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Citation: Physics Today **68**, 8, 40 (2015); doi: 10.1063/PT.3.2881
View online: https://doi.org/10.1063/PT.3.2881
View Table of Contents: https://physicstoday.scitation.org/toc/pto/68/8
Published by the American Institute of Physics

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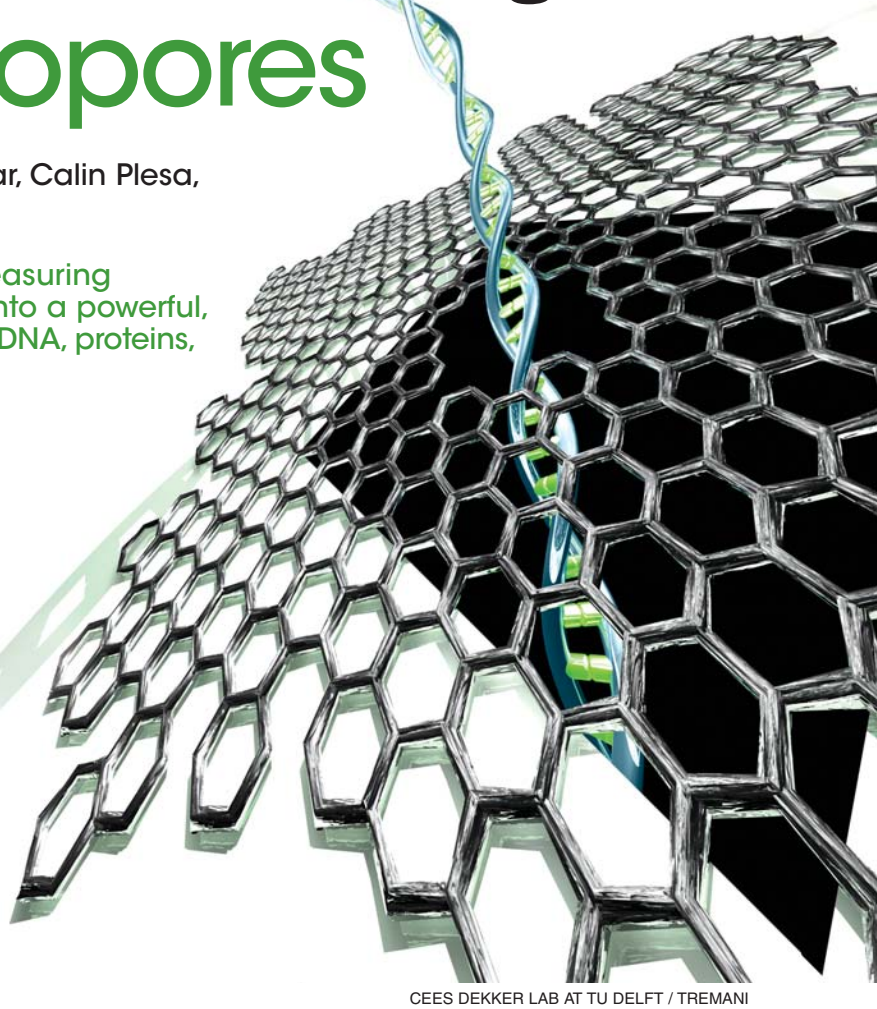
A 70-year-old idea for measuring blood cells has evolved into a powerful, versatile tool for studying DNA, proteins, and other biomolecules.



CEES DEKKER LAB AT TU DELFT / TREMANI

In the 1940s Wallace Coulter set about finding a way to quickly count blood cells, which at the time was a slow and inefficient process. His approach was to pass cells, one by one, through a small hole connecting two compartments filled with electrolyte solution. Simultaneously, he applied a voltage across the compartments and measured the ionic current through the hole. As a cell passed through the hole, it would partially block the flow of electric charges, and the current would drop by an amount proportional to the volume of the cell.

Coulter's technique worked out wonderfully and revolutionized cell counting. The holes in Coulter's devices were roughly 10 μm in diameter, slightly larger than the size of the cells being probed. But as time passed, improved fabrication techniques led to smaller holes, which allowed smaller analytes to be investigated.

