetric concentration between two chambers. Later, Wanunu *et al.* reported asymmetric ionic concentration measurements [52]. The ionic concentration ratio of chambers was adjusted from symmetric (1M/1M) to asymmetric (4M/0.2M) ratio in their system. Large asymmetric concentration drove more cations from one chamber to another; hence area near pore located in the high salt side was concentrated with positive charge (see Fig. 3a). The established polarity dragged more DNA traveling through the pore against ion diffusion, resulting in elongation of the translocation time. Moreover, concentrated cations enhanced DNA capturing and increased the throughput of the system. They showed that the 4M/0.2M ratio of asymmetric concentration prolonged translocation speed down to 0.14 mm/s and 30-fold increase in capture rate. Thus, the asymmetric concentration can enhance both resolution and sensitivity to the measurement. More recent nanopore device developed this concept by directly utilized positive surface charge material, hafnium oxide (HfO_2), as a membrane rather than tuning the ion concentration (see Fig. 3b). It reported the best record for slowing down DNA translocation speed to 6 μm/s [53]. A strong effect of the pore size onto translocation speed was observed about 100 times improvement comparing between 1.7 and 1.4 nm. HfO_2 also possessed comparatively higher chemical stability than standard silicon membrane,

and thus the device is more robust and reliable [53, 54]. In addition, graphene also showed the ion selective function. Graphene surface can be furnished with carboxyl group by oxidative electrical pulse method. It possibly provides a better control of molecule transportation as reported in the simulation study [55]. Some research applied modified-surface graphene nanopore membrane for desalination process [56]. One possible scheme for future research would focused on modifying and engineering surface charge molecules on nanopore membrane to overcome translocation speed.

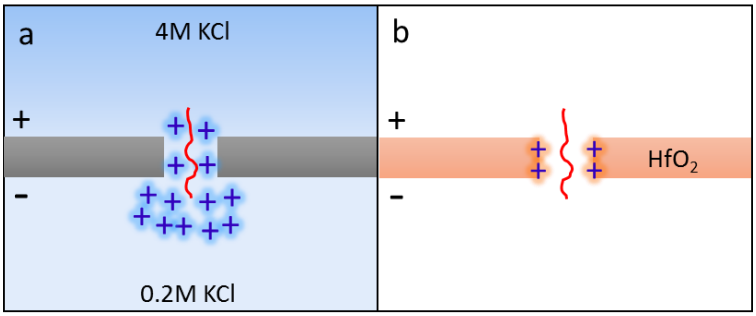

Fig. 3. (a) The asymmetric concentration of KCl (4M/0.2M) forced more potassium ion toward low concentration side, which would promote a strong interaction of DNA inside pore; (b) Positive surface of HfO_2 membrane showed the similar effect to attract DNA in the pore.

Other materials and structures had been integrated with conventional nanopore membrane to decelerate translocation speed. Nanofiber mesh structure (see Fig. 4a) had been introduced to slow down the speed to about 0.48-0.85 mm/s [57]. Entropic cage structure was reported to trap the DNA; however, the structure is not appropriate for DNA sequencing purpose [29]. Later, translocation speed of 2.2 μm/s had been achieved by coating nanopore with nanobeads structure, which presented a 2000 times improvement compared to standard nanopore devices (see Fig. 4b) [58]. This speed actually exceeds the requirement for DNA sequencing (60 μm/s); however, not all DNAs can be translocated at that speed. The imperfection of nanobeads structure leaked a bunch of DNAs travelling very fast through the pore. Apart from small random success, this low throughput device required the development on DNA capturing at the pore.

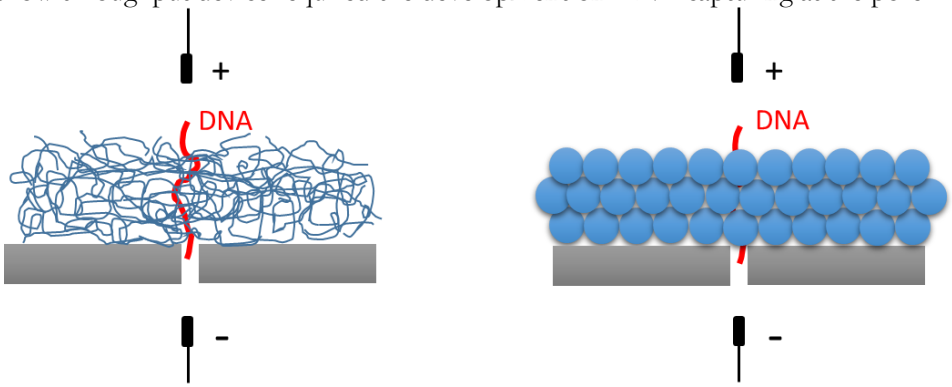

Fig. 4. Illustration of nanofibers (left) and nanobeads layer (right) on the membrane nanopore to retard DNA travelling speed.

