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On “Three decades of nanopore sequencing”

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Sir,

The “historical perspective” by Deamer, et al. does not mention several key ideas, methods, and experimental results that were essential to the study and possible practical use of nanopores for single molecule sensing and macromolecule characterization. It also contains several factual errors. To address these, we discuss why the notion of sequencing DNA with a nanopore was not “implausible”, what was required to make it even feasible to consider, and how the science and technology advanced during the early stages of the field.

During the late 1980s, we both started working in Adrian Parsegian’s laboratory at the NIH, which pioneered the use of pore-impermeant polymers to estimate the physical properties of ion channels¹. There, during a collaboration with Lindsay Bashford and Charles Pasternak from London (1988), one of us discovered serendipitously how to keep the alpha-hemolysin channel open indefinitely and we found that a nanopore could be used to sense ions, quantify their concentrations, and measure the kinetic rate constants that describe the interactions between the ions and the pore.^{2,3} In our description of those results, we suggested that single nanopores could be used “*to study the kinetics of fast chemical reactions in a single microscopic (in fact, nanoscopic) ‘cuvette’*”² One of us traveled to Bayley’s lab at the Worcester Foundation for Experimental Biology, asked if he could provide us with purified alpha-hemolysin, showed him these results, and established a collaboration to extend our nanopore-based single molecule sensors work.^{4,5}

In 1989, inspired by work on macromolecular transport through the nucleopore complex in John Hanover’s lab at the NIH (a few doors down from Parsegian’s), even knowing that the chances of success would be slim (*see below*), one of us tried to detect DNA with single nanopores formed by the Voltage Dependent Anion Channel (provided by Marco Colombini at the University of MD, College Park). Unfortunately, the results were inconclusive because that channel exhibited voltage-dependent gating^{1,6} (which made it difficult to separate any transient ionic current blockades that might be caused by nucleic acids). Regrettably, they were also consistent with what one would expect for the mean residence time of a single molecule in a nanometer-scale pore. Specifically, nanopores are so short that Einstein’s one dimensional diffusion equation suggests that a single molecule would spend only ~ 100 ns in the pore - far too brief a time to be detected by any electrophysiology patch clamp amplifier.^{7–10} In addition, even if infinite bandwidth amplifiers were available, the flux of ions through the pore in that short time interval would be woefully inadequate to characterize the

molecules.¹⁰ Importantly