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The last two decades have seen a renaissance of the Coulter counter concept. The principle remains essentially the same, but nanopores—holes with a diameter of merely a few nanometers—have shrunk the length scale from that of single cells to that of single molecules. When DNA molecules are added to one side of the pore and an electric field is applied, the resulting electrophoretic force on the negatively charged DNA can pull the molecule through the pore in a head-to-tail fashion, leading to an observable blockade in the ionic current, as shown in figure 1. Many of the initial nanoscale experiments used α-hemolysin, a small pore-forming toxin produced by the bacterium *Staphylococcus aureus*, as the channel for ion flow.[1] As shown in figure 2, the pore can naturally insert itself into a lipid bilayer that is nonpermeable to ions. Only 1.4 nm across at its narrowest point, an α-hemolysin pore is just wide

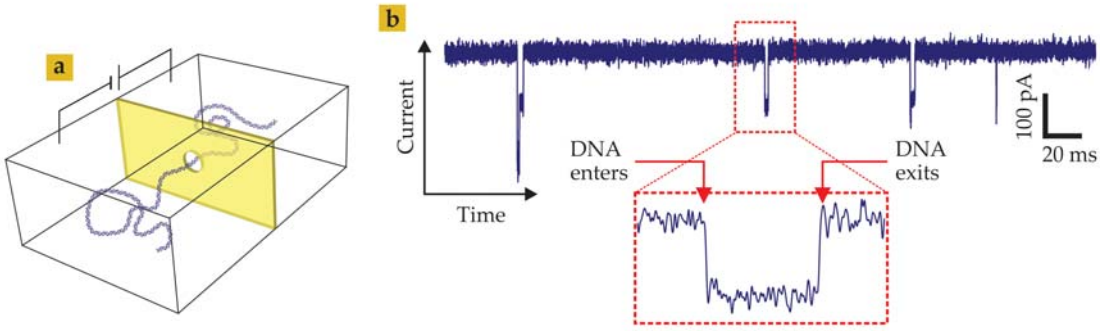

Figure 1. Threading DNA through a nanopore. **(a)** A negatively charged DNA molecule immersed in electrolyte solution can be coaxed through a nanopore by an electric field. **(b)** Experimental current trace of DNA molecules stochastically captured into a pore. The transiting molecule momentarily blocks the flow of ionic current through the pore by an amount that depends on the volume of the molecule within the pore constriction.

enough to allow the passage of small molecules. Large macromolecules such as single-stranded DNA can also pass, provided they are threaded through the pore in a single-file conformation.

In the 1990s several research groups—including those of David Deamer (University of California, Santa Cruz), Daniel Branton (Harvard University), John Kasianowicz (NIST), and Hagan Bayley (then at Texas A&M University, now at Oxford University)—began probing whether the different bases on a DNA strand might block measurably different amounts of ionic current as they pass through a nanopore. If so, the pattern of current generated by a DNA strand threaded through a nanopore might provide a linear readout of the strand's base sequence.[2]

Such an approach to DNA sequencing is attractive due to its conceptual simplicity. Conventional sequencing methods use a shotgun approach whereby a long strand is fragmented into small pieces—each perhaps 100 base pairs or so in length. Those strands are then independently analyzed, either by a combination of gel electrophoresis and chemical analysis or by fluorescence techniques. Nanopore sequencing, by contrast, can potentially offer very long read length, high speed, and low cost; it is label-free; and it can be done at the single-molecule level.

Although significant challenges remain to turn that vision into a practical reality, the goal appears to be within reach. (For more detailed reviews of nanopore-based DNA sequencing, see references 3 through 6.) Meanwhile, nanopores have also developed into a powerful tool for probing single-molecule biophysics phenomena, such as DNA–protein binding and pore-translocation processes that occur naturally in cells. In this article we describe the basic biophysical mechanisms underlying pore translocation of biopolymers, particularly DNA. Then we discuss its broader use as a tool not only to sequence genetic material but also to study the physics of charged macromolecules under nanoconfinement and in nonequilibrium conditions.

A zoo of pores

Nanopores can be broadly classified into three categories: biological, solid state, and hybrid, as shown in figure 2. Biological pores, studied for several decades now, typically consist of transmembrane proteins harvested from living cells. Since the initial experiments with α-hemolysin, biochemists have explored a range of biological pores, including the protein MspA, produced by *Mycobacteria*; ClyA, produced by *Escherichia coli*; and the anthrax toxin.