The DNA translocation speed in different systems and set ups was summarized in Table 1. Translocation speeds in the table could not be solely justified among each other because the effect of pore diameters and DNA length have not been normalized. Herein, the overview would be at least simplified for preliminary comparison. Nevertheless, the ionic blockage current measurement seems arduous to attain the information and resolution of basepair sequencing. Other measurement approaches have been proposed, e.g. tunneling current detection, optical nanopore sensor, and force detection, which will be discussed shortly in the next section.

Table 1. List of selected ionic current blockage experiments in nanopore. Each system specified with DNA samples, pore diameters, and the result of translocation speeds.

Summary of translocation speed with different ionic current blockage set up				
Modification	**Sample**	**Pore diameter (nm)**	**Speed (mm/s)**	**Reference**
Earliest solid state nanopore	10 kbp dsDNA	10, 3	10, 3.4	[19]
Small nanopore with 200mV bias voltage	400 bp dsDNA	2.7 to 5	0.45	[26]
Very large pore with 200mV bias voltage	48.5 kbp dsDNA	30 to 60	0.15	[47]
Add 50% Glycerol to KCl solution	3 kbp dsDNA	4 to 8	1.7	[33]
Use glutamate as ionic solution	20.7 kbp dsDNA	20	0.7	[48]
Temperature decrease down to 0 C°	6kbp dsDNA	4.0	0.26	[54]
Asymmetric ionic concentration (4M/0.2M)	8 kbp dsDNA	3.5	0.14	[52]
Hafnium membrane	89-mer ssDNA	1.4	0.006	[53]
Graphene membrane	7 k-mer ssDNA M13	10	0.13	[42]
Nanofiber mesh structure	10 kbp dsDNA	6	0.48	[57]
Nanobeads array structure	5.3 k-mer poly(dA) ssDNA	2 to 3	0.002	[58]

4. Other Measurements Approaches

4.1. Transverse Electronic Current

In 2005, the idea to determine the exact electronic properties of DNA was proposed by measuring the current in transverse direction via nano-electrode gate tunneling [59]. This method required a couple of transverse electrodes embedded on the nanopore membrane. The detection volume is much smaller compared to that of the previous method. The ionic blockage current determines bulk ion conductivity, while tunneling current measures the information directly at the gap. DNA has two twisted chains of nucleotides where each nucleotide constructs with deoxyribose, nitrogen base, and phosphate group. The DNA code is determined according to the sequence of four different bases; adenine (A), guanine (G), cytocine (C), and thymine (T) attached along the chain. Two strands of polynucleotides adhere together by hydrogen bonds between complementary basepairs, either A-T or C-G. In principle, it was expected to read a direct signal when each basepair is passed through the nanoelectrode gap at once (see Fig. 5). The transverse electronic current technique showed a better ability to discriminate single nucleotide by measuring its actual electronic property. Theoretical study of molecular electronic of DNA between nano-electrodes gap has shown its potential toward DNA sequencing [60, 61]. From the simulation results, adenine (A) base on DNA strand demonstrated a higher current than guanine (G), cytocine (C) and much higher than thymine (T) owing to their distinct electronic and chemical characteristic of four bases. Later, the conductance of different bases

was found to be related to their geometries rather than electronic structures [62]. The larger base provided a shorter distance for charge carrier, and consequently, a higher conductance. The tunneling current level was ranked accordingly; A > G > C > T in their report. Unexpectedly, their results were somehow complied with previous simulation. Thus, electronic properties and geometries of bases lead a synergic effect on the DNA conductivity.

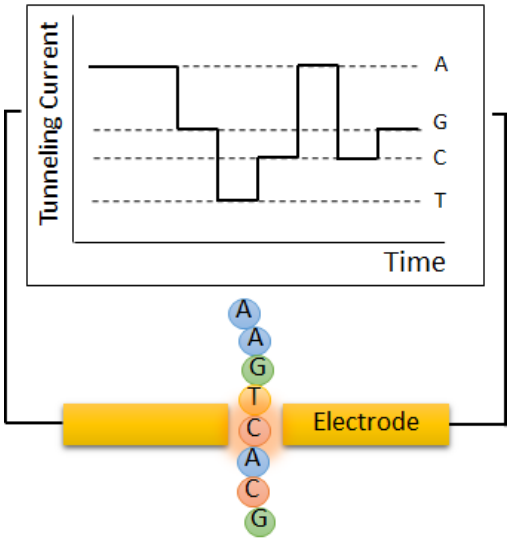

Fig. 5. Concept of tunneling current measurement on DNA nanopore sequencing. The sequence of nucleotide bases (i.e. Adenine-A, Guanine-G, Thymine-T and Cytocine-C) on DNA strand passed through small nanogap electrodes. The tunnelling current was measured at the gap; ideally presented different signal level among four nucleotides.

