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THE REALM OF THE NANOPORE

Interest in nanoscale research has skyrocketed, and the humble pore has become a king.

Jennifer Griffiths

A little more than 10 years ago, a group of scientists made a discovery that would revolutionize the way DNA was sequenced—or so they hoped. In their 1996 paper (*1*), the team, which included John Kasianowicz of the U.S. National Institute of Standards and Technology, Daniel Branton of Harvard University, and David Deamer of the University of California Santa Cruz, showed that they could use an electric field to drive single-stranded DNA and RNA molecules into the aperture of a proteinaceous transmembrane ion channel and use the pore as a sensor. "It was very clearly allowing us to detect individual [DNA] molecules and [to] begin to learn something about the character of these molecules," says Branton. "The very first thing we noticed was that we could obviously determine the length or the size of the molecule

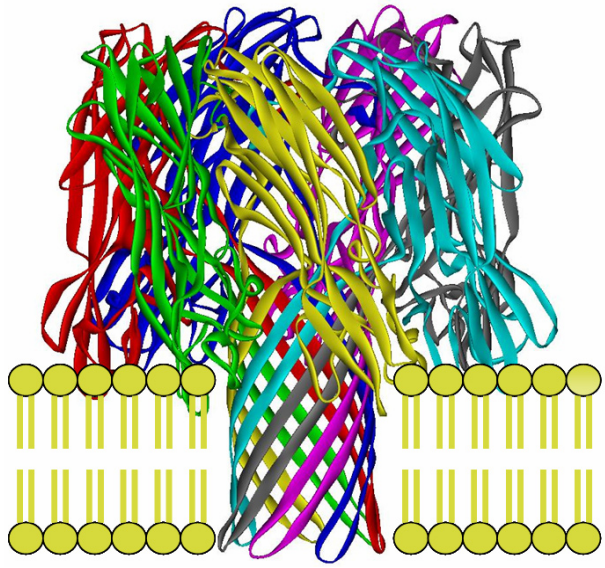
The primary strength of nanopores is that they provide localized measurement in a volume comparable to the size of a molecule.

in this way, because a longer strand takes a longer time to get through the pore."

Encouraged by their results, the scientists suggested at the end of that paper that if single strands of DNA could be detected by a nanopore, it was possible that buried within that signal was information about individual bases in the chain. If that information could be decoded, then a new single-molecule method of sequencing would be the result.

Kasianowicz says, "That study significantly piqued others' interest because of the possibility of rapidly sequencing DNA using a single nanopore. However, despite the rapidly expanding number of scientists working in the field of nanopore-based sensors, no one has yet accomplished that feat."

Still, the many researchers working with nanopores have made tremendous strides in understanding and manipulating the proteinaceous pores and have even started creating their own—termed solid-state or synthetic nanopores—using nanofabrication techniques. "Nanopores provide localized measurement in a volume comparable to the size of a molecule; that's primarily their strength," says Andre Marziali of the University of British Columbia (Canada). "So whereas initially everyone was excited about DNA analysis, now there are a lot of applications for molecular detection in general." The consensus is that the achievements in the field are quite remarkable, even if the original goal of sequencing DNA is still out of reach.



α-Hemolysin situated in a lipid bilayer membrane. (Adapted with permission from Ref. 11. Copyright 2004 Taylor & Francis, Ltd.; www.informaworld.com.)

Harnessing hemolysin

The draw of nanopore sensors, says Michael Mayer of the University of Michigan, is in their composition. "All you need is a hole, [an] electrolyte, two electrodes, a voltage source, and some way to measure current," he says. "If you think about other techniques where you measure individual molecules, that's outrageously simple."

The experimental setup for a proteinaceous nanopore experiment consists of two chambers of buffer that are separated by a lipid membrane into which an ion-channel protein has been inserted. A voltage is applied across the membrane; this drives ions and molecules of a charged analyte, such as DNA, through the pore. "At the simplest level, when a DNA molecule enters the pore, you see a blockage of the ion current," explains Marziali. The duration and amplitude of that blockage signal give a readout of some physical parameter of the analyte.

The pore used by Kasianowicz, Branton, and Deamer for their original experiments was α-hemolysin, a relatively large ion channel from *Staphylococcus aureus* with a 1.5 nm pore restriction—just the perfect size for single-stranded DNA molecules to slide through in single file. The wild-type protein has seven identical subunits that are soluble upon expression yet self-assemble into a lipid bilayer.