Just over a decade ago, several groups, including the group of Jene Golovchenko at Harvard and the group of one of us (Dekker) at Delft University of Technology, fabricated solid-state nanopores by drilling a hole into a thin insulating membrane using a focused ion or electron beam.[7] Nowadays, synthetic pores can also be formed by way of voltage-induced dielectric breakdown. The membrane may consist of any of a wide variety of materials; most common is silicon nitride, but aluminum oxide, silicon oxide, hafnium oxide, and, more recently, two-dimensional layered materials such as graphene, boron nitride, and molybdenum disulfide are also used. An alternative approach to fabricating solid-state pores is to use a laser pipette puller to fashion glass nanocapillaries, which results in tip openings as small as tens of nanometers in diameter.

A newer development has been the creation of hybrid pores. The first hybrids were created by capturing α-hemolysin proteins in SiN pores, but recent efforts have focused on assembling pores via DNA origami and then docking them into solid-state pores or lipid bilayers.

Translocation basics

Regardless of the type of pore, biopolymer translocation generally occurs by way of the same basic process, illustrated in figure 3. First, a polymer must be captured at the entrance of the pore. Many biopolymers, including DNA, are highly charged, so an applied electric field imparts an electrophoretic force that pulls the molecule toward the pore. At the same time, the polymer undergoes diffusion, which tends to move it away from the pore. The competition between those two effects defines a capture radius, typically around 1 micron, about the pore mouth: Once a molecule drifts within the capture radius, electrophoresis takes over and the molecule is guaranteed to eventually reach the pore; at further distances it may diffuse away.

The second step is nucleation—the insertion of one end of the polymer strand into the pore. A key

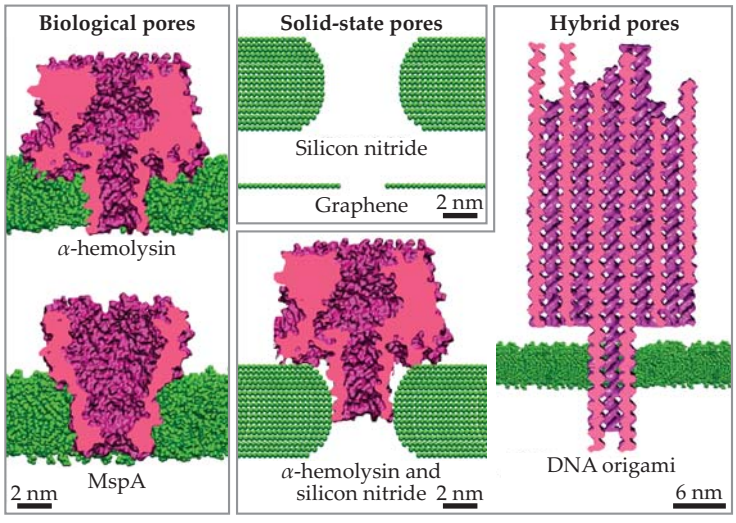

Figure 2. Nanopores can be grouped into three major categories: biological pores, such as *α*-hemolysin and MspA, inserted into lipid bilayers; solid-state pores, drilled into thin membranes of silicon nitride, graphene, or other materials; and hybrid pores consisting of biological pores docked into solid-state pores or DNA-origami pores docked into lipid membranes or solid-state pores. Actual silicon nitride membranes are typically thicker than shown here. (Images courtesy of Aleksei Aksimentiev and Hendrik Dietz.)

feature distinguishing biopolymers from the blood cells of the classic Coulter counter is that biopolymers have enormous conformational entropy. At room temperature, the conformation is continuously changing, and the biopolymer will typically approach the pore mouth as a randomly coiled blob, with neither of its two ends necessarily having the correct orientation for insertion into the pore.

For the molecule to enter the pore, one of its ends must explore space within the coiled blob to find the pore entrance. That step requires crossing a considerable entropic barrier on the order of 10 $k_B T$, where k_B is Boltzmann's constant and T is temperature. The precise value of the barrier depends on the polymer length, the electrolyte concentration, the nature of the pore–polymer interactions, and other details. When the chain is squeezed into the narrow path of the pore, its range of allowed conformations is considerably reduced, and it therefore encounters a further conformational entropic barrier of roughly 2–3 $k_B T$ for proceeding with the translocation event.

After entering the pore, the chain transits the pore through a drift-diffusion process. Depending on the pore chemistry, the exiting chain may need to peel away against an attractive force from the pore, a step that would be associated with an uphill free-energy change.

A complex environment

Although the basic steps change little from one pore or polymer to the next, several factors affect the detailed dynamics of a translocation event. Let's walk through the different elements that come into play.