Oshiro *et al.* has experimentally demonstrated the nucleotide measurement using scanning tunneling microscope (STM) [63]. A tunneling current was observed when nucleoside modified tips are in contact with a complementary nucleoside-furnished surface. Nucleoside is a nucleotide without phosphate groups. This result encouraged other research groups to use STM to study DNA's electronic properties. The determination of hydrogen bond stiffness of basepairs (i.e. A-T, C-G) was reported using STM between thiolate bases modified tunneling tip and nucleosides on gold planar surface [64]. Eventually, Tsutsui *et al.* presented the first nucleotide detection between nanopore electrode gap [65]. They observed different tunneling currents of three nucleotides solutions, i.e. thymidine monophosphate (TMP), guanosine monophosphate (GMP), and cytidine-monophosphate (CMP). GMP presented a larger current than CMP and TMP respectively in which HOMO-LUMO gap of guanine > cytocine ≈ thymine. The result agreed with the previous theoretical study [61]. AMP has more complicated characteristic of measured current possibly due to the nonspecific binding with gold, so it had not been measured and compared [66, 67]. The transverse current measurement had been conducted to measure strand of lambda DNA [68]. The detecting resolution was merely comparable to the conventional ionic blockage, which still unable to discriminate single basepairs information. Thus, the transverse electronic measurement also required DNA dynamic controllability.

Graphene was then introduced to the transverse current sequencing in an attempt to confine detection area better as shown in Fig. 6. Theoretical simulations of graphene nanopore exhibited an enhancement in tunneling signal toward DNA measurement comparing to those of conventional nanogap electrode materials [69-71]. Translocation speed can be slightly slowed down by the strong interaction between graphene and polynucleotide. The measurement space was more confined comparing to that of conventional membrane; however, graphene pore exhibited a larger noise caused by small molecules interruption which fluctuated DNA signal. At the moment, the challenge is to manage the reproducibility and reliability of measurement and fabrication of graphene sheet pore as a nanogap sensor. So far, DNA tunneling current measurement had never been conducted experimentally using graphene nanopore. Recent study measured graphene tunneling current characteristic by mechanically adjusting the junction between two graphene sheets [72]. This finding showed the possibility toward DNA sequencing using graphene nanogap in the future.

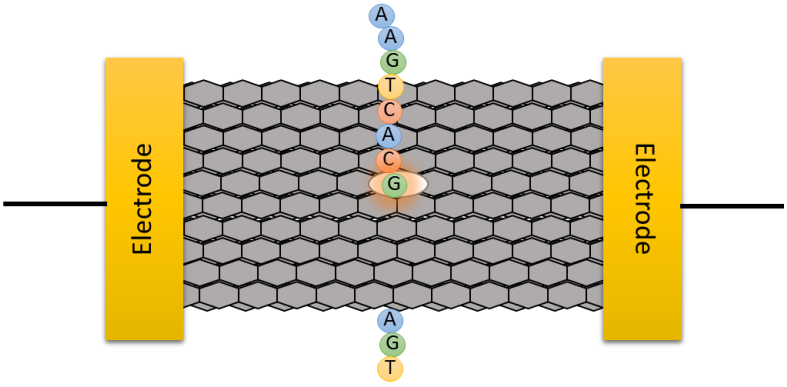

Fig. 6. Schematic of graphene nanopore for tunneling current measurement. Graphene sheet as a conductive material can directly be connected with 2 measured electrodes. The DNA was passed through the pore fabricated on graphene sheet.

