NANOPORES: ELECTRONIC TOOLS FOR SINGLE-MOLECULE BIOPHYSICS AND BIO-NANOTECHNOLOGIES

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Nanopores – nanometer-sized holes in ultrathin membranes – are driving a revolution in the life sciences and in medicine, where high-performance detection systems must achieve greater speed, sensitivity and accuracy. In this short review, we present the principles of solid-state nanopore-based single-molecule detection and discuss the challenges, which must be overcome in order for solid-state nanopores to transition from a leading candidate under development to a disruptive technology.

INTRODUCTION

Dynamic phenomena in confined geometries, including the transport of charged biopolymers through nanoscale pores, are fundamental processes of life (e.g., the passage of mRNA through the nuclear pore complex; the secretion of proteins across cell membranes). Elucidating the fundamentals of biomolecular transport through nanopores is a fertile field of research for many physicists, and strategies for controlling molecular capture and passage will find numerous technological applications.

The last decade has seen significant advancements in nanofluidic devices to develop biomimetic systems and to study transport processes at the single-molecule level [1,2]. These studies have shed some light on the underlying mechanisms of polymer dynamics in pores [3]. In particular, exciting results have been obtained through the study of passage of nucleic acids through solid-state nanopores (ssNP) [4–7]. Nanometer-sized holes in thin dielectric membranes, ssNP have emerged as a versatile tool to investigate a wide range of phenomena involving DNA and proteins. They provide a confined space within which single molecules can be captured and electrically interrogated with high throughput. The basic concept behind single-molecule analysis using nanopores, illustrated in Fig. 1, is the following: when a small voltage bias (~100 mV) is applied across a nanopore in a membrane separating two chambers filled with a liquid electrolyte (typically 1M KCl), a charged molecule can be electrophoretically driven into the pore, resulting in a transient change in ionic current (~sub-nA) which provides information (e.g., length, size, charge, shape, and dipole) about the translocating molecule. In addition to single-molecule DNA sequencing [8], this concept exhibits great promise for other applications, including molecular counting [7,9]; scanning of local structures along DNA molecules [10] for specific protein detection [11], sequence-specific PNA binding detection [6], and investigation of single biomolecular interactions [12]. In the present article, we review the principles of nanopore-based single-molecule detection, the main hurdles in developing nanopore-based technologies for life sciences and medical applications, as well as the progress our laboratory has made toward providing innovative solutions to challenges in the nanopore field.

SUMMARY

We review the principles and challenges of nanopore-based single-molecule detection for life sciences and medical applications, as well as the progress our laboratory has made toward providing innovative solutions.

Nanopore Signal, Passage Speed, and Capture

In a typical nanopore experiment, an applied voltage V drives ions through a small pore of diameter d_{pore} in a thin membrane of thickness L, separating two fluidic reservoirs filled with an electrolyte of conductivity σ. The passage of a biomolecule with diameter $d_{analyte}$ results in a current change expressed as $\Delta I = \frac{V\sigma\pi d_{analyte}^2}{4L}$. The nanopore size requirements strongly depend on the biosensing application of interest. The pore size should be as close as possible to the analyte of interest, with a preference for thin membranes [7] so as to maximize the signal and the spatial resolution of the nanopore.

In the case of nucleic acid sequencing, for example, it should be possible to read the sequence of individual bases in a DNA molecule as minute variations in the current signal [13], since each DNA base has a unique geometry. In practice, a combination of background noise and signals from neighboring bases prevents the collection of sufficient ions per base, largely due to the rapid passage of DNA through thin solid-state nanopores (>10 bp/μs). By prolonging the time that a molecule spends within the nanopore, more ions per DNA base would be detected,

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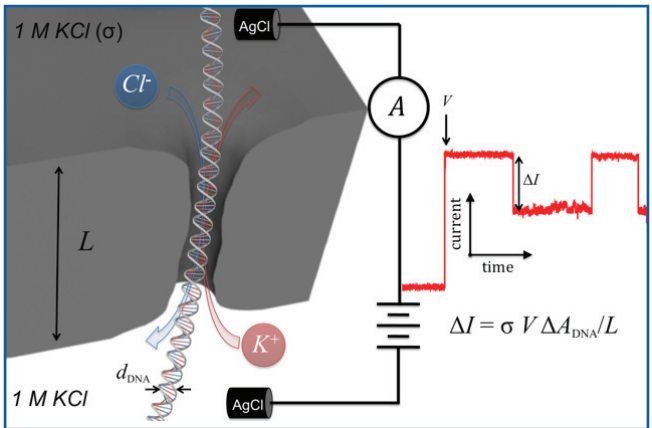