▶ **Polymer charge.** Most biopolymers have intrinsic electric charge. A strand of DNA, for instance, has one negative charge at each base along its phosphate backbone. In solution, however, the strand is constantly surrounded by a cloud of small, positively charged counterions. That results in DNA having an effective charge that's generally less than its chemical charge—and the effective charge is what determines the electrophoretic force due to an electric field.

In principle, a counterion—say, singly ionized potassium or sodium—will adsorb at a negatively charged site on the phosphate backbone whenever the charge separation is less than the so-called Bjerrum length. (For aqueous solutions at room temperature, the Bjerrum length is about 0.7 nm.) That adsorption, however, comes at the cost of the counterion's translational entropy. A compromise between those two competing interests results in a polymer conformation in which, on average, some fraction of the DNA's charge is neutralized by counterion condensation.

The precise fraction depends on the specifics of the system, such as the identity of the counterions, the flexibility of the DNA chain, and—in pore-confined chains—the extent of confinement and the dielectric heterogeneity at the pore surface. DNA translocation experiments can serve as a means to measure the effective charge of DNA and to explore its various contributing factors. Indeed, single-molecule studies measuring the electrophoretic force on DNA held in a nanopore have estimated the effective DNA charge to be about a quarter of the nominal chemical charge of one electron per base.

▶ **Hydrodynamics and electroosmotic flow.** As a biopolymer drifts to and through a pore, each repeat unit is subject to a frictional force from the background fluid, as in the classic Stokes problem of a spherical particle dragged through a fluid. Unlike in the Stokes problem, however, the frictional force is accompanied by Coulomb forces exerted by the surrounding electrolytes, and the force on any given repeat unit influences, in a self-consistent manner, the trajectories of all the other repeat units in the connected polymer chain.

Polymer physicists have advanced powerful approximate theoretical methods to describe the cooperative hydrodynamic drag on a charged polymer in an electrolyte solution. How good are those theories? Nanopores are novel tools for assessing the various models. Although many details remain to be ironed out, nanopore experiments have shown the general concepts of single-molecule polymer hydrodynamics in nonequilibrium conditions to be reliable.

With crafty pore design, one can often manipulate the hydrodynamics of biopolymer translocation. If the pore walls bear permanent charges, a thin layer of counterions will self-assemble near the wall in order to maintain local electroneutrality. Under an applied electrical field, those counterions move toward their favorable electrode—and drag some water along with them. The resulting flow, called electroosmotic flow, can be quite strong and can influence the manner in which the biopolymer crosses the pore. In a typical scenario—such as a DNA strand translocating across a negatively charged SiN nanopore—positive counterions will move