Another promising idea is applying a long conduit membrane; i.e. nanochannel structure, with embedded the nanogap electrode, at the point of detection (see Fig. 7). Small channel can overcome DNA entropic barrier by unfold and confine the DNA better than nanopore due to its geometry. Besides, higher contact area between DNA and the channel surface possibly slow down the DNA translocation by increasing the interfacial interaction. Transverse current detection can be employed at the nanogap to detect whenever the nucleotide strand passes through. The real time DNA (1.1 kbp dsDNA) detection through 9 nm width nanochannel was conducted; however, the translocation speed was unexpectedly high (4 mm/s) [73]. Other research showed that lambda DNA (48.5 kbp) could travel at 0.083 mm/s through their nanochannel device [74]. Nanochannel structure showed a promising scheme to confine the DNA dynamic; however, inevitable drawbacks of nanochannel are the high possible because of sample clogging and the leakage of the channel. Noise was also accumulated along a conduit channel. Anyhow, number of theoretical studies of DNA sequencing in nanochannel model continued to study and demonstrated its potential toward DNA sequencing [75-77]. Nano funnel structure integrated with nanochannel was also proposed to facilitate the DNA translocation as it can suppress the electric field effect onto the DNA [77].

4.2. Optical Detection

The optical detection is another promising method for the nanopore based DNA sequencing technology proposed by Meller group [78]. The principle is to map each basepair from DNA strand on two-unit code oligomers by biochemical conversion. The arrangement of two-unit code strands constructed four representative color sets for four nucleotide's bases (A, T, G, and C) (see Fig. 8a). Those code strands were hybridized with two small strands of DNA designed for fluorescent emission mechanism [79, 80]. They are oligomers tagged with fluorescent label molecules and self-quenching molecules. At first, the quencher molecules halted the fluorescent emission, and the hybrid DNAs stayed dark. When the hybridized strands (between code strands and two fluorescent-mechanism strands) translocated through nanopore, they will unzip and strip each fluorescent strand off one by one. The nanopore diameter is constrained to about 2 nm in size since the cross-sectional diameter of DNA is 2.2 nm. Figure 8a illustrates the concept of unzipping optical nanopore sequencing. The DNA unzipping mechanism has extensively been studied both in biological nanopore [81-84] and solid-state nanopore [85]. After the fluorescent tagged oligomers were unzipped away from quencher strands, those free fluorophores can emit light upon excitation. The two-color code was then translated back to the target DNA sequence for each nucleotide. The optical read out of DNA in the solid-state nanopore was reported in the real experiment [86]. The DNA analytes were tagged with fluoropores (FRET tags); showing the fluorescent signal when DNA unzipped the tag strands. The fluorescence signal was detected by the total internal reflection fluorescence (TIRF) microscope. Simultaneous measurement of ionic current blockage and fluorescent detection agreed with each other. The optical signal showed slightly slower speed owing to unzipping process; however, large background noise interfered with sample signal from undesired photoluminescent of membrane itself. The enhancement of optical signal was proposed by using an array of synthetic nanopores in parallel measurements [87]. They measured two color codes and

presented the ability to read single nucleotide base but with 10% identification error. Others suggested the application of plasmonic structures for unzipping method with a report of simulation results [88, 89]. In their report, surface-enhanced Raman signal could separate the information of different nucleotides along DNA strand. The real fabrication of nanopore embedded in plasmonic nanowell could actually enhance DNA signal about 10 times in comparison with previous study [90]. Nanoslit structure has also been introduced in Raman spectroscopy to promote optical signal showing the distinct spectrum shift of four different bases (see Fig. 8b) [91]. The nanoslit was fabricated by nanomachining processes; starting from electron beam lithography to create pattern of nanoslit, KOH anisotropic wet etching to make inverse pyramidal cavity shape, BHF etching to open the nanoslit structure and Au sputtering. Au nanoslits were then used to test with 4 different nucleotide sample solutions (dAMPs, dTMPs, dGMPs, and dCMPs). Those results showed a high potential toward DNA nanopore sequencing. Optical measurement showed a successful read-out of separate base pairs; nonetheless, it required complicated sample preparation steps and large instruments set up. This technique would lie somewhere in the middle of traditional sequencing and nanopore sequencing, although the process is faster and more compact in comparison with the bench-top laboratory system.