One reason that α-hemolysin was originally chosen was that it is very stable and well behaved. "As soon as we made this protein, we could tell it was going to be a relatively simple, or cooperative, protein to work with," says Hagan Bayley of Oxford University (U.K.), whose group has studied α-hemolysin extensively. "Some membrane proteins are very, very unstable, and I think in retrospect, with this particular protein we have been lucky, because we've worked on a lot of other membrane proteins, and none of them have been quite as friendly as α-hemolysin."

Despite the generally accommodating nature of α-hemolysin, a few kinks had to be worked out before it could be used as a nanopore sensor. Nature designed the protein to open and close depending on pH or applied potential, a property that would have to be eliminated to prevent false blockage signals. The other problem was that the analytes just moved too quickly through the pore. Kasianowicz says that he spent a lot of time in his early α-hemolysin research finding conditions in which the pore stayed open and the analytes moved slowly enough that the protein could be used as a sensor. Once those problems were solved, α-hemolysin became the protein of choice as a sensor for ions, DNA, and proteins.

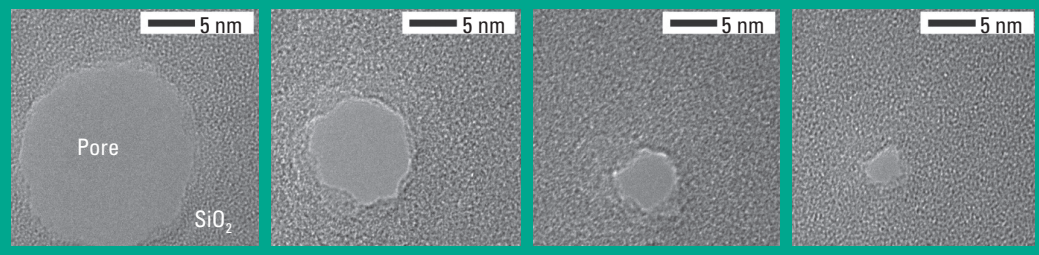
Over the years, α-hemolysin has maintained its dominance in the field. Other pore proteins have been pursued, but none so far has displaced α-hemolysin in terms of popularity. "It's really amazing," says Mark Akeson of the University of California Santa Cruz. "If somebody had to engineer a . . . pore, they'd make α-hemolysin, basically."

Improving on nature's design

That's not to say that biochemists haven't been trying to make α-hemolysin even better. Bayley's group has been working for years on modifying the protein. "Much of our work . . . in the field has been centered around protein engineering—taking this molecule and changing its properties, either by using molecular genetics or targeted chemical modification," he says.

It turns out that α-hemolysin is particularly amenable to mutagenesis. The β-barrel structure of the transmembrane domain tolerates all kinds of modifications to its sequence.

According to Bayley, his group has successfully engineered up to 7 arginines per subunit (49 total for the whole channel) into the sequence without a problem. "You simply couldn't do this with an α-helical protein," he says. "It would explode if you put so many charged side chains right in the middle of the protein."



(Left to right) Images over time of a silicon oxide pore in a transmission electron microscope. The electron irradiation causes the pore to shrink gradually to a size of ~3 nm (*12*).

As an example of another successful α-hemolysin modification, Bayley points to his group's experiments in which they adapted the interior of the pore to noncovalently bind cyclodextrin. "That was a big breakthrough, because it's known that cyclodextrins will bind organic molecules in solution," he says. Recently, Bayley and colleagues showed that they could trap ribonucleoside and deoxyribonucleoside 5′-monophosphates with their cyclodextrin–hemolysin construct and distinguish the different nucleobases from the signals produced (*2*). These results may have implications in the future for DNA sequencing.

Nanopores go synthetic

Protein pores obviously have been very successful, but they have their drawbacks as well. The general feeling among researchers is that synthetic nanopores are the way of the future. "We always learn a lot from α-hemolysin," says Liviu Movileanu of Syracuse University. "But solid-state nanopores show a long, grand promise."

One major problem with the α-hemolysin system is its lack of stability—but, for once, it's not the protein's fault. "The funny thing is that the protein itself can be extremely robust," says Zuzanna Siwy of the University of California Irvine. "But the membrane, unfortunately, is very unhappy. So the whole thing, although it's beautiful, is hard to translate to everyday life—to make a device out of it—just because of the lipid bilayer membrane."