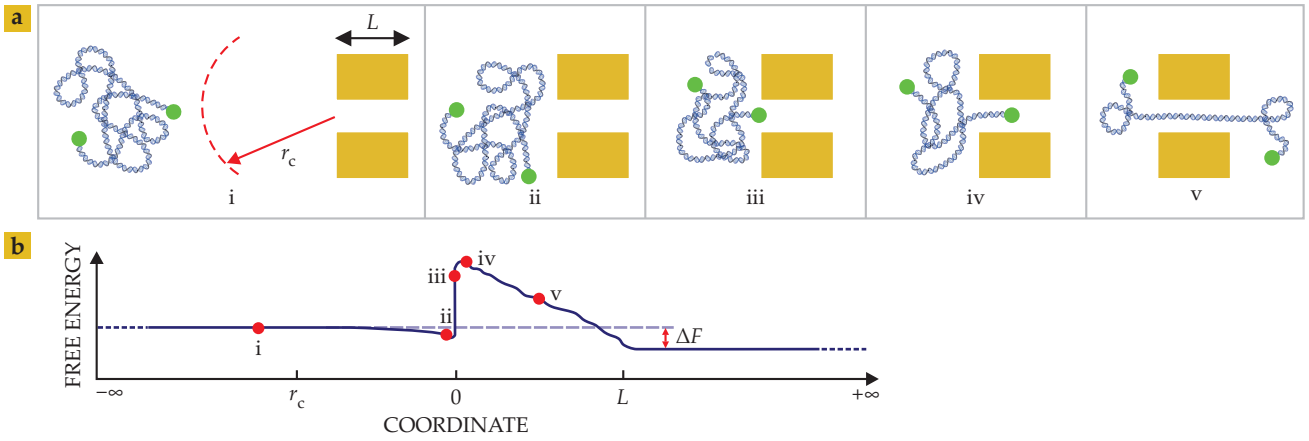

Figure 3. The threading of a biopolymer strand through a nanopore of length *L* proceeds through the stages shown in **(a)**: (i) The charged polymer drifts into the capture zone, defined by the radius r_c, and is pulled toward the pore by an applied electric field; (ii) the biopolymer arrives at the pore with its ends unregistered with the pore entrance; (iii) one end of the strand aligns with the pore entrance and enters the pore; (iv) the strand begins to uncoil as the end moves through the pore; and (v) the strand crosses the pore through a combination of electrophoretic drift and diffusion. Each step corresponds to a different stage on a free-energy landscape **(b)**. The coordinate values 0 and *L* correspond to the points when the chain's head and tail enter and exit the pore, respectively. The transitions between the various stages are typically associated with a free-energy barrier of 10–15 k_BT. Here, ΔF is the net free energy gain by the translocating molecule.

counter to the flow of DNA and thereby slow the strand's transit.

▶ **Polymer topology and entropic barrier.** Whether and how a biopolymer transits a nanopore depends on the associated free-energy barrier. That barrier in turn depends on the biopolymer's topology—whether the polymer is single stranded or double stranded; linear, knotted, or ring-like; and so forth.

The free-energy landscape is also affected by pore–polymer interactions, which can vary widely between biological and solid-state pores. The charge distribution in a biological pore is typically heterogeneous, with positive and negative charges located at specific positions on the pore wall. In contrast, the charge distribution at the wall of a solid-state pore can be uniform.

A strong electric field at the pore entrance can also deform a biopolymer, such that the chain doesn't maintain equilibrium as it approaches and crosses the pore. As a result, the ionic-current signals collected during a translocation event can contain richly detailed information about nonequilibrium effects at the single macromolecule level.

Considerable theoretical efforts have been made to describe how the above factors work together to dictate the experimentally observed signatures of translocation events.[8] Most theories are based on quasi-equilibrium assumptions within the Fokker–Planck formalism, whereby the free-energy landscape is computed under the assumption that chain conformations equilibrate on a time scale shorter than the translocation time. That assumption is valid for short strands, but nonequilibrium effects clearly come into play for longer ones, such as kilobase-length DNA strands, which have been shown experimentally to have equilibration times much longer than their translocation times. When the translocating polymer possesses intrinsically structured domains, as do so-called multidomain proteins, the free-energy barrier can exhibit a rich structure that can potentially be used to decode the molecule's structural details.

Sequencing DNA

Nanopores are well suited to a large number of biosensing applications, a few of which are illustrated in figure 4. Most discussed is their potential for a next generation of single-molecule DNA sequencing devices—indeed an application of momentous importance. To read out the sequence of a DNA strand as it passes through a nanopore, one needs to reliably associate specific current levels with specific bases. To first approximation, the magnitude of the current blockade is proportional to the size of the base occupying the pore. The size differences among the four canonical bases—adenine, guanine, cytosine, and thymine—are significant enough to yield current variations of around 10 picoamps in biological pores. Such variations are easily detectable using state-of-the-art amplifiers.

Because the measured signal is essentially the convolution of the signals of all bases within the pore cavity, the identification of single bases requires pores with a shallow constriction that can be occupied by only one or a few bases at a time. As a result, protein pores with thin orifices and pores in monolayer-thin materials such as graphene are preferable to pores having thick, tunnel-like orifices. In practice, even the best pores sense a moving window of several bases, and raw current data must be deconvoluted to recover the actual sequence.

One key challenge in nanopore sequencing is controlling the speed of the translocation. In solid-state pores, typical free translocation velocities for double-stranded DNA range from 200 to 50 000 base pairs per millisecond. That's extremely fast, considering that most amplifiers currently in use have a resolution of about 10 μs. (Some newer implementations can achieve 1-μs resolution.) In other words,

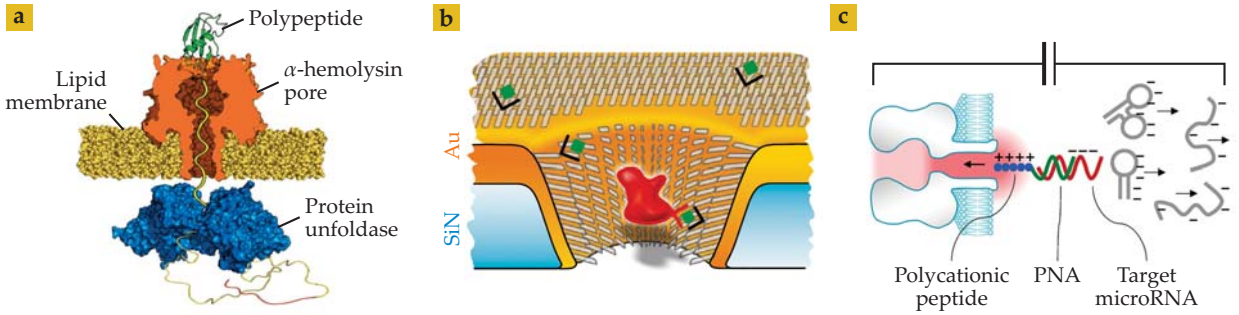

Figure 4. Nanopore biosensors take a variety of forms. **(a)** A protein-unfolding enzyme, ClpX , can be used to pull a polypeptide chain linearly through an *α*-hemolysin pore. The technique suggests the possibility of using nanopores to perform protein sequencing. (Adapted from ref. 11.) **(b)** A gold-plated nanopore is transformed into a biosensor by integrating an analyte-specific receptor (green and black) directly into the pore. Binding between the receptor and the analyte (red) manifests as a prolonged partial blockade of the ionic current through the pore. (Adapted from ref. 17.) **(c)** Short, specific strands of RNA (red) can be detected using as bait a complementary strand of peptide nucleic acid (PNA; green) attached to a positively charged peptide (blue). The isolated RNA strands are negatively charged, but the peptide-PNA-microRNA complexes are positive. The polarity of the voltage drop across the pore can therefore be selected to drive the targeted strands of RNA toward the pore and all other strands (gray) away from the pore. (Adapted from ref. 18.)

under typical conditions, between 2 and 500 bases will fly through a solid-state nanopore in the shortest resolvable time unit. Accordingly, the signal is averaged over many bases and sequence information is lost.

Strategies for slowing down translocation include increasing the viscosity of the electrolyte solution, decreasing the temperature, tuning DNA–pore interactions, and maintaining asymmetric salt conditions across the pore. With biological pores, complex pore–polymer interactions often have the effect of slowing down translocation. An interesting innovation has been to dock at the pore entrance specialized enzymes that permit DNA to pass only by way of a slow, ratchet mechanism.[9] Due to the above factors, biological pores typically exhibit much slower translocation speeds of 0.1 to 700 bases/ms for single-strand DNA.[5]

A biological pore is at the heart of Oxford Nanopore's MinION, which recently became the first nanopore sequencing product on the commercial market. Average read lengths for the device range from 5 to 10 kilobases, although much longer reads are possible. Approximately the size of a USB flash drive, the MinION has potential to open up the ability to perform sequencing directly at remote locations in the field. That portability could have important applications in areas such as epidemiology.

To gather sufficient sequence data in a reasonable time, any nanopore-based sequencing device must simultaneously record data from many pores in parallel. The MinION sequencer employs several hundred pores in parallel, and future developments may allow even higher parallelization. The first results from the MinION, however, indicate high base-identification error rates—about 15% in a recent report.[10] Although it is impressive to see that the first nanopore-based DNA sequencer is starting to produce results, it remains to be seen whether it can truly penetrate the sequencing market.

Solid-state nanopores may offer a way to overcome some of the limitations of biological nanopores, such as their fixed diameter and the limited stability of the pore and the encompassing lipid bilayer. Furthermore, they lend themselves naturally to integration into solid-state devices equipped with microfluidics and electronics for signal processing. In addition to the fast DNA translocation speeds, however, solid-state nanopores currently suffer from relatively high noise levels—due to high substrate capacitances—and a lack of an atomically defined pore constriction. Thus far, those challenges have prevented DNA sequencing with solid-state nanopores.

Hybrid pores hold promise to combine the best of both worlds—the membrane stability, device compatibility, and scalability of solid-state pores and the atomically defined pore constriction and biological engineering available for protein and DNA-origami nanopores. It is still too early, however, to assess the impact of the hybrid approach.

Beyond sequencing

So far, the main driver behind the nanopore electrophoresis experiments has been the societal need to sequence DNA rapidly and inexpensively. But nanopores are useful for other kinds of biosensing. Because they probe each base's structure, they are well suited to detect base modifications such as methylation and hydroxymethylation, as several studies have shown. Another advantage of the structure-based readout is that the nanopore technique should be directly applicable to RNA and is likely adaptable to other polymers such as polypeptides.

Recently a group at the University of California, Santa Cruz, demonstrated that the protein-unfolding enzyme ClpX can be used in the manner illustrated in figure 4a to thread an amino-acid chain through a nanopore, which suggests the possibility of protein sequencing.[11] Full *de novo* protein sequencing is a much harder problem than DNA sequencing, however, because it requires distinguishing between 20 different amino acids rather than just 4 bases. Still, early steps are being made in that direction.

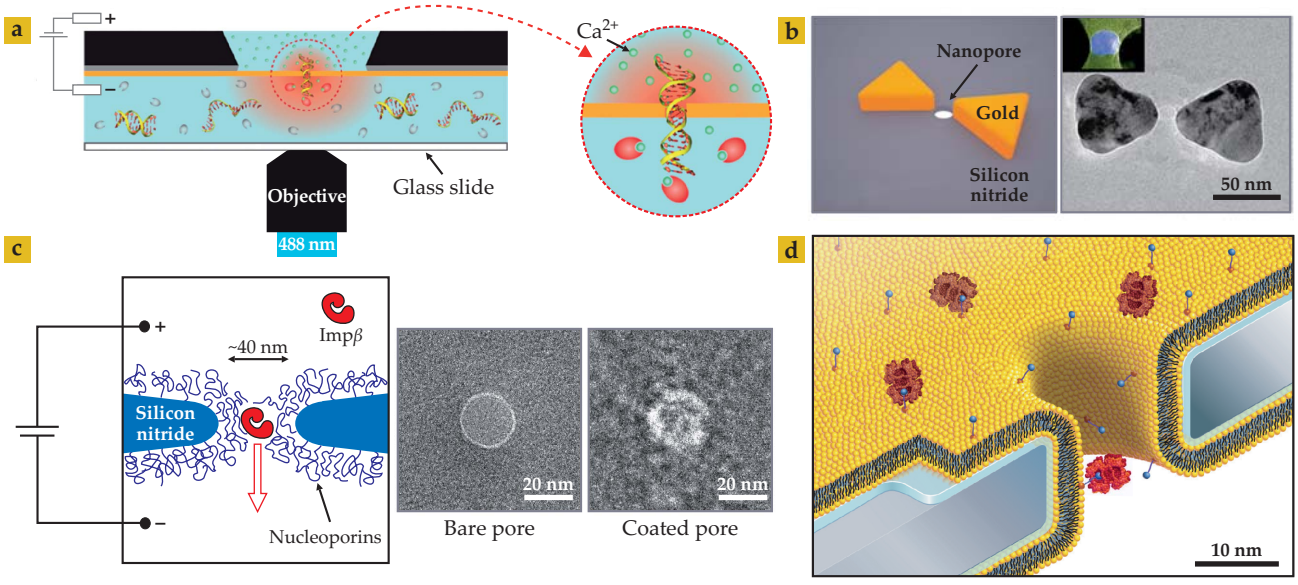

Figure 5. New tools in the biophysics kit. **(a)** DNA translocation through a nanopore can be detected optically using a calcium-sensitive dye (red) and calcium conditions that are asymmetric about the nanopore. The fluorescent dye is excited with a 488-nm laser. (Adapted from ref. 13.) **(b)** A plasmonic bowtie antenna affixed to a solid-state nanopore facilitates manipulation and Raman optical detection of translocating biomolecules. The antenna, shown at right in a transmission electron microscope image, concentrates incident light to create a plasmonic hot spot with a large electromagnetic field at the entrance of the 10-nm-diameter pore, shown in a false-color close-up in the inset. (Adapted from ref. 14.) **(c)** Solid-state pores coated in nuclear pore proteins (nucleoporins) have been shown to selectively regulate the transport of proteins, such as the transport receptor importin-β (Impβ), in a manner similar to naturally occurring nuclear pore complexes. The transmission electron microscope images at right show a nanopore before and after coating. (Adapted from ref. 15.) **(d)** A nanopore coated with a lipid-bilayer membrane (yellow) can concentrate analytes (red) onto the membrane surface and slow down their translocation through the pore. (Adapted from ref. 16.)

Nonsequencing applications are also being pursued. For instance, nanopores can be used as biosensors to detect specific analytes. A typical approach is to tune the pore–analyte interaction using a bait–prey system, in which a binding group for the analyte is directly integrated into the pore, as shown in figure 4b. The binding group (the bait) can be, say, an antibody or a single-stranded DNA oligomer that complements a sequence in a translocating DNA strand (the prey). Binding extends the residence time of the analyte in the pore, such that the presence of the analyte is signaled by a prolonged current blockade.

The binding group in a bait–prey system need not be fixed to the pore. The system in figure 4c, for instance, detects a short, negatively charged RNA strand by using as bait a complementary strand of peptide nucleic acid (PNA)—a synthetic analogue of DNA. Latched to the PNA strand is a positively charged peptide. On binding, the net charge of the RNA-PNA-peptide complex becomes positive, and, as a result, only bound RNA strands are pulled toward the pore.

Scientists are also using nanopores to study DNA–protein complexes, with the eventual goal of being able to determine the protein's binding location and identity and to probe the complex with force spectroscopy.[12] Furthermore, nanopores may be exploited to characterize and separate synthetic macromolecules. The various charged macromolecules used in the health care and materials industries typically start out highly polydisperse. As of yet, there are no robust techniques to characterize the length distributions of water-soluble polymers. Researchers have recently shown that nanopore electrophoresis could offer an effective way to determine the lengths of the various molecules in a milieu, which could greatly impact separation science.

A biophysics playground

If nanopore sequencers can indeed match the accuracy of current state-of-the-art DNA sequencers, they will offer distinct advantages such as label-free electrical readout and single-molecule sensitivity. More generally, nanopores are also emerging as powerful and broadly applicable tools for biophysics at large. To illustrate that point, we mention a few noteworthy examples.

Nanopores allow one to study the physics of charged macromolecules under confinement and in nonequilibrium conditions. Those studies can be expected to yield generalized equations of Brownian motion that account for the electrophoretic mobility, effective charge, and diffusion coefficients of charged molecules. Emerging data on the effects of counterions on DNA translocation open a new way of exploring the polarizability of DNA and the electrolyte ions inside nanopores. That exploration could, in turn, lead to yet another way of interrogating the structure of confined water.

Polymers are topologically correlated, and the formation of knots must be a common feature. Nanopores should be able to detect, measure, and characterize the properties of various kinds of knots, including the forces required to tighten them. When proteins are used as the analytes, a nanopore can serve as a tool to probe their folding and unfolding kinetics. One may be able to probe whether multidomain proteins unfold sequentially, one domain at a time, or all at once. Unfolding and folding pathways can also be compared.

Furthermore, it may be possible to encode either nanopores or analytes with charge patterns, which could then be read out by observing translocation dynamics. Such patterning may be designed to promote ratcheting and stochastic resonances and thereby provide novel ways to study nonequilibrium effects.

Although ionic current remains the most popular detection modality in nanopore experiments, new modalities are being explored. They include optical methods involving the detection of fluorescent dyes attached to analytes or, as illustrated in figure 5a, the use of ion-sensitive dye gradients across the pore.[13] Plasmonic nanostructures can be integrated into the membrane to focus light onto the nanopore, as shown in figure 5b, and may allow Raman spectroscopy of translocating analytes.[14] Researchers are also pursuing alternative electrical methods of detection, including measuring the tunneling current between nanoelectrodes positioned opposite one another across the pore's diameter and, in graphene, measuring the electrical current in the membrane plane. Nanopores can also be combined with other single-molecule measurement techniques, such as optical tweezers, to measure the force on a macromolecule within the pore, which greatly enhances their capabilities for biophysics experiments.

Extending nanopores to the study of biological systems is a new emerging area. Transport across nuclear pore complexes is being studied using biomimetic nanopores such as the one in figure 5c.[15] Likewise, lipid-coated nanopores have been used to concentrate analytes onto membrane surfaces and to slow down translocation, as illustrated in figure 5d.[16] The many cellular processes involving molecular transport through small pores—the injection of phage genetic material into a host, mitochondrial transport, the transport of proteins to the endoplasmic reticulum, or the transport of messenger RNA across nuclear pores, for instance—are far more complex than DNA translocation through a nanopore. Yet the basic ingredients of capture, nucleation, and threading are ubiquitous—they are just dressed up by other biochemical features that endow selectivity and control. Despite the challenges, we expect that the nanopore community will bridge the gap between artificial and biological systems by methodically increasing the complexity of nanopore electrophoresis experiments.

The field of nanopore studies has grown tremendously in the past decade, branching out into seemingly every direction. We expect that it will continue to impact both sequencing and biophysics for many years to come.

Cees Dekker and Calin Plesa acknowledge support from the Netherlands Organisation for Scientific Research as part of the Frontiers of Nanoscience program and the European Research Council. Murugappan Muthukumar acknowledges support from the National Institutes of Health, the Air Force Office of Scientific Research, and NSF.

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