

TOPICAL REVIEW

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Topical Review

DNA sequencing by nanopores: advances and challenges

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Abstract

Developing inexpensive and simple DNA sequencing methods capable of detecting entire genomes in short periods of time could revolutionize the world of medicine and technology. It will also lead to major advances in our understanding of fundamental biological processes. It has been shown that nanopores have the ability of single-molecule sensing of various biological molecules rapidly and at a low cost. This has stimulated significant experimental efforts in developing DNA sequencing techniques by utilizing biological and artificial nanopores. In this review, we discuss recent progress in the nanopore sequencing field with a focus on the nature of nanopores and on sensing mechanisms during the translocation. Current challenges and alternative methods are also discussed.

Keywords: DNA sequencing, translocation, biological nanopores, artificial nanopores, carbon nanotubes, fluorescence

(Some figures may appear in colour only in the online journal)

1. Introduction

All information required for the functions of living systems is contained in genomes of organisms and plants, and to decipher this information one can use a process which is known as DNA sequencing [1, 2]. DNA is a biological heteropolymer consisting of four nucleotide monomers: adenines (A), guanines (G), cytosines (C) and thymines (T). The specific order of how these nucleotides are arranged essentially codes all biological phenomena. The process of DNA sequencing is a precise determination of the amount and distribution of these nucleotides in DNA molecules [1, 2]. It has a very strong impact in various biological fields, including human genetics [1, 3], plants and agriculture [4], bioinformatics [1, 3, 5],

studies of microbial species [5], cancer and viruses [4, 6], and many others. Progress in DNA sequencing always leads to revolutionary advances in the diagnosis and treatment of various diseases [4, 5].

Large sizes, genetic variations and complexity of biological genomes require efficient sequencing methods in order to properly characterize them. For example, the human genome consists of approximately three billion bases. Sequencing a large amount of bases is time consuming and quite expensive using current methods [2]. The increasing demand for faster and cheaper genome sequencing resulted in the development of advanced sequencing technologies [1–3, 7]. However, the existing methods of DNA sequencing are still not well optimized from the point of view of cost and speed. The ‘\$1000 Genome’

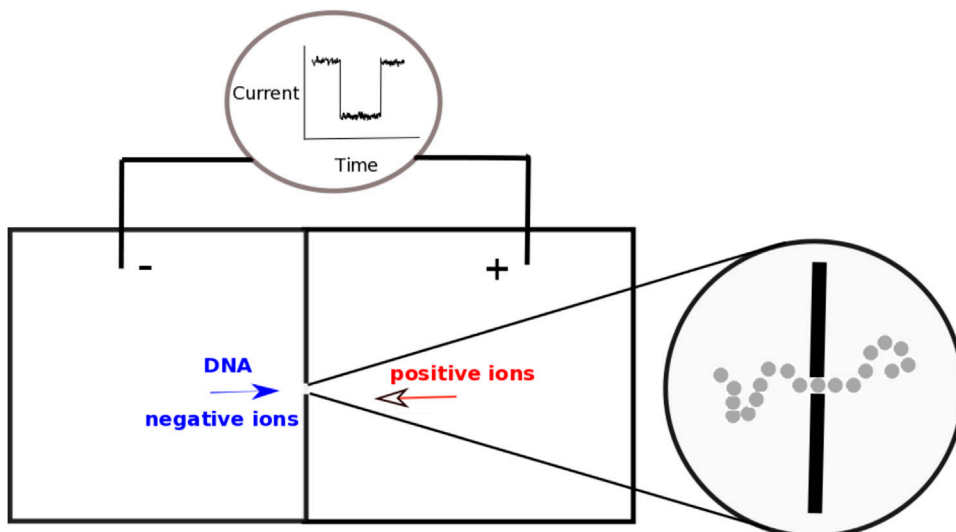


Figure 1. The principle of nanopore ionic measurements. Small ions migrate toward the membrane due to the bias voltage across a pore. The current through the channel decreases while the DNA molecule is in the pore.

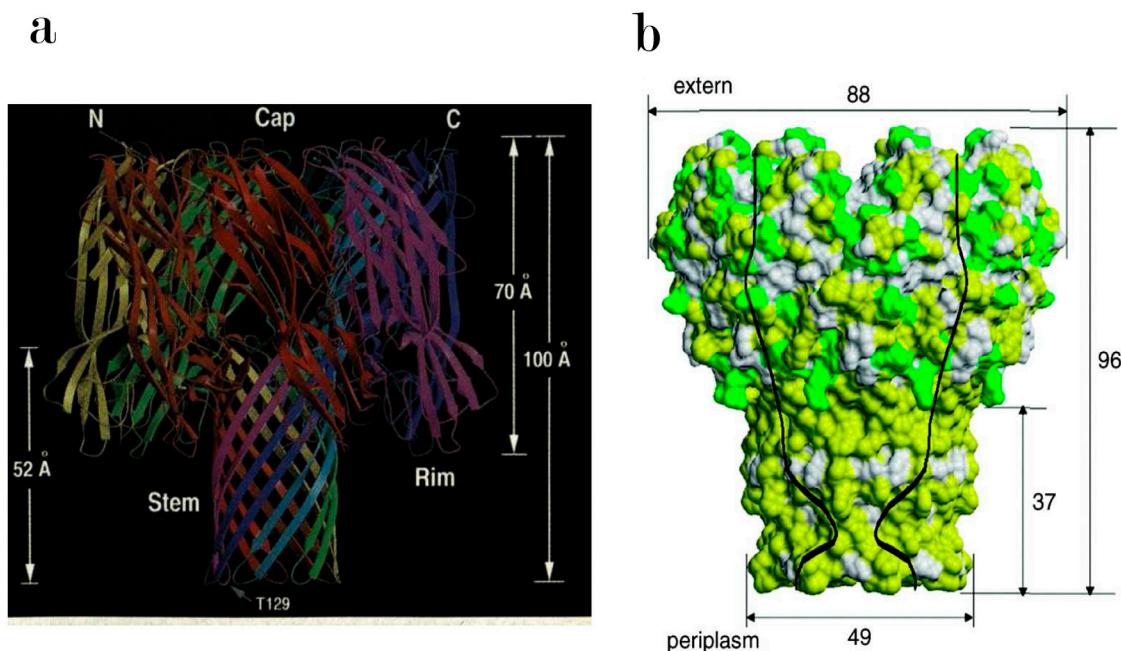


Figure 2. Structures of biological nanopores: (a) α -hemolysin (from [26]. Reprinted with permission from AAAS), (b) MspA (from [27]. Reprinted with permission from AAAS).

project was introduced by the National Institute of Health in order to make technological breakthrough in solving the problem, expecting that this should facilitate the development of a new level of personalized medicine [8]. The goal is to achieve a full genome sequencing in a patient within 24h for a cost of less than \$1000. As a result, in recent years significant efforts were made in the search of cheaper and faster sequencing methods.