Fig. 1 Schematic representation of a DNA polymer with cross sectional area $\Delta A_{DNA} = \pi d_{DNA}^2/4$, where $d_{DNA} \approx 2.2$ nm, translocating through a 3 nm pore in a 10 nm thick membrane (L) immersed in 1M KCl, with conductivity σ under an applied voltage V.

thus enabling sequence detection. A number of other applications also stand to benefit from prolonged passage times and the resulting enhanced discrimination ability needed for the multiplexed detection of biomarkers. By modifying the nanopore, its environment, or the analyte itself, researchers have reduced the speed of translocations by nearly 10-fold [14]. Despite this success in reduced passage speed, the propagation dynamics of polymers through pores (i.e., inter- and intramolecular velocity fluctuations) remain relatively unexplored experimentally. Simulations have suggested that the wide distribution observed for the translocation velocities for identical molecules can be attributed to drag-induced velocity fluctuations [15], and a recent experimental study mapping coarse velocity profiles observed that polymers accelerate at the end of the translocation process [16]. In light of these studies, it is evident that controlling passage speed and motion is an active area of research and that a more detailed velocity transfer function is still required to accurately convert temporal signals into positional information.

Nanopore analysis generally requires hundreds or thousands of single-molecule events to build reliable statistics. Enhancement of the typical ssNP capture rate (~$1 s^{-1} nM^{-1}$) is vital for applications requiring timely detection, particularly of low abundance targets. However, few experimental studies have investigated in detail the two-step capture mechanism of charged polymers by ssNP [17,18], in which a molecule diffuses from bulk solution toward a point near the nanopore mouth where field-driven funneling takes over [19]. The characteristic length scale at which the molecule's motion crosses over from almost purely diffusive to drift-dominated motion is called the capture radius — i.e., the "event horizon" (the point of no return) to express the fact that molecules cannot escape and are translocated through the nanopore. For a given applied voltage V and a molecular electrophoretic mobility μ, there exists a region of nonzero electric field outside the pore that defines the capture radius as $r^* = \frac{d_{pore}^2 \mu V}{8LD}$, which is typically on the order of a few microns. Molecular arrival in this capture radius is typically dictated by the diffusion constant D and is thus linear in analyte concentration. However, for low voltages or short DNA fragments, the electrical pulling force barely surpasses the entropic barrier to molecular translocation. Thus, the capture rate shifts from a linear dependence with voltage to an exponential one, dominated by barrier crossing rate as opposed to arrival rate in the capture radius. In this entropic regime, molecular size and conformation also become important. Large pores offer higher capture rate, but their reduced sensitivity means that alternative rate-maximizing approaches are preferred. Alternatives include the use of a salt concentration gradient across the pore, which has been shown to enhance the capture rate by as much as 20 [17], and dielectrophoretic focusing to preconcentrate DNA in the vicinity of the nanopore detector [20]. Despite these developments, many aspects of the nanopore capture process have yet to be investigated in a systematic way, since seemingly identical pores often behave drastically differently.

Challenges

Despite intense research efforts and the many exciting developments described above, a number of hurdles remain for nanopore-based technologies to realize their full potential in life science and medical diagnostic applications. Overall, the field is facing three major technical challenges: (*i*) specificity of the signal; (*ii*) control of speed during biomolecular passage through the nanopore; and (*iii*) fabrication of solid-state nanopores in a low-cost, scalable way that is compatible with manufacturing. In our group, we believe that answers to these obstacles are, in one way or another, tied to the technique used to fabricate solid-state nanopores.