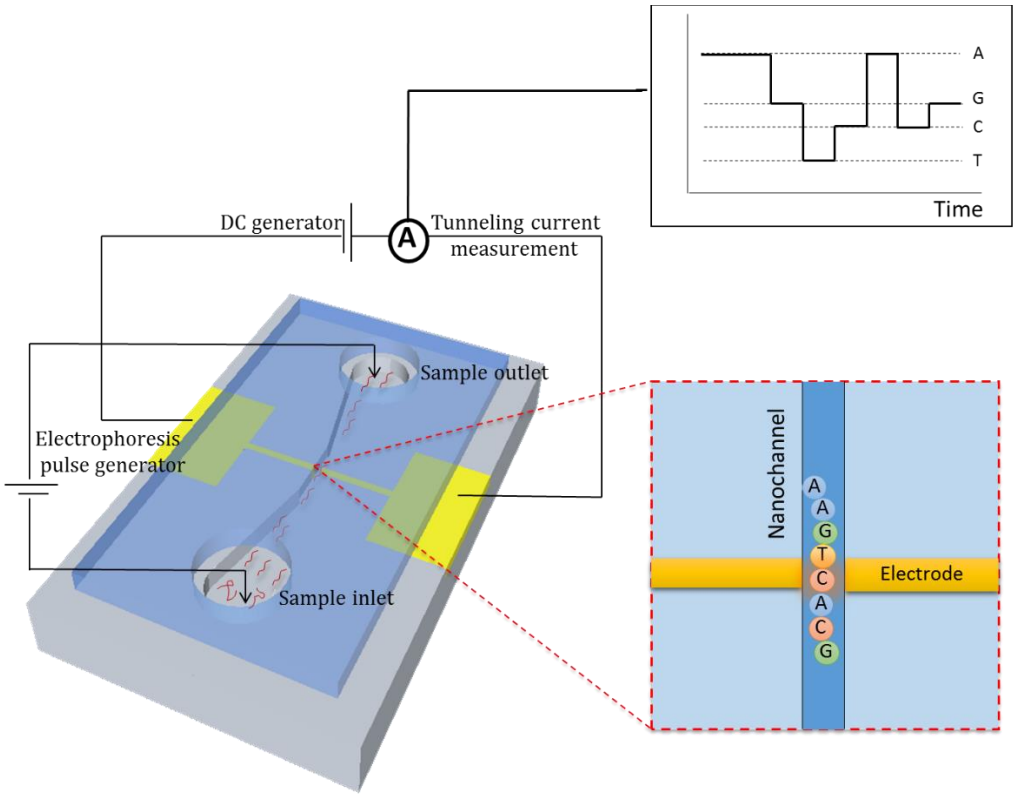

Fig. 7. Tunneling current measurement of DNA passed through nanoelectrodes gap, which is embedded in nanochannel device. The principle of detection is same as that of the transverse current nanopore sensor.

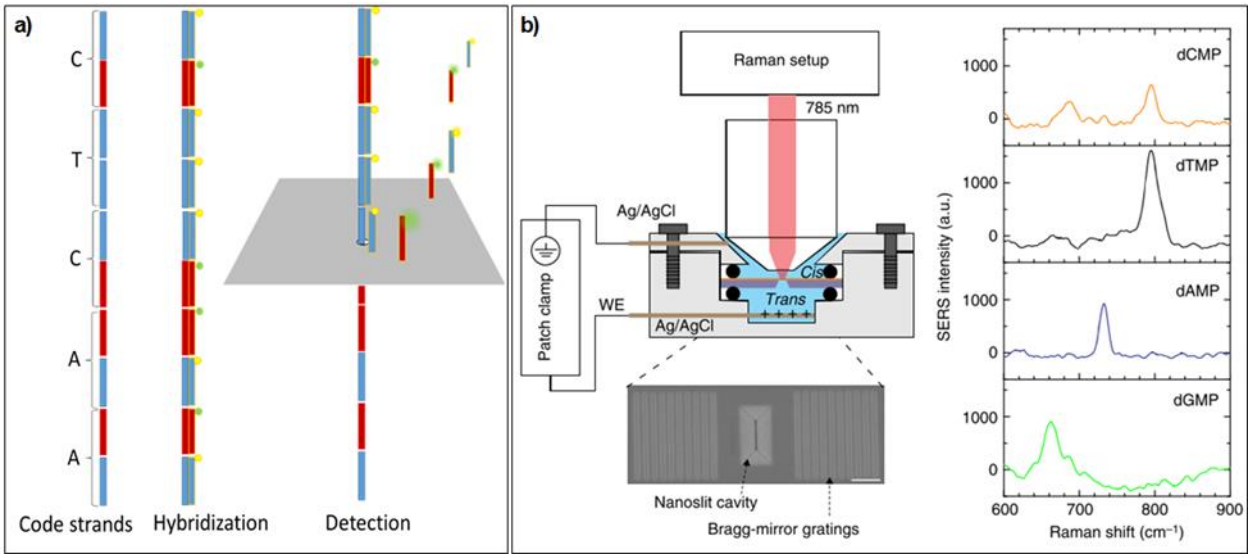

Fig. 8. (a) The nucleotides mapped with 4 four arrangements of two-unit code oligomers. Those two code oligomers were hybridized with the fluorescent strand and quencher strand. When the hybridized DNA passed through the pore, the optical signal is detected by fluorescent emission. (b) The measurement setup of nanopore device with nanoslit cavity to enhance Raman signal. They showed distinct Raman peak shift for 4 four different nucleotides [91].