Some efforts have been made to stabilize the membrane, but instead, many people in the field are moving to purely synthetic nanopores. "I find that these proteinaceous pores are limited," says Gregory Timp of the University of Illinois Urbana–Champaign. "We find it useful to work in a range of pHs and temperatures and at very high voltages, and so, consequently, proteinaceous pores don't fit the bill."

Synthetic nanopores also provide flexibility in terms of size. "One of the advantages that we have in a synthetic pore is that we get to determine the horizontal and vertical," says Timp. "We get to say how thick the membrane is, and we get to say what diameter the pore is." This is important because although the α-hemolysin pore is the perfect size to admit single-stranded DNA, for researchers who might want to study double-stranded DNA or even proteins, the popular protein pore is just too small.

Fabricating nanopores

Mainly for these reasons, many in the field have been making the transition from biological to synthetic pores, and some new researchers with expertise beyond biochemistry have joined the game. Solid-state nanopores "get a little closer to the realm of physics in the kinds of questions and the devices that you make," says Jene Golovchenko of Harvard University. "More and more people with physics backgrounds are involved with it, so it's kind of an interdisciplinary field now."

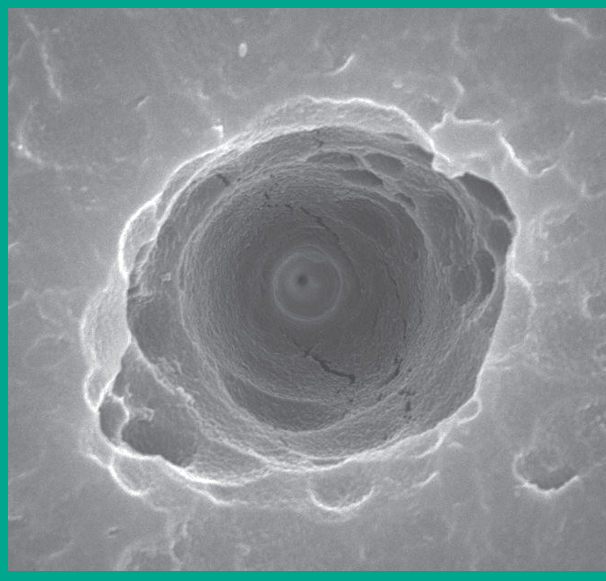
The physicists bring their expertise in nanotechnology to the field. Nature makes it look easy with α-hemolysin, but reproducibly fabricating such small pores can be tricky. "It's an extremely hard problem," says Golovchenko. "We have to develop new methods of making things . . . and understanding what's going on at the nanoscale."

Several competing methods exist for fabricating nanopores. "We don't right now have an established methodology to drill nanopores very accurately and precisely at single-atom resolution," says Movileanu. "Everybody is doing it in their own way. . . . The skills are different; the materials are different; the people are different."

In one method for making nanopores that is related to microlithography, scientists use a beam of electrons or ions to bore a hole through an inorganic silicate. In the electron beam method, "we start off with a very high voltage transmission electron microscope and focus the electron beam down to a few tens of angstroms, and we sputter material off the pore at ~1 atom/s until we remove enough so that we can see through it," Timp explains. "We're using effects that our friends in transmission electron microscopy would call bad," he adds. "They call this beam damage; we call it a feature!"

A competing method for fabricating nanopores is called track etching. The process consists of two steps. In the first, a thin, plastic membrane is bombarded with heavy ions. "These massive ions [are] going at tremendous velocities with fantastic kinetic energy," says Charles Martin of the University of Florida. "They just crash right through the plastic film and create a damage track." In the second step, the damage track is chemically etched to create the pore.

According to Martin, the track–etch method is better than competing methods at creating reproducible pores. His group addressed the reproducibility issue in a recent paper (*3*). "My students and I recognized that if this field of artificial nano-



Electron micrograph of a conical polycarbonate membrane pore. (Adapted with permission from Ref. 13.)

pore resistive-pulse sensing is to go anywhere, then reproducible fabrication of the nanopore sensing element is essential," Martin says. He also points out that the tracked membrane is available commercially, making it easier for anyone to start making these pores. "You don't need a room full of multi-million-dollar microfabrication machines," he says. "The track–etch method offers resistive-pulse sensing to the masses!"

Selective pores

One area where the α-hemolysin nanopores have a significant head start is selectivity. Work by the groups of Kasianowicz and Bayley has led to proteinaceous nanopores that can detect specific analytes, but the solid-state pores are lagging far behind.

An advantage of proteins such as α-hemolysin is that they can be precisely manipulated on the basis of structural and sequence information. "The power of the protein that folds in a well-defined shape is that you know exactly where your modified group will end up," says Cees Dekker of Technische Universiteit Delft (The Netherlands).

Instead of a chemical functionality being placed at a precise location, as is possible with proteinaceous pores, the derivatization of solid-state pores is a more global process. "We do not yet have fabrication or chemical methods of inserting a chemical group in the place we want," says Siwy, who, along with Martin, pioneered the biofunctionalization of synthetic nanopores. "We just put them on the whole surface, so it's less elegant." Still, Martin says that this work takes a big step toward what has been accomplished through functionalization of α-hemolysin.

Researchers are working on methodology to more precisely place functional groups on solid-state pores. "I think that there's a lot of action surrounding the idea of atomically precise pores, and I think that's warranted," says Timp. If these efforts are successful, solid-state pores may soon catch up with proteinaceous pores in terms of selectivity.

Progress toward sequencing

Recently, the U.S. National Human Genome Research Institute announced an initiative aimed at reducing the cost of sequencing an individual human genome from ~$10 million to ≤$1000. These "$1000-genome" grants fund researchers working on all aspects of technology that may speed up and reduce the cost of sequencing. Single-molecule approaches, such as nanopores, are attractive for this purpose because they don't require amplification of the DNA with expensive reagents.

This initiative has revived the idea of making a nanopore device to sequence DNA, although perhaps not as it was originally envisioned in 1996. Work over the years has shown that scientists are unlikely to identify a single nucleotide with ionic current blockage measurements. Only ~100 ions transverse the pore in the time that a single nucleotide occupies it, resulting in a signal that is just too weak to allow researchers to discriminate among the bases.

But researchers are now starting to get creative and dream up new methods to extract DNA sequence information from nanopores. Some are trying to detect DNA passage by using signals other than ionic current. "One big challenge is to be able to put sensors on the pore or very close to the pore," says Rashid Bashir of the University of Illinois Chicago. "The goal there would be to add another dimension of sensing—another modality, not just current, but a field effect or something else—and work toward temporal and spatial resolution of a single base."

One example that does not use ionic current comes from the group of Amit Meller of Boston University (*4*). In their method, a single strand of DNA is hybridized to individual fluorescently labeled oligonucleotide probes. As the DNA is fed into the nanopore, the probes are stripped off sequentially and each flashes a different color of light, which the researchers detect by total internal reflectance microscopy.

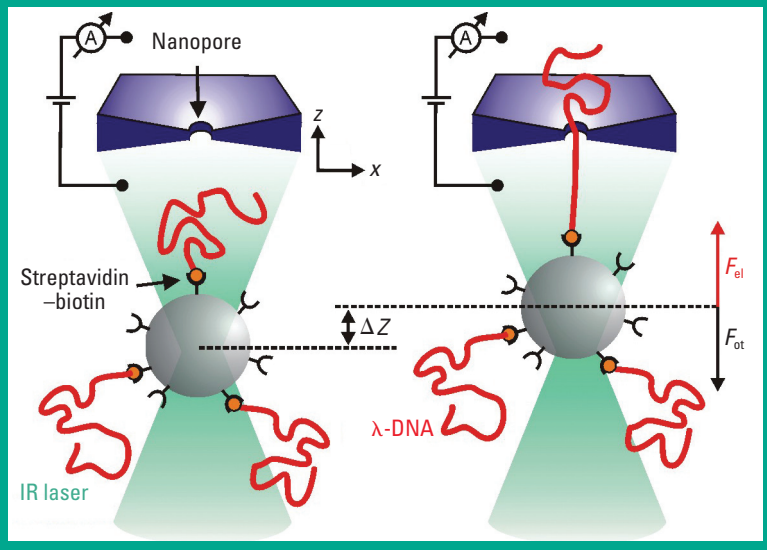
Another approach, which is being developed by NABsys, a company founded by Xinsheng Ling of Brown University, combines nanopores with an older technique—sequencing by hybridization—to create hybridization-assisted nanopore sequencing (HANS). In HANS, 100,000 bp segments of single-stranded genomic DNA are hybridized with a library of 6-mer DNA probes. The segments are run through a solid-state nanopore, and the hybridized, double-stranded segments give a distinct signal. The location of each probe can be deduced from a current-versus-time readout, and the full genomic sequence is reassembled from this data. Ling says that the advantage of the technique is that it bypasses the single-nucleotide-identification requirement. "I believe there is no need to kill ourselves to try and detect single nucleotides if we can already detect multiple nucleotides," he says.

These aren't the only nanopore sequencing ideas out there, by far—the $1000-genome grant awards list reads like a who's who of the nanopore research field, showing that the nanopore sequencing concept is far from stagnant.

Nanopores for biophysics

Meanwhile, nanopores are helping to unravel other scientific questions. "There are definitely lots of applications, even beyond the DNA sequencing," says Meller. Some of these applications address fundamental problems in biophysics.

For example, several independent groups of researchers, including those led by Kasianowicz, Marziali, Akeson, and Meller, have used nanopore force spectroscopy to derive biophysical properties, such as association rate constants and DNA unzipping kinetics (*5*–*7*). This technique takes advantage of the restricted pore opening by feeding a noncovalently bound complex into the channel. Because the complex is too large to pass through the pore, it stalls until the force of the applied voltage overcomes the binding interaction energy. Researchers can extract information about a system's biophysical properties from the nanopore data.



A nanopore/optical tweezers experiment. A focused laser beam is used to trap a DNA-coated bead near a solid-state nanopore. An ionic current directs the DNA into the nanopore. When the DNA enters the nanopore, an electrical force (F_{el}) is exerted on the bead, which is pulled over a distance ΔZ until the optical force (F_{ot}) and F_{el} are balanced (*8*).

Meanwhile, Dekker and colleagues are combining solid-state nanopores with other biophysical techniques (*8*). "Our fundamental interest [in nanopores] is that we now use them as a tool to probe local forces and use them as local actuators," says Dekker. "Because we combined these solid-state nanopores with other single-molecule techniques such as optical tweezers, we now can manipulate the single molecule in the neighborhood of that solid-state nanopore." Manipulating the DNA in the vicinity of the nanopore has allowed Dekker's group to measure the force that is applied to the DNA during translocation through the nanopore. Eventually, they may be able to probe local structures along the DNA, such as proteins bound to certain regions of the sequence.

Nanopore proteomics?

Although researchers have been probing DNA with nanopores for many years, only somewhat recently have they started to use them to analyze proteins. Mayer says that his group originally chose to work on nanopore protein analysis because, compared with the body of DNA work, relatively little research had been done with proteins. "Quite often, proteins can cause some problems," he says. "They can be sticky and [have] other issues."

Still, researchers are pushing ahead. "We've been using [nanopores] to interrogate the structure of prion proteins for mad cow disease applications," says Marziali. "Other groups have translocated proteins and α-helices and made early, early steps toward protein analysis, as well. I think there are potentially some excellent applications."

As with DNA, selectivity of the pores for a given protein can be an issue. Martin's group has addressed this problem with their functionalized synthetic pores (*9*). "High selectivity was achieved because we attached protein recognition agents such as antibodies to the nanopore," he says.

Ling is looking even farther into the future. "It would not be a far-fetched statement that you might be able to see the nanopore proteomics field coming out soon," he says. In fact, Kasianowicz and colleagues have recently demonstrated that they can measure the mass of polymers in solution with α-hemolysin, thus essentially using a nanopore as a mass spectrometer (*10*).

Nanopore research has made huge strides in the past decade, and researchers are proud of their achievements. "We're controlling matter on a subnanometer scale, and we're using it for fun things like controlling DNA molecules and trying to figure out what DNA sequences are," says Timp. "I can't emphasize enough how exciting that is, because it's only recently that it's become possible to do that kind of stuff."

This progress is the result of a sustained, organized effort to open up nanopores as reliable new tools and create new methods to actually do chemistry on a single-molecule scale, says Golovchenko. "It's a really challenging endeavor. But of course, it's a very big payoff worthy of such a sustained effort."

Jennifer Griffiths is an associate editor of Analytical Chemistry.

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