State-of-the-art in ssNP Fabrication

The desired diameter of a nanopore depends strongly on the application of interest, but should generally match that of the analyte (1.5-2 nm for ssDNA detection and sequencing; 2.5-5 nm for short dsDNA and RNA markers; 5-20 nm for long DNA fragments, genomic screening and protein analysis). Nearly all applications require stable, accurately sized pores in mechanically robust membranes, with thinner membranes favored due to the corresponding increase in ionic current signal [7] and spatial resolution. Until recently, state-of-the-art techniques for achieving such features employed a focused beam of high-energy particles to drill a hole in a ~20-50 nm thick solid-state membrane, followed by a manual-shaping step with a defocused beam to achieve the desired size. The typical techniques – transmission electron microscope (TEM), ion sculpting apparatus, or helium ion microscope (HIM) – involve very expensive instruments (>$1M) that are located in dedicated facilities and call for extensive training and highly qualified personnel for their operation. Further, these techniques require vacuum, and the resulting nanopores must undergo cleaning and wetting steps in order to facilitate immersion in liquid

for sensing. Such time-consuming preparation steps significantly reduce yield and reliability, since pores are frequently damaged during one of many handling steps (especially in sub-30-nm thick membranes) and present high noise levels associated with poor wetting, or size changes due to harsh chemical treatment.

The Invention, Nanopore Fabrication by Controlled Breakdown (CBD)

We have invented a simple, rapid, and cost-effective fabrication method capable of creating individual solid-state nanopores directly in a neutral KCl solution. The method relies on application of a sustained electric field across an insulating membrane in solution near its dielectric breakdown strength (~0.5-1 V/nm) by applying a relatively low voltage ($<$10V across ~10 nm membranes), which induces a localized leakage current through the otherwise insulating membrane. By monitoring the resulting sustained leakage (tunnelling) current, the fabrication of a single nanopore is detected as an abrupt increase in the current, which we attribute to the onset of ionic current. Control of nanopore size in thin membranes is obtained through feedback control used to terminate the application of the electric potential and to limit the damage to the membrane after the initial dielectric breakdown event. The equipment required for fabricating ssNP by CBD is a simple computer-controlled current amplifier circuit to monitor the current and control the electric field strength. Figure 2 highlights the dramatic difference in complexity and cost between CBD and TEM-based nanopore fabrication methods.

The CBD process itself is based on the physics of dielectric breakdown, a phenomenon which has been extensively studied in the context of dry-state semiconductor devices [21]. The underlying kinetics of the process are only partially understood and are theoretically quite complex. Qualitatively, the breakdown process proceeds as follows: as a high electric field is applied to a material near its dielectric strength, the material is no longer fully insulating and will support a leakage current. This leakage current induces damage to the material, in the form of localized charge traps between which electrons or holes can tunnel if they are sufficiently close together. Once a path of these traps exists which electrically connects both sides of the dielectric material, a highly conductive path is formed with a diameter on the scale of ~1 nm, which will sustain a sufficiently large current to locally damage the material. Such percolation-based models of dielectric breakdown predict a Weibull distribution of time-to-breakdown, a feature which we have observed experimentally [22]. In the case of wet breakdown, the kinetics are also influenced heavily by the nature of the ionic solution used to carry the electrical current, being particularly sensitive to the pH, indicating that the electrochemistry at the membrane/solution interface also plays an intrinsic role in the breakdown process. In semiconductor devices, this damage is catastrophic and generally results in the destruction of the device under test, and as such is the eventual failure mode