4.3. Force Detection

Optical tweezers has been applied to manipulate and measure mechanical properties of single molecules [92, 93]. The analyte was attached to the microbead, which controlled the position by laser beam. The optical trapping concept was adopted for nanopore DNA sequencing since 2006 [94]. In the vicinity of the pore, the voltage bias drove the DNA through the pore by an applied electric field. The optical trap force was measured and it correlated well with the travelling distance of bead against an electric force. The measuring force could determine the effective charge of DNA according to its length. In addition, they observed the simultaneous force and ionic current response when DNA passing through the pore (see Fig. 9) [94]. Optical tweezers not only control the translocation of DNA, but also waver single DNA back and forth through the pore [95]. Trepagnier *et al.* applied optical tweezers to move the DNA molecules backward and forward in the vicinity of nanopore with only 10 mV, which is about 10 times less than the bias voltage required to drive DNA through the nanopore in the first place. This result provided the ability to conduct multiple measurements of same DNA molecules. They can manage the movement of DNA by varying the voltage in the DNA flossing. In addition, optical tweezers were applied to other analytes, such as protein coated DNA [96] and dsRNA [97]. Recent studies utilized optical tweezers together with lipid bilayer coated membrane to suppress electroosmotic flow speed [98]. Force detection provides the advantage toward the DNA translocation dynamic; nevertheless, system is comparatively large and complex. In addition, the capturing of long DNA strand is troublesome via optical trapping.

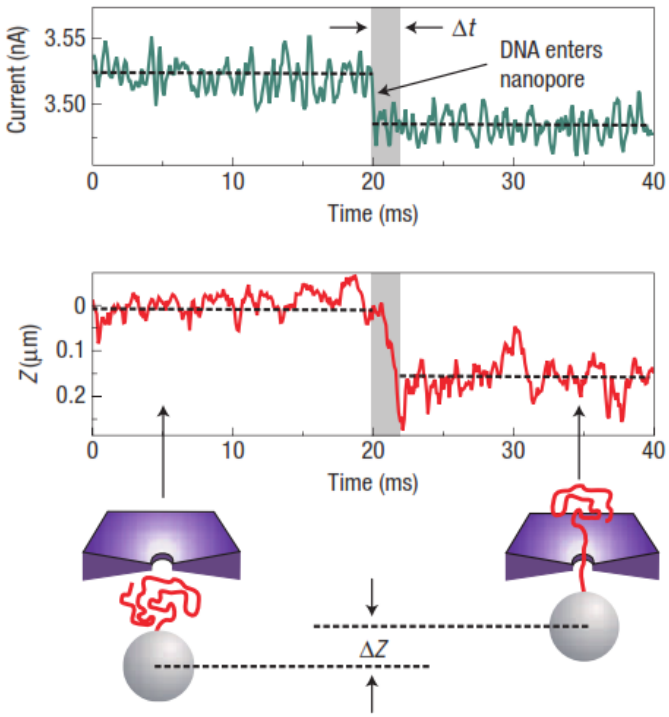

Fig. 9. The result comparison between ionic current measurement and displacement measuring of DNA by optical tweezers before and after DNA travel through the pore [94].

5. Synthetic Nanopore Fabrication

The synthetic nanopore adopted the conventional micro/nanofabrication techniques to create an artificial pore on the membrane materials [99]. The first solid-state nanopore was fabricated using ion beam sculpturing technique on silicon nitride material [18]. Afterwards, various materials were introduced as nanopore membrane materials, e.g. semiconductors (Si, SiO_2, Si_3N_4, SiC, Al_2O_3, ZnO, BN), metals (Au, Al, Ni, Mg) and polymers (polyimide, polyethylene terephthalate and polycarbonate). Likewise, number of fabrication techniques were applied upon variety of materials, such as particles beam sculpting, controlling deposition, focus beam drilling, glass nanopipettes, and track etching, etc. Particles beam sculpting method has been used for nanopore fabrication by using ion beam either open the small pore that prepared with back etching (Fig. 10a) or closing the large pore that was made by FIB or wet etching (Fig. 10b) [18, 24, 100-109]. The size reduction by atomic layer deposition (ALD) has similar principle with the sculpting method, unless the pore closing step is done by uniform coating [21, 110, 111]. Evaporation of metal has been applied to shrink nanopores as well [112, 113]. Meanwhile, direct drilling is a single step process that supplied a small ion or electron beam to drill a very thin substrate at once to create small pore (Fig. 10c) [46, 47, 100, 114]. For glass materials, the nanopore can be constructed by laser heating to draw the hole accompanied with mechanical force to open the pore (Fig. 10d) [115-117]. Polymer pores are usually fabricated by ion track etching (Fig. 10e) [118-120]. At first, high energy ions bombarded the membrane material to create a nanopore track, then applied wet etchant to remove the damaged area resulting in an open-pore structure. The organic pore structure can also be fabricated and controlled by thermal decomposition [42, 43]. Other than those conventional techniques, some groups introduced elastomeric membrane as a size-tunable nanopore by applying a mechanical strain on polymer, i.e. polyurethane (Fig. 10f) [121-123]. This size-alterable nanopore allows a real time diameter adjustment to enhance signal and increase the reproducibility of the detection result. It entails a higher reliability of the nanopore size distribution data, which is crucial in the analysis of the nanopore sensor. The conventional fix-sized nanopore researches required several pore fabrication in order to attain pore-size differentiation data that would increase sampling errors. Recently, a promising synthetic nanomaterial has been proposed as a candidate for nanopore based sensor, namely graphene, owing to its exceptional electrical and mechanical properties. Graphene thickness is especially

comparable to the DNA basepair unit (0.34 nm), lending a better potential to read signal from each DNA nucleotide. Fabrications of small pores using electroburing or particles beam lithography process on graphene membrane have been reported [124-129]. Nanopore fabrication processes together with the sensor development are, currently, on-going to achieve the actual basepair DNA-sequencing. Many fabrication techniques have been introduced according to the proposing materials and new measurement schemes.

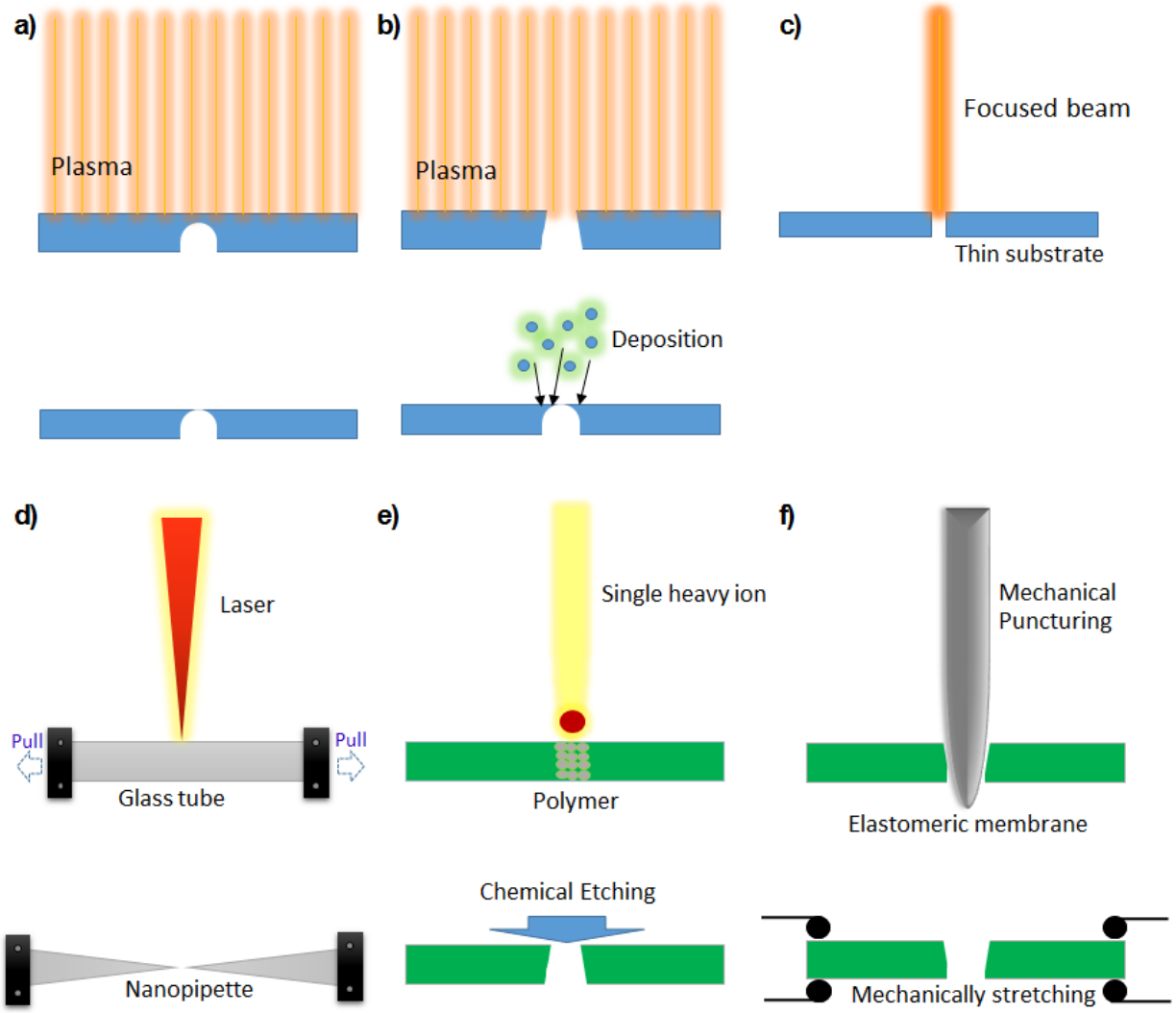

Fig. 10. (a) Particle sculpturing method can open a small pore in the thin region. (b) Large pore can be closed either by ion sculpting or deposition. (c) Direct drilling was used to make a small pore on the thin membrane. (d) Glass pipette created nanopore on glasses by applied laser and mechanical pulling simultaneously. (e) Ion track etching applied heavy ion to create nanopore track and the pore was opened by wet etching. (f) Elastomeric membrane nanopore was fabricate by mechanical punching, the size controllable nanopore was prepared for further adjustment.

6. Summary and Future prospects

Nanopore based DNA sequencing surpasses the conventional sequencing technology in many aspects. Despite those benefits, the most undeniable challenge toward this novel technology is the controlling of DNA translocation speed in the nanopore which prevents the ability to discriminate single nucleotide on the strand of DNA. The development of solution moved toward the manipulation of the DNA translocation in the pore. Many solutions were proposed to overcome such an obstacle, e.g. tuning nanopore membrane, modifying experimental parameters or employing other alternative measurement systems. The analogy of each method was summarized in Table 2. Ionic blockage measurement is the simplest way for device

fabrication and experimentation; however, the resolution was almost impossible to provide single nucleotide sequencing detection. Optical detection by far reported a DNA sequencing ability, though the tradeoffs would prevent it from portability and industrialization. Force detection was applied to solve translocation problems; however, it was not a standalone approach. Currently, the most promising option is the transverse tunneling current measurement, which provides high resolution, direct measurement of basepair, small system, and ease of quantification. Cooperating with some structure, i.e. nanochannel or surface modification could enhance the controllability of translocation dynamic in the future. Recently, the new idea was proposed to use nanochannel for genome mapping rather than flow sequencing [130]. DNA will be fixed in the channel and measured each section of freeze DNA by a sequence of consecutive detection points. This concept might be skeptical in a way to control a very adjacent detection point and DNA confining. Each system has its own strengths and drawbacks; however, their potential toward DNA sequencing technology has been emphasized. The development of a device is still ongoing to overcome the translocation dynamic of DNA. Novel material and the modification of surface would expect to finally achieve DNA nanopore sequencing technology. In near future, the cooperation of many methods should accomplish the nanogap DNA sequencing technology to attain high resolution, high throughput, low-cost, and simple operating system.

Table 2. The comparison of nanopore detection methods.

Method	Advantages	Drawbacks
Ionic Blockage measurement	• Easiest way to set up the system • Well developed and contain availability of references • Small system	• Indirect measurement method as measuring the bulk ionic solution • Low resolution in compare with other detection methods
Transverse Current measurement	• Higher resolution to discriminate the basepair at the nanoelectrode gap • Gain better opportunity to control the translocation of the base pair through the pore by adjustment of transverse field	• Fluctuation of the current measurement due to DNA conformation change • Large noise caused by DNA interaction and electronic signal of other species
Optical Detection	• Higher resolution for the separation of nucleotides as the unit codes • Be able to slow down the DNA translocation by unzipping process	• Required small pore less than the diameter of the dsDNA (2.2nm) for unzipping process • Consume huge amount of time • Very complicated steps • Required large operating system
Force Detection	• Allow the ability to control the translocation velocity through the nanopore	• Difficulty in capturing the DNA at the pore entrance • Required complicated set up of DNA- bead and optical tweezers

Acknowledgement

This work was supported by Engineering Research Grant, Faculty of Engineering, Chulalongkorn University (No. 002/2562) and International School of Engineering. MEMS and Nanotechnology laboratory facilities to carry out this work is greatly acknowledged.

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