One of the most promising new approaches to DNA sequencing, that meets the challenges of the cost and speed, is the use of biopolymer translocation via nanopores, which is known as nanopore sequencing [9–13]. Nanopore sequencing is a label-free and amplification-free single-molecule

approach, in which DNA molecules are driven through channels, producing signals that allow researchers to identify the corresponding sequences [9, 10]. It was proposed in the middle of 1990s independently by several research groups [12, 14–16]. The basic scheme of the nanopore translocation experiments is shown in figure 1. The idea of this approach is that individual nucleotides in the DNA molecules can be differentiated with characteristic changes in the trans-membrane currents and dwell times when the voltage-driven bi-molecule passes through the pore that connects two chambers with electrolyte solutions [12, 14–16]. Because DNA molecules are charged, voltages are applied to drive them across the nanopore. While the biopolymer is not in the channel,

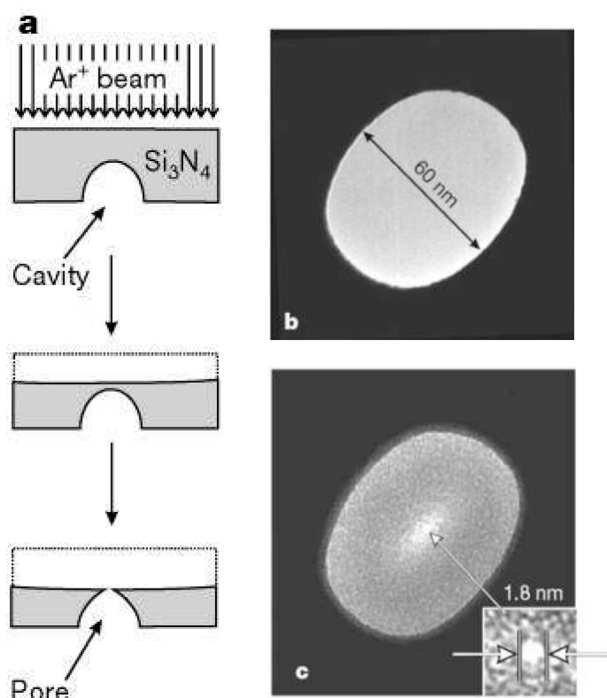


Figure 3. (a) Schematic illustration of the solid-state nanopore fabrication by ion beam sculpting. (b) TEM image of initial 61 nm diameter pore made by FIB in a 500 nm Si_3N_4 membrane. (c) TEM image of the same sample after Ar^+ ion-beam exposure (reprinted with permission from [34]).

the basic current due to small ions in both reservoirs can be sustained. Once a DNA molecule enters the pore, the fluxes of ions are decreased (mostly due to geometric constraints), and these blockages are measured both in terms of the currents and in terms of the translocation times [12, 13]. Since these parameters result from complex interactions between DNA and the nanopore, it is expected that the passing current should directly correlate with the sequence of translocating biopolymers.

Nanopore sequencing is a complex process that utilizes both experimental and theoretical methods in order to determine the chemical composition and the arrangement of monomers in the DNA molecules [7]. It has been argued that the nanopore sequencing could be faster and more efficient than other techniques due to its inexpensive sample preparation, direct procedure, minimal requirements for chemical modifications or for enzyme-dependent amplifications. This eliminates the need for additional enzymes such as polymerases or ligases during the readout process, and the purified fluorescent reagents are also not needed [17, 18]. At the same time, a significant number of issues should be resolved before the method can be widely applied [10, 18–20]. This review briefly discusses recent advances in the DNA nanopore sequencing, as well as the existing challenges and possible alternative approaches. A large number of comprehensive reviews, commentaries and a book cover in detail various aspects of the nucleic acids translocation through the channels and nanopores [7, 11, 13, 18, 20–24].

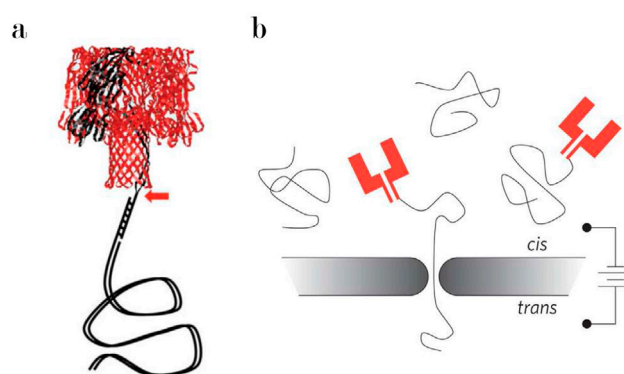


Figure 4. (a) An example of the hybrid nanopore system: α -hemolysin heteroheptamer with a 3 kbp dsDNA attached via a 12-nucleotide oligomer to one protein subunit. (b) Experimental setup, in which protein-conjugated dsDNA is electrophoretically translocated through a narrow solid-state nanopore (reprinted with permission from [49]).

2. Nature of nanopores

The main component of the nanopore sequencing method is nanopores. Because the signal produced during the translocation is a result of interactions between the confined DNA molecules and the channels, it is important to understand the physical–chemical properties of the nanopores that are employed in these experiments. There are two main classes of nanopores currently used in the biopolymer translocation studies: biological and artificial pores. Below, we discuss and compare the performance of the different types of nanopores for DNA sequencing.

2.1. Biological nanopores

First biopolymer translocation experiments utilized biological nanopores that were made from protein membrane channels such as a toxin protein α -hemolysin [7, 12, 15, 16, 20, 25]. This protein spontaneously inserts into membranes forming a cylindrical pore. It is always found in the open configuration, supporting the fluxes of small ions. In the narrowest part of the channel the diameter is close to 1.5 nm so that only single-stranded DNA or RNA molecules can pass it [7, 12, 15]. The structure of this nanopore is shown in figure 2(a).

There are many advantages in using α -hemolysin nanopores for DNA sequencing. The channel is quite stable under different chemical and thermal conditions in the system: it can work when pH is ranging from 2 to 12 and for the temperatures up to 100 °C. The biological nature of pore also allows researchers to make chemical modifications via mutations, enhancing specific interactions with translocating polymers [7, 12, 20, 25]. This is the reason why α -hemolysin is the most commonly used nanopore. Experiments show that α -hemolysin is able to distinguish different types of nucleotides [7, 12], and furthermore, show that different nucleotides can produce different blockage currents [19].

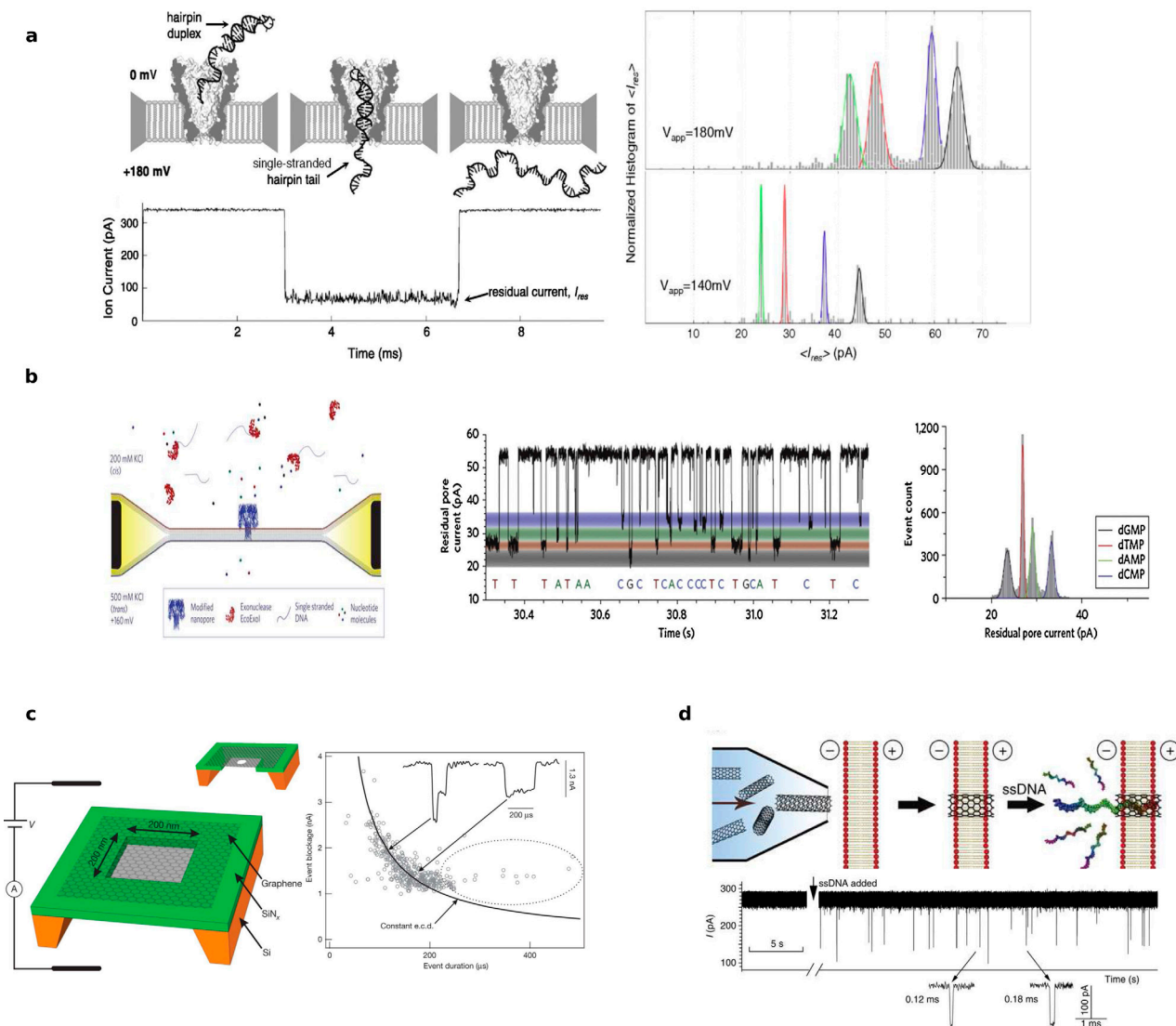


Figure 5. Examples of ionic current measurements in biological and solid-states nanopores. (a) Left: DNA translocation through the nanopore MspA. Right: differentiation of DNA homopolymers with biological nanopores. Green, red, blue and black spikes denote poly (dT), poly (dC), poly (dG), poly (dA), respectively (reprinted with permission from [29]). (b) Left: experimental setup for the an exonuclease attached to the top of the α -haemolysin pore through that sequentially cleaves dNMPs (gold) off the end of a DNA strand. Middle: single base recording from the pore with amino-cyclodextrin modified pore upon addition of mono-nucleotides showing dGMP, dTMP, dAMP and dCMP discrimination. Right: corresponding residual current histogram of nucleotide binding events (reprinted with permission from [54]). (c) Left: graphene membrane in SiN_x suspended across a Si frame. Right: event blockage versus event duration when DNA driven through a 5 nm-diameter graphene pore by an applied voltage bias of 160 mV. Scatter plot of showing that folded DNA (left of inset, deep blockade level) and unfolded DNA (right of inset, shallow blockade level) can be distinguished. The solid line represents a constant event charge deficits (reprinted with permission from [61]). (d) Top: illustration of the insertion of a SWCNT into lipid bilayer for translocation the DNA. Bottom: typical ionic current signal recorded when DNA was added to the cis side of a SWCNT nanopore (reprinted with permission from [60]).

Despite extensive applications in multiple experiments, there are several drawbacks of using α -hemolysin for DNA sequencing. As one can notice from the structure (see figure 2(a)), between 10 and 15 nucleotides can occupy the cylindrical β -barrel region. The resulting interactions with the nanopore are due to all of them, and it is difficult to deconvolute the obtained signals with a single nucleotide precision [7]. These observations stimulated a search for a better nanopore where a single nucleotide resolution can be obtained. It was found that another membrane protein, *Mycobacterium smegmatis* porin A (MspA), can successfully function as a nanopore for DNA sequencing after proper

genetic engineering [7, 28]. It has a funnel shape, as shown in figure 2(b), and the diameter at the constriction is close to 1 nm [29]. This channel is more stable under extreme external conditions than the α -hemolysin. It was shown that MspA still functions in strong acids and bases, as well as at high temperatures (but below 100 °C, similarly to α -hemolysin) [20]. MspA achieved excellent spatial resolution in some DNA sequencing experiments [20, 29]. However, the translocation was too fast to clearly distinguish single nucleotides without errors, and additional DNA cutting enzymes had to be added to the system, which significantly complicated DNA sequencing experiments [29].

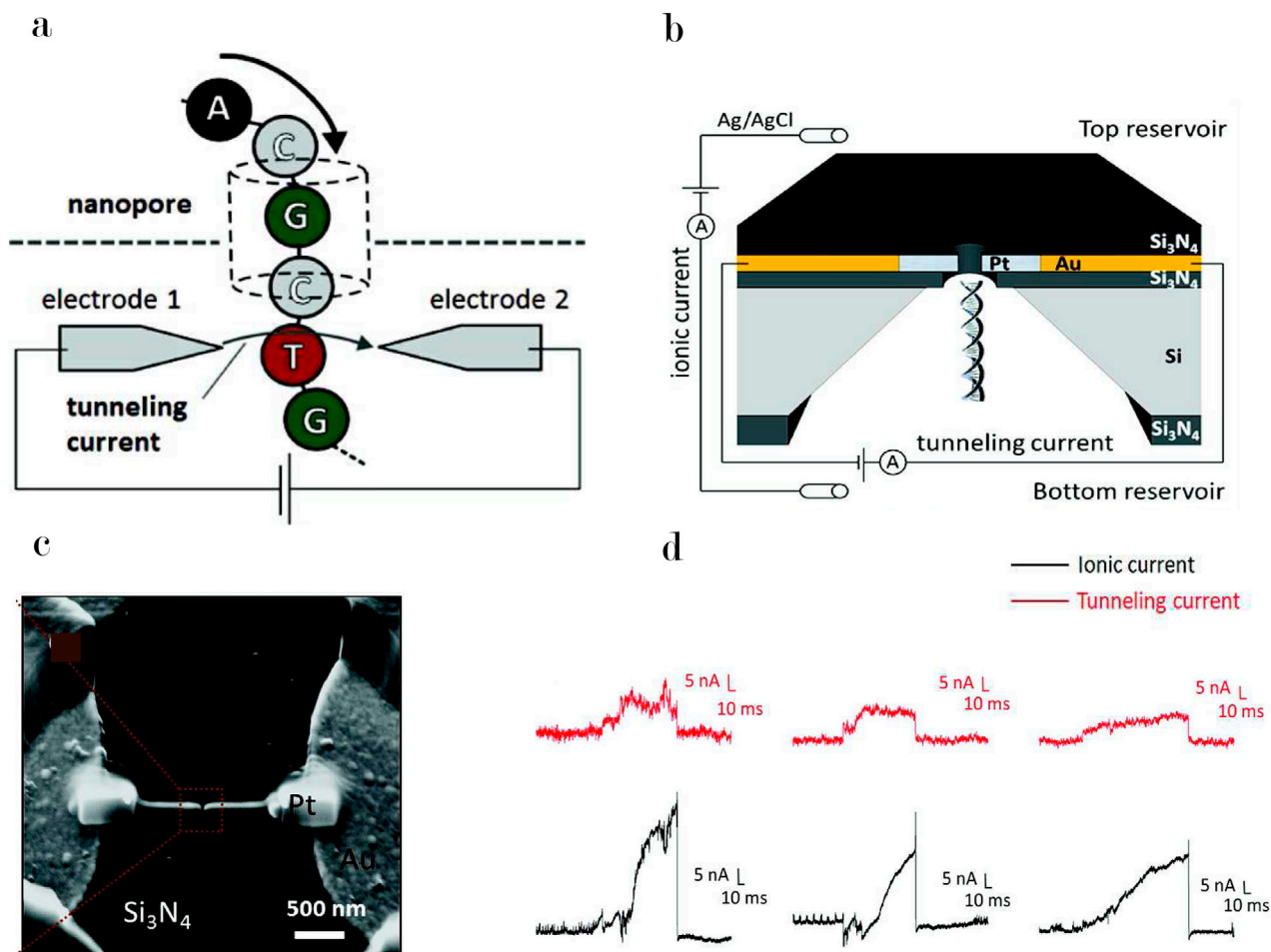


Figure 6. (a) Principles of the transverse current measurement. (b) Schematic of the device when two platinum electrodes were integrated near a SiN_x nanopore by electron beam deposition. (c) SEM image of the Pt nanoelectrodes fabricated by EBID. (d) Detection of DNA events by ionic and tunneling current (reprinted with permission from [67], copyright 2011 by the American Chemical Society).

Both α -hemolysin and MspA nanopores are narrow channels so that only single-stranded DNA or RNA chains can pass through them [7, 12]. To investigate double-stranded DNA chains, it was proposed recently to explore the engineered bacteriophage phi 29 protein channel [30]. It has a larger diameter, closer to 3.6 nm, and higher conductance than other biological nanopores. This significantly improves the resolution of translocation measurements. Phi 29 is also chemically stable, it can withstand large voltages, and it has many more possibilities for mutational changes [20, 30]. However, no successful DNA sequencing has been reported so far. The main problem here is how to interpret the observed experimental signals and how to connect them to the sequence of translocating DNA.

A powerful new pathway for creating new biological nanopores was recently proposed by Movileanu and coworkers [31]. By utilizing genetic engineering methods, the ferric hydroxamate uptake component A (FhuA) β -barrel protein was converted into a large-conductance biological nanopore with controllable physical-chemical properties. In principle, this method can be applied to any β -barrel protein channel to make them powerful single-molecule sensors. Although this investigation shows a great promise for sensing biological molecules, there has been no DNA sequencing reported with this approach yet.

Generally, biological nanopores have many advantages in application for DNA sequencing, including the simplicity of devices, their flexibility, sensitivity and the ability of chemical modifications via mutations [7, 10, 32]. They are very convenient for investigation of biological processes that involve interactions of DNA with other molecules [7]. However, devices based on biological nanopores also suffer from many problems such as restricted sizes and limited stability with respect to modifications in physical parameters. In addition, they are quite fragile, and the observed signals strongly depend on chemical conditions (such as pH, temperature, and salt concentration), complicating the interpretation of them. Therefore, the prospects of the nanopore sequencing method based purely on biological pores currently seems to be limited [33]. At the same time, there are recent developments, which we discuss below, that give some promise to a successful DNA nanopore sequencing with biological pores.

2.2. Solid-state nanopores

The limitations of biological nanopores stimulated significant experimental efforts in developing artificial nanopores in solid-state membranes [33–35]. In comparison with biological

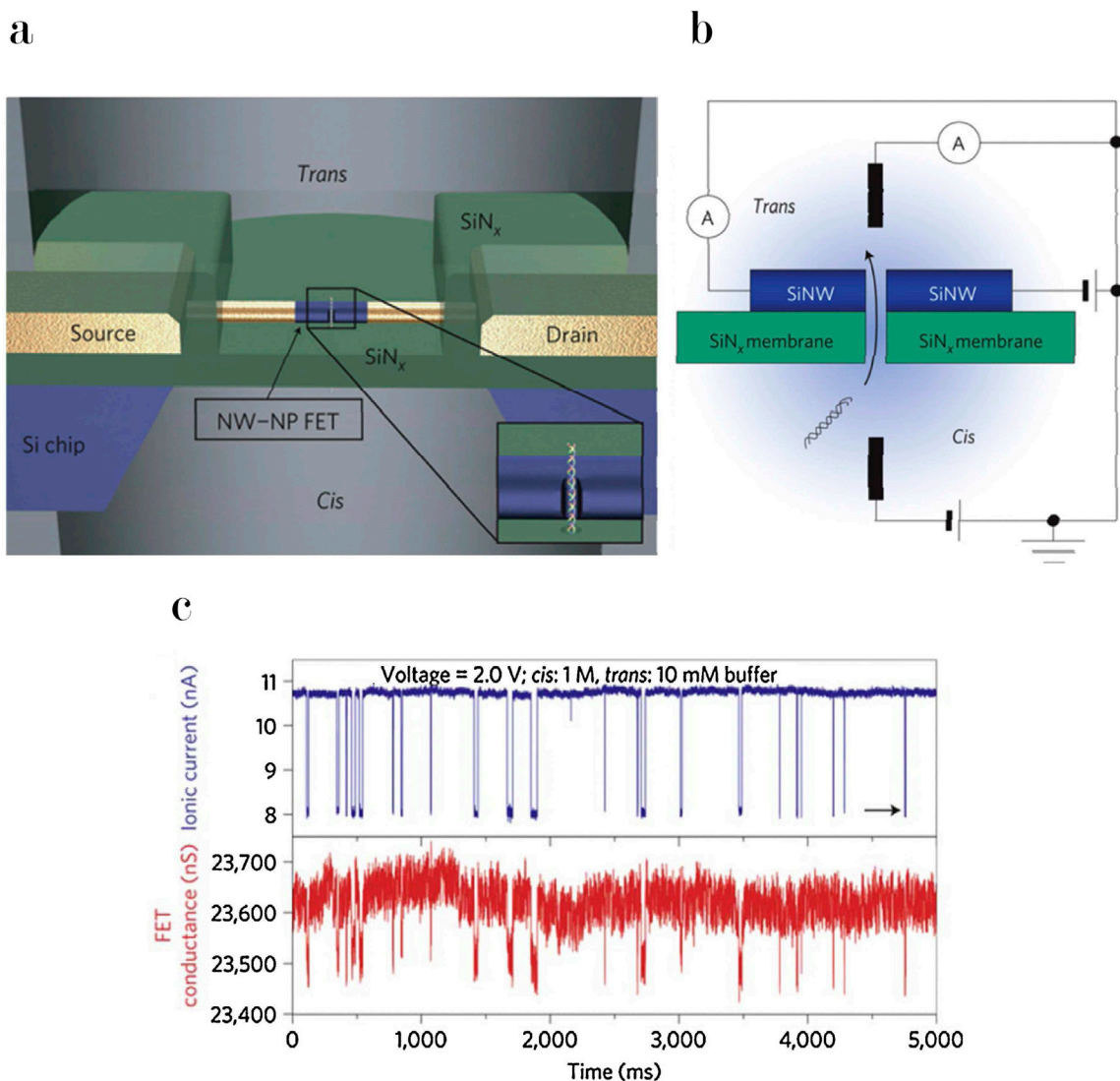


Figure 7. (a) Schematic of the nanowire-nanopore measurement. (b) Schematic of the sensing circuit (c) Simultaneously recorded ionic current and FET conductance signals of translocation of 2.6 kbps linear double-stranded DNA at 2.4 V voltage in KCl buffer with different concentration in both chambers (reprinted with permission from [71]).

channels, the solid-state nanopores are more robust, durable, and chemically, mechanically and thermally stable [33, 36]. They provide a very convenient, controllable and reproducible method for investigating polymer translocation phenomena at different conditions, which can also be easily coupled with other single-molecule techniques. Solid-state nanopores can be fabricated in different sizes and shapes [34].

First solid-state nanopores were created in 2001 from a silicon nitride (Si₃N₄) membrane by utilizing focused beam of argon ions [34], and this is illustrated in figure 3. Artificial nanopores were also produced using SiO₂, SiC and Al₂O₃ films because these dielectric materials have relatively low mechanical strength (so that they can be manipulated to produce holes), robustness, good insulating properties and high chemical stability [20, 33, 36]. Another advantage of these materials is that nanopores can be easily modified chemically or mechanically [36]. It was found that Al₂O₃ had better resolution and lower noise, while slowing down the translocation of DNA molecules due to stronger electrostatic interactions [19, 20]. Two main fabrication techniques,

such as electron beam machining and focused ion beam milling, have been mainly used for creating these synthetic channels [20, 33, 36]. However, these solid-state nanopores have not been yet successfully tested for DNA sequencing [33].

A new recent approach to develop synthetic nanopores is to utilize single-layer materials such graphene, boron nitride (BN) and molybdenum disulfide (MoS₂) [20, 37–40]. A potential advantage of this method is the fact that these ultrathin membranes have a thickness comparable with the size of DNA nucleotide (~0.3 nm), and this should significantly improve spatial resolution in sequencing measurements. (see figure 8 for an example of graphene nanoribbon transistor nanopore) In addition, these materials possess extraordinary electrical and mechanical properties. However, there are many issues in application of these artificial nanopores for DNA translocation, including clogging the pores due to strong interactions with DNA nucleotides, strong adsorption of DNA chains to the membrane outside of the channel, and large noise levels produced by small holes [41]. Significant cleaning and

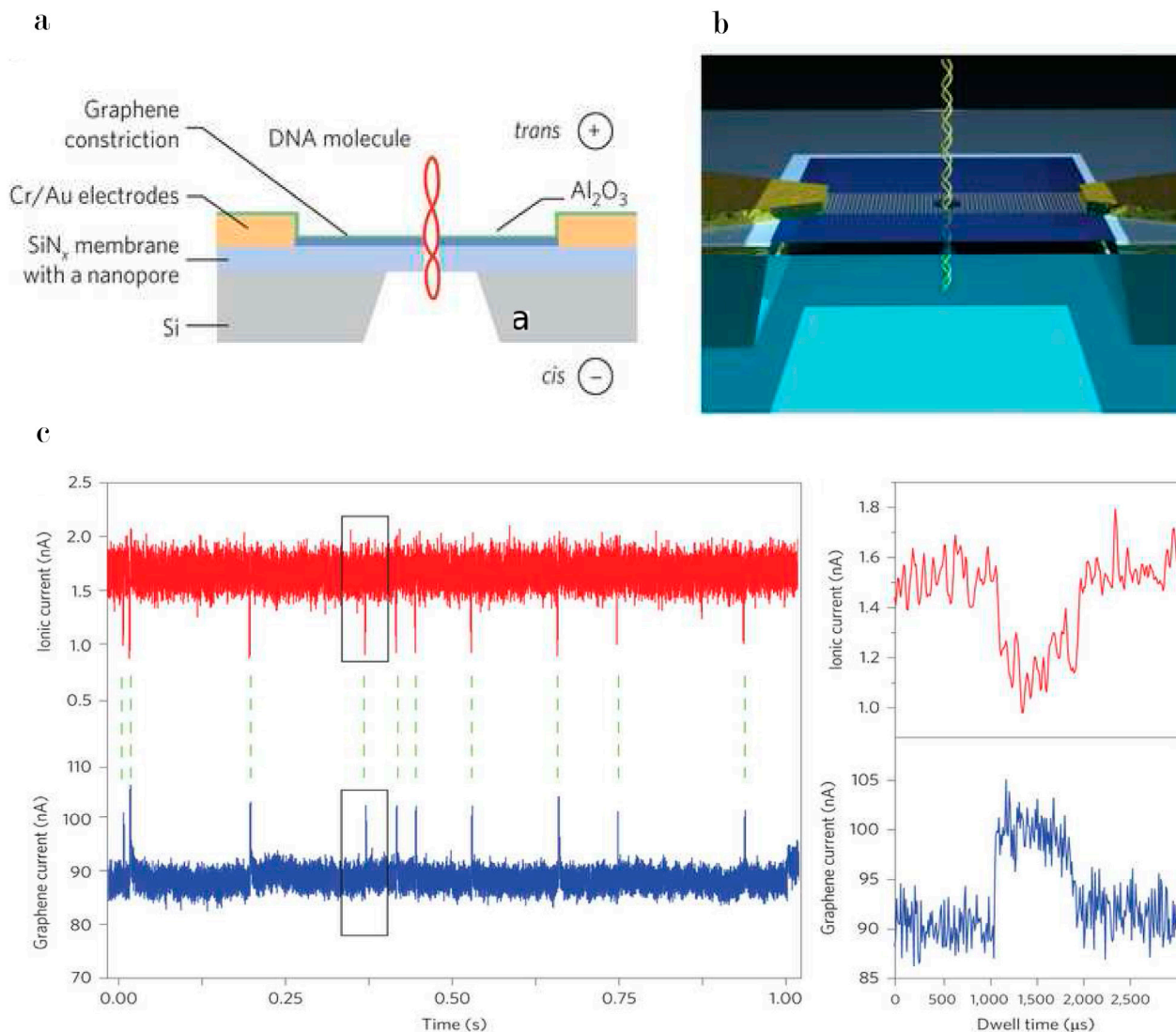


Figure 8. (a) Schematic of the GNR transistor nanopore. (b) Artistic representation of the device. (c) Simultaneously recorded ionic current and electrical current flowing through the GNR during translocations of pNEB (213 bp-long) DNA in 10 mM KCl (transmembrane voltage, 200 mV; graphene source-drain voltage, 20 mV). Ionic current is displayed in red and graphene current in blue. Green lines indicate the position of the correlated events in the ionic and graphene current (reprinted with permission from [76]).

chemical modifications of the surfaces are needed [42–44]. Because of these effects, the interpretation of the translocation signals is problematic.

Synthetic nanopores have many superior properties that make them convenient for performing the DNA sequencing. These include chemical and thermal stability, mechanical strength, controlled geometry and the ability to be integrated with other nanoscale single-molecule devices [20, 33]. Solid-state nanopores also stimulated the development new sensing techniques, and this will be discussed below [45]. However, the number of shortcomings is also significant. The DNA translocation velocity is too high for extracting signals that can clearly decipher the DNA sequence [10], although several attempts have been made to improve this [46]. Another serious obstacle toward using solid-state nanopores is the lack of chemical specificity to discriminate between analytes of approximately the same size. This means that different nucleotides would interact very similarly with the internal surfaces

of these nanopores. In addition, the preparation of solid-state nanopore frequently leads to undesired chemical modifications of the surface outside the nanopore, and this might also strongly affect the translocation dynamics [7, 21]. These observations suggests that existing solid-state nanopores are still not ready for doing high-quality DNA sequencing, and significant improvements are needed.

2.3. Hybrid nanopores

To overcome limitations of biological and solid state nanopores, several research groups suggested combining the best properties from both types of nanopores [47, 48]. Thus, the method of hybrid nanopores was created. The idea is to attach specific biological recognition groups inside the solid-state nanopores. This should substantially improve the chemical specificity, while still preserving mechanical and chemical stability. An example of such systems is shown in figure 4. Several

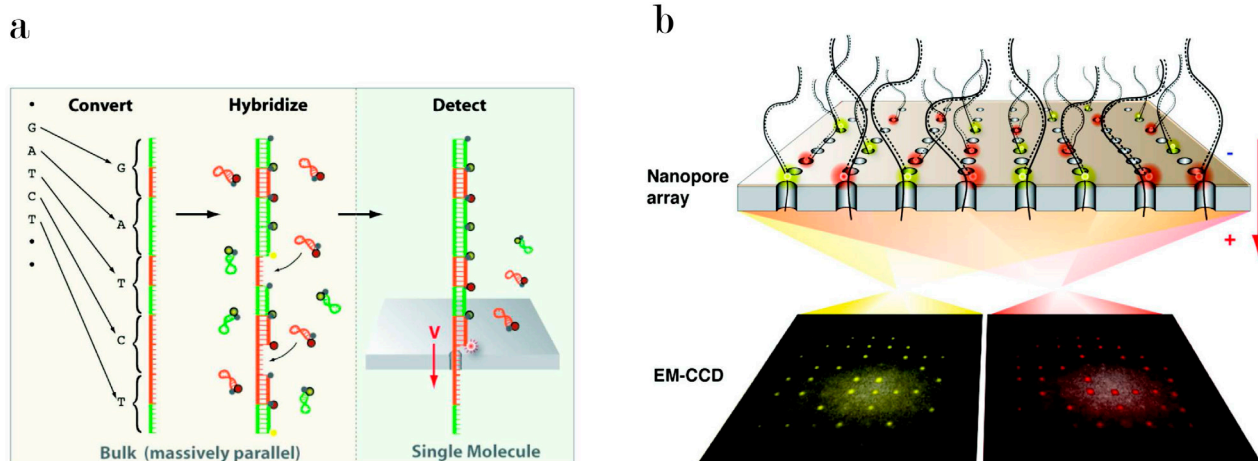


Figure 9. (a) Bulk conversion of each nucleotide of the target DNA sequence to a known oligonucleotide, followed by hybridization with molecular beacons. (b) Schematic illustration of the conceptual parallel readout scheme that enables simultaneous readout of an array of nanopores (reprinted with permission from [77]).

hybrid nanopores have been created [21, 47–49], however, no reports of using them for DNA sequencing have been found.

3. Sensing mechanisms in DNA translocation through nanopores

The success of DNA nanopore sequencing procedures strongly depends on the mechanism of sensing, i.e. on how the signal can be produced and how it can be uniquely connected with the structure of the translocating DNA molecules. Several sensing mechanisms have been proposed and explored in recent years, although with different degrees of success. Here we discuss and compare main methods of reading off the DNA sequences in the translocation via nanopores.

3.1. Ionic current blockades

The most widely applied technique in the sequence detection is a measurement of ionic currents during the translocation [10, 12, 16, 18, 33]. It is argued that the biopolymer threading through the channel blocks the fluxes of small ions by geometrical exclusion, and this change in the current is recorded as a function time. The main idea of this sequencing method is the assumption that modulations in the ionic currents strongly correlate with the chemical nature of the nucleotide at the narrow part of the nanopore. Figure 5 shows several examples of using biological and solid-state nanopores for detecting the DNA translocation through the channels based on the ionic current measurements.

A large number of experimental studies on DNA nanopore sequencing using ion-current blockages have been performed [50, 51]. However, many challenges in the field have not yet been resolved. Some investigations clearly show that the secondary structures of nucleic acids might affect the level of ionic blockage during the motion via the α -hemolysin pores [7, 52]. This might be related to the structure of α -hemolysin (see figure 2(a)), where the interactions between DNA and the protein channel are strong in the extended cylindrical β -barrel region. Several attempts were made to add specific recognition

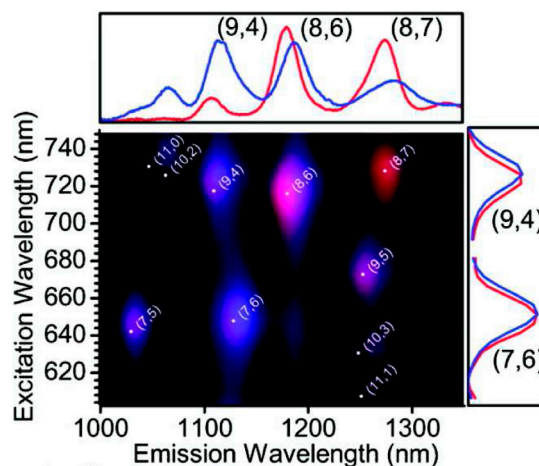


Figure 10. Two-color overlay graph of 2D PL maps of empty tubes (red) and one containing mainly water-filled SWCNTs (blue) (reprinted with permission from [99]).

groups inside the α -hemolysin pores to increase the sensitivity and resolution [32, 53, 54]. One of them is illustrated in figure 5(b) [54].

The problems with the resolution in the DNA sequencing using α -hemolysin pores was the reason for developing the engineered MspA nanopores, where essentially only single nucleotides could interact strongly enough with the channel to change the ionic current [29]. Similar to α -hemolysin, several attempts have been made to increase the sensitivity of this pore by constructing a MspA mutant [28], immobilizing DNA with neutral constriction [55] and controlling DNA translocation by phi 29 DNA polymerase [9, 56]. However, even in these cases the modulations in the ionic currents were very close for different nucleotides, and the noise in the current itself, as well as due to spatial fluctuations of the DNA molecules in the nanopores, caused significant errors in detection [57].

But the use of artificial non-biological nanopores did not improve much the prospects of the successful DNA sequencing. Solid-state nanopores, despite being very stable mechanically and chemically, are not as sensitive as the protein

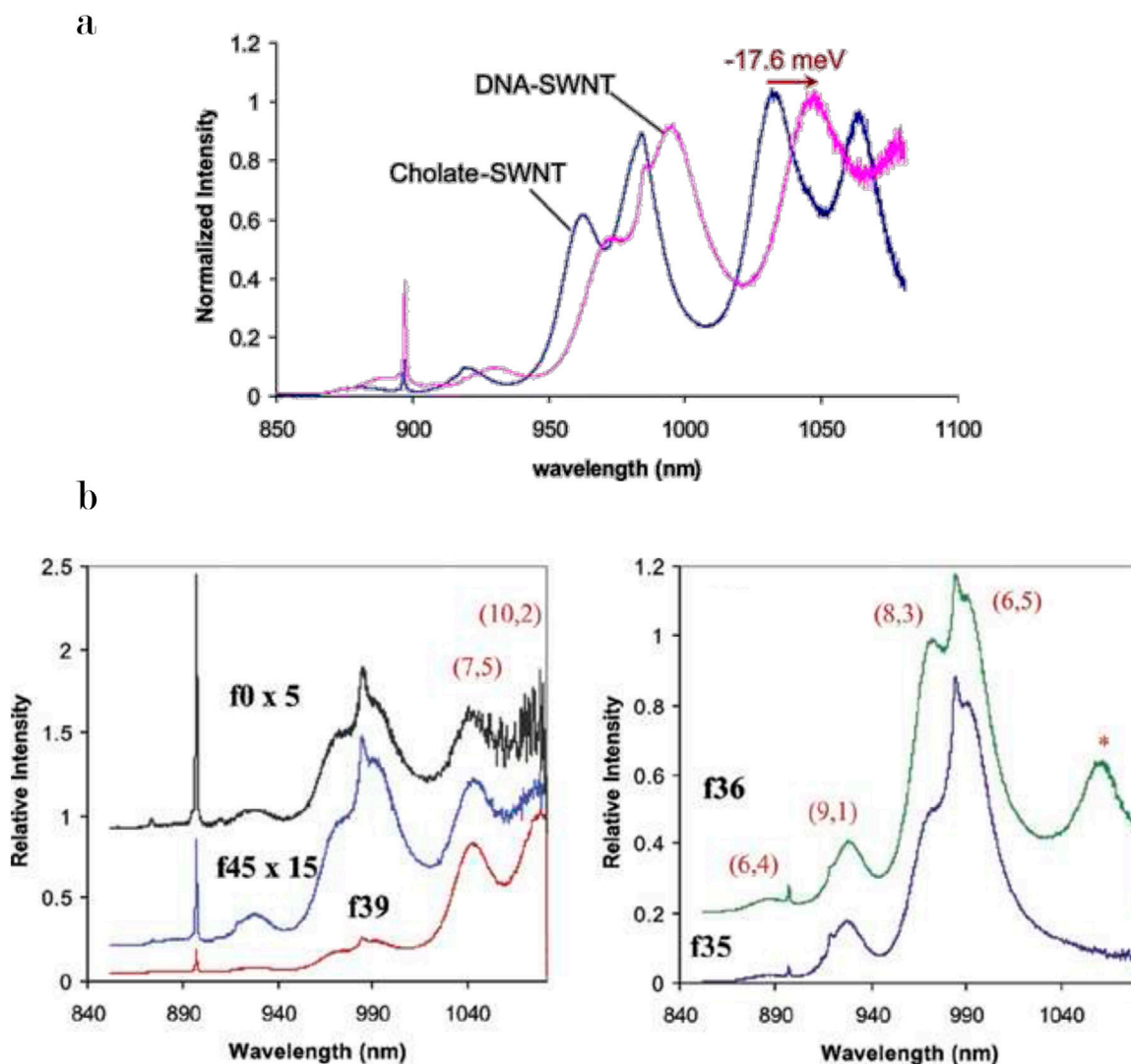


Figure 11. (a) NIR fluorescence spectra significant red shifting of DNA-SWNT fluorescence as compared to the fluorescence of cholate-SWCNT (reprinted with permission from [103]). (b) Partial fluorescence spectrum of eluted column SWCNT-DNA fractions from chromatography column at 785 nm excitation (reprinted with permission from [104]).

channels due to the nature of materials from which they are made [23, 51]. In addition, it is not clear how to increase such selectivity. However, two specific recently developed types of solid-state nanopores might present a better possibilities for DNA sequencing: ultra-thin graphene nanopore and carbon nanotube nanopores. They are shown in figures 5(c) and (d). Ultra-thin thickness of a graphene membranes makes the DNA translocation responses very sensitive to the nucleotide physical properties, although the hole diameter that is drilled in graphene is still too wide for precise recordings of the translocation. This large diameter produce a low signal to noise ratio, which is not very useful for developing high-sensitivity sequencing approaches. Note, however, that recent experimental advances allowed to produce smaller pores in graphene [58]. Single-walled carbon nanotubes (SWCNTs) are currently considered as promising candidates for the nanopore devices due to their wide variety of sizes, unique physical-chemical properties and large conductance changes during ssDNA transport [59], even though using them in nanopores is in its early stages and needs more investigations. Recently,

short SWCNT inserted into lipid bilayers (figure 5(d)) were utilized to study the detection of modified 5-hydroxymethyl-cytosine (5 hmC) in ssDNA, which may have implications for high-throughput screening of the genomic DNA [60].

3.2. Transverse current measurements of DNA translocation

In the current-blockage method the ionic fluxes parallel to the translocating DNA chains are measured and interpreted. Recently an alternative approach for DNA nanopore sequencing has been proposed, which is based on the measurement of the electrical current perpendicular to the DNA transport direction during the translocation [62–64]. Transverse electrical conductivity is expected to uniquely depend on the nature of each nucleotide in the nanopore [65]. The physical nature of these current has been theoretically investigated and experimentally visualized by the scanning tunneling microscopy [62, 66]. It was suggested that these currents are due to tunneling effects, or they might be related to the properties of semiconductor materials coupled to the nanopores.

3.2.1. Transverse tunneling current. In transverse tunneling current measurements, two electrodes are placed in the nanopore, and the transverse voltage is applied across them as it is shown in figure 6(a). Electrons jump from one electrode to another along the nanometer distance inside the nanopore. Passing DNA bases modulate the potential barrier between the two electrodes, resulting in a tunneling current that decays rapidly with distance. This quantum effect of current change leads to the enhanced spatial resolution and it also provides a superior molecular specificity [67]. Figure 6 shows the result of the first device that has been made with the embedded nanojunctions electrodes for the sensing of DNA translocation. These results are promising because the nanoelectrodes did not need any insulation, and all signals were recorded at the low sampling frequency [51].

Measuring electron tunneling currents flowing between perpendicular electrodes during the translocating of DNA is considered a promising approach for the nanopore sequencing techniques [68]. However, several challenges exist. First of all, nanoelectrode fabrication inside a nanopore is an extremely difficult technological step, which is a main barrier for application of these DNA sequencing devices. In addition, the method must ensure that the DNA molecule always traverses the pore in a specific orientation due to the high sensitivity of tunneling current to atomic scale changes of orientations and distances [18, 68]. In other words, the tunneling current will depend on the relative orientation of the nucleotide with respect to the electrodes. All these effects significantly lower the resolution of this DNA nanopore sequencing method.

3.2.2. Transverse semiconductor current. A related approach utilizes the changes in the conductance of the semiconductor material, which is attached to the nanopore, when DNA molecules translocate through the channel. It is known that nanowires and carbon-nanotube field-effect transistors (FETs) are frequently used as chemical and biological sensors because of their high intrinsic speeds, and high sensitivity [69, 70]. Lieber's group suggested to directly couple FET with the nanopores for DNA translocation analysis [71]. Figure 7 represents a schematic view and results from the silicon nanowire as the nanoscale FET with SiN_x membrane integrated with the nanopore. Simultaneous recordings of the nanowire FET conductance (figure 7(a)) and the parallel ionic flux when *cis* and *trans* chambers are filled with solutions of different ionic strength verify that this method correctly measures the translocation events. However, there is a significant level of the noise in FET transistors, which diminishes the value of this approach [71].

As a way to improve this method, it was suggested to integrate graphene nanogaps [72] and/or graphene nanoribbons (GNR) [73, 74] with SiN_x membranes into the nanopore for investigating the translocation phenomena as illustrated in figure 8. It has been shown that passing a DNA through a graphene-embedded nanochannel changes the conductance of the coupled nanoribbon significantly. This is due to high π - π stacking interactions between the carbon atoms and in graphene and nucleotides in DNA [73]. However, the signal-to-noise ratio of the graphene electrodes still needs to be significantly improved

for achieving single nucleotide resolutions. In addition, the mechanisms of sensing using coupled nanoelectrodes are still not fully understood [75].

3.3. Optical recognition

A very elegant method that is not based on measurements during the translocation has been developed recently [77]. Instead, an optical sensing mechanism is utilized here, as shown in figure 9. The method consists of the following steps. First, the original target DNA sequence is biochemically converted to a larger oligonucleotide molecule where each of the original A, C, G and T nucleotides are now coded with combinations of two specific predefined unique sequences (see figure 9). After this, the single-stranded oligonucleotides are hybridized with specific fluorophore groups. In this double-stranded molecule the fluorescence is quenched. Then the double-stranded molecules translocate through narrow solid-state nanopore, which forces them to unzip and to remove the fluorescent molecular beacons. This unquenches the fluorescence and produces a series of two-color photon bursts which are recorded. Because the original nucleotides are uniquely binary coded, the sequence of the target DNA can be easily read out [77]. The great advantage of this method is the ability to recognize DNA sequences using massively parallel readout.

This is probably the most promising approach for DNA sequencing because it combines a very high sensitivity, fast parallel read out of the signals and the abilities to improve by utilizing multi-color schemes. However, this method still has several challenges before it can be efficiently applied in the DNA nanopore sequencing. The errors in identification of nucleotides are of the order 10%, and the target DNA conversion is quite complex [10]. The ability to convert and read large genomic fragments with a high fidelity using this approach has not yet been demonstrated [10].

4. Challenges based on current experimental methods

In the last twenty years significant progress has been achieved in developing DNA nanopore sequencing methods for practical applications. Several experimental studies have clearly shown that protein nanopore devices can in principle perform DNA sequencing [9, 29]. There is also a commercial nanopore device, called MinION, from Oxford Nanopore Technologies that was introduced in 2014, which has been tested by several research labs [78–81]. However, the errors from this device are still significant, and the improved data analysis is required [81]. Although the device is portable and convenient for on-site laboratory use [82], it still has a higher error rate in comparison with current biochemical sequencing platforms [79].

Experimental challenges encountered in DNA nanopore sequencing have stimulated significant computational studies that greatly assisted researchers in describing the complex processes during the translocation of DNA molecules through channels [83–85]. These theoretical investigations provided

an important molecular picture on the mechanisms of DNA sequence detection. However, the application of computational methods is limited to not very long biopolymers and to time scales that are frequently too short in comparison with what is observed in experiments. In addition, there are questions on the precision of available force fields that are utilized in calculations.

Thus, despite multiple efforts and some promising developments, several crucial issues in DNA nanopore sequencing are still not fully resolved. We discussed several specific issues above by reviewing various methods. But generally there are two main problems for all nanopores: very high speed of the translocation and low signal sensitivity [10, 18, 20].

In the current-measurement experiments, DNA molecules translocate through the channels with a speed of ~ 1 base/ μs , which is too high for reliable detection of different nucleotides. The high speed of DNA translocation leads to the presence of a small number of ions in the nanopore that are used to identify the nucleotide. Therefore, signal to noise ratio decreases and distinguishing between the four nucleotides becomes more difficult. Thermal fluctuations make the measurements even less reliable, especially in such short amount of time [57]. It has been suggested that it would be reasonable to reduce the velocity of the ssDNA translocation to one nucleotide (~ 3 Å) per millisecond, which is three orders of magnitude slower in comparison with the current speeds. This would allow to individually identify the nucleotide sequence a DNA molecule containing one million bases in 20 min. One way of slowing down the translocation is to add bulky groups to DNA via chemical modifications [86], or to couple the translocation with other single-molecule methods like optical traps [21]. Experiments show that this goal is close to be reached for biological nanopores, while the polymer translocation in solid-state pores is still too fast.

Another critical problem is increasing the sensitivity and chemical specificity for all types of nanopores. It was improved in α -hemolysin biological nanopores by a combination of site-specific mutagenesis or the incorporation of DNA processing enzymes into the nanopore [25], chemical labeling of the nucleotides [87], and the covalent attachment of an aminocyclodextrin adapter [32]. In this approach, the enzyme attaches to the DNA in the bulk solution, reducing the speed of the motion as the enzyme brings the DNA molecule through the nanopore [88]. For solid-state nanopores the sensitivity (the ability to detect a low signal) was improved by optimization of the solution conditions (temperature, viscosity, pH) [25], chemical functionalization [89], surface-charge engineering [46], varying the thickness and the composition of the membranes [90, 91], and the use of smaller diameter nanopores [90] for polymer-pore interactions to increase the accuracy (the ability to discern different bases). However, even for these improved conditions there is not enough spatial and temporal resolution needed to obtain high-quality structural information at the single base level [18, 20, 50, 92].

Furthermore, almost all nanopore sequencing experiments measure electric currents through the system, and the sequencing information is obtained by analyzing these data.

But this significantly limits the ability to improve the spatial and temporal resolution, as well as the reliability of the reading out the information on the chemical composition of the nucleic acids translocating through nanopores.

5. Potential measurement methods based on carbon nanotubes

Although significant progress in DNA nanopore sequencing has been made in recent years, the main goal of high-speed and high-resolution single-nucleotide recognition of translocating DNA molecules has not been fully achieved with current measurements of ionic fluxes [18, 20, 50, 92]. This has encouraged researchers to look for new materials and approaches for nanopore analysis of nucleic acids. One of the promising new routes is the use of single-walled carbon nanotubes (SWCNTs). Nuckoll's group has already used SWCNTs for DNA translocation measurements using the current blockades [59]. But a new idea not explored before is to use spectroscopic properties of SWCNT and changes in them due to DNA/SWCNT interactions. It is reasonable to assume that the interactions between DNA and carbon nanotubes affect the spectra in a way that depends on the chemical composition and sequence of nucleotides. One should also mention another important advantage of optical detection over the ionic current measurements in terms of speed and sampling rates.

It has been shown theoretically and experimentally that absorption and emission properties of a SWCNT strongly depend on physical and chemical properties of the surrounding environment [93–95]. The surrounding media of a nanotube results in a dielectric screening effect that rescales excitation and emission transition energies between the valence and conductance sub-bands [96, 97]. There are several works describing the effect of the outer environment on nanotube emission [98]. However, little is known about the effect of the environment inside the CNT. Cambre *et al* [99] reported that individual and water-filled SWCNTs could be differentiated by their photoluminescence (PL) spectra. They showed a distinguishable red shift in the optical wavelength of filled SWCNT in comparison with empty ones as shown in figure 10. Therefore, when a nanotube is filled by a denser medium the excitation energy of the SWCNT decreases [99].

In addition, several theoretical and experimental studies have also revealed that the presence of DNA in the local environment of SWCNTs influences their PL properties [100, 101]. Hydrophobic forces and π -stacking interactions between ssDNA and SWCNT result in helical wrapping of ssDNA around the carbon nanotube surfaces [101]. Four DNA bases have strong adsorption affinities to a graphite surface with different magnitude [102]. This has been demonstrated also by the modulation of the fluorescence signal emitted by a DNA-SWCNT. Figure 11(a) shows that peak positions of DNA-SWCNT fluorescence have as much as 15.6 nm red shift in comparison with that of sodium cholate-SWCNT complex [103].

Optical features of DNA-wrapped SWCNT have helped scientists in recent years to understand the nature of DNA

assisted SWCNT separations. Utilizing DNA-SWCNT hybrids with specific DNA sequences to separate nanotubes has become one of the most efficient separation techniques [105]. It is reported that the hybrid displays a different response in absorption and fluorescence spectroscopy as compared to SWCNT alone [104–107]. Strano *et al* have shown that DNA sequences rich in guanine (G) and thymidine (T) amino acids adsorb strongly to carbon nanotubes. Fractions eluted from ion-exchange column show a small shift in their fluorescence spectra (see figure 11(b)). Each fraction has different densities of nucleotides due to different structures of ssDNA on the outside of the separated SWCNTs [104].

These results suggest that there is a dependence of SWCNT properties on the sequence of DNA inside the tube, which can be measured. Basically, the carbon nanotube-DNA PL spectra wavelength and intensity could be changed based on the sequence of DNA, and this effect might work as integrated detecting mechanism for nanopores sequencing. Previous works have reported some outstanding results on fabrication of carbon nanotube nanopore chips that make this path easier [59]. Furthermore, enhancing the SWCNT's photoluminescence quantum yield through incorporation of defects has been shown recently [108]. This is an encouraging outcome for the development of new photoelectronic sensors based on carbon nanotubes.

The use of SWCNT spectral features for DNA nanopore sequencing is a promising route that should be explored more. However, one has to mention that measuring DNA translocation by this approach has not yet been demonstrated. Another big question that needs to be addressed is how to solve the problem of spatial resolution. Because the exciton length in SWCNT is of order of 100 nm, these experiments would probably probe the collective effect of hundreds bases that are within of this interval. It will be important to develop theoretical methods of reliably obtaining the DNA sequence from spectral measurements as described above.

6. Future outlook

The field of nanopore sequencing has witnessed unprecedented advances in measuring the chemical composition and structure of nucleic acids. But still there are many fundamental and technological problems before the final goal of fast and precise detection of every single base in any DNA or RNA molecule can be achieved. These challenges stimulate the development of new ideas and methods. We believe that the following research directions might be promising in resolving these issues.

In most current experiments, the nanopores provide mostly the 'passive' confinement for the transport of biological molecules. It will be essential to explore more the interactions between the nucleic acids and the pores, and how they influence the measurements. This also includes the changes in the geometrical shape and the chemical modifications that can be tuned to achieve the highest efficiency in nanopore sequencing. Varying the geometry of the nanopores can be associated with entropic interactions in the system, while the chemical changes influence directly the inter-molecular interactions.

The second direction is to create new methods that will simultaneously measure several sequence-dependent properties during the biopolymer translocation. Currently, almost all existing methods probe only one of the characteristics such as the current (in most cases) or spectroscopic signal (rarely). The ability to have several outputs in one experiment will significantly improve the spatial and temporal resolution of the system as well as the analysis of experimental data. Finally, it will be important to couple the existing nanopore sequencing methods with existing single-molecule spectroscopic methods such as optical traps, magnetic tweezers, fluorescence correlation spectroscopy and many others. This will uncover new microscopic features of various complex biological processes that could not be measured separately by each of this techniques. Another interesting idea is to generalize the existing methods for determination the chemical composition and sequence of the protein molecules.

Detection of chemical sequence of DNA with single-nucleotide resolution remains one of the most important scientific and technological problems. Using nanopores for DNA sequencing has many advantages, such as easy sample preparation, minimal chemical modifications, the ability of massive parallel measurements and coupling to other single-molecule methods. Several methods achieving single-nucleotide discrimination in DNA molecules have been presented. However, many scientific and technological issues remain unsolved. These include high speeds of translocation, low sensitivity and chemical specificity, and low spatial and temporal resolution. This suggests that new materials and new techniques are needed in order to reach the ultimate goals. We expect that future studies of DNA nanopore sequencing will bring many new exciting discoveries.

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