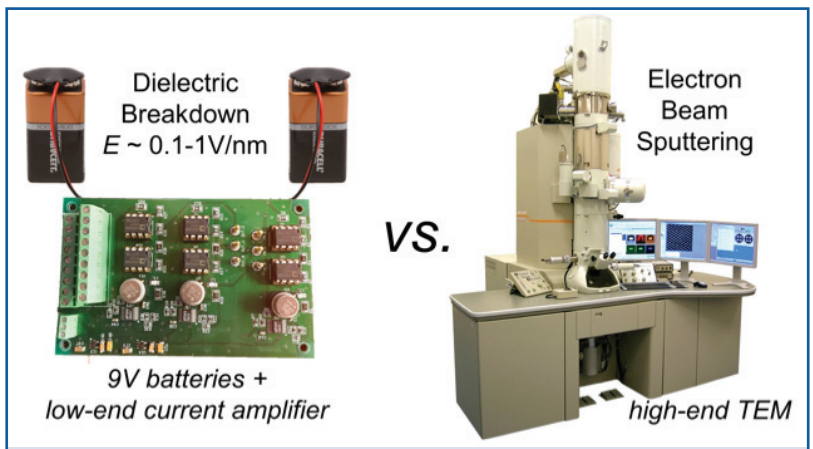

Fig. 2 Nanopore fabrication by controlled breakdown (CBD) only requires application of a relatively low voltage ~10V to generate a high electric field in ~10 nm thick membranes. A simple battery-powered current amplifier circuit controlled by a computer can be used to fabricate ssNP. Note that the circuit shown is capable of forming 3 nanopores on separate membranes in parallel, and the circuitry can easily be scaled up. The alternative method requires a ~$1M, field-emission transmission electron microscope, and can only make one pore at a time.

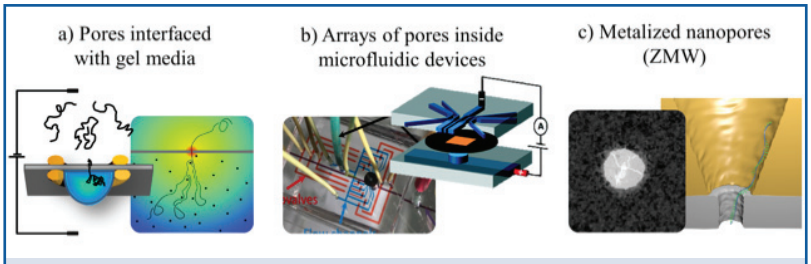

Fig. 3 Examples of research projects. a) A nanopore is interfaced with a gel medium (agarose or acrylamide) on one side of the membrane to reduce the translocation speed during passage (in collaboration with Prof. Gary Slater, Physics, uOttawa); b) a Si chip containing a single free-standing membrane is encapsulated within a microfluidic chip enabling the fabrication of an array of individually addressable nanopores for lab-on-a-chip applications (in collaboration with Prof. Michel Godin, Physics, uOttawa); c) a nanopore is opened in a metalized membrane by CBD thus forming a pore inside a zero-mode waveguide for improved optical detection.

for most computing hardware. For this reason, most of the research on the subject has been performed with the goal of avoiding it entirely. In a liquid environment, however, the damage can be precisely controlled by quickly (within ~0.1s) turning off the voltage once a sudden increase in the leakage current is detected, which signals the formation of the conductive path discussed earlier. Pores formed using CBD are generally on the order of just a few nanometers in diameter, and 2 nm pores can be made reproducibly [23]. Once a pore is formed, its size and electrical characteristics can be precisely tuned using intermediate voltages to controllably enlarge and condition the pore [24]. Because the pore is made in the same solution that is used for single-molecule sensing, it is immediately wetted and ready for experiments. This simple advantage enormously increases the yield compared to particle-beam fabrication techniques, which require multiple handling steps and pre-treatment before wetting is possible.

CBD offers unique opportunities to design and reliably fabricate certain advanced solid-state nanopore devices – such as multichannel [25] or multilayer constructs [26] – that would otherwise be prohibitively challenging, extremely low yield, or simply unfeasible. Over the last few years, we have published articles on the basic principles of the CBD method [27], its reliability and precision [23], and its ability to fabricate pores in multilayered metalized membranes [26]. We have also considered the kinetics of dielectric breakdown in liquid [22] and the propensity for integration into microfluidics for array formation and lab-on-a-chip applications [25]. Further, we have performed nanopore force spectroscopy measurements studying the stretching transition of the B- to the S-form of short dsDNA fragments [23], and worked on strategies to manipulate molecular transport using electrically gated nanopore transistors [28], gold-oligo interactions [26], and nanopores interfaced with gel media [14]. Figure 3 illustrates some of these projects.

CONCLUSION

The CBD nanopore fabrication method profoundly impacts our capacity to build advanced nanodevices and to perform complex nanopore measurements. These advances bring us closer to delivering real-world solid-state nanopore technologies, with significant implications for the study of fundamental biological processes and for the investigation of biomolecular building blocks like DNA, RNA, and proteins, one molecule at a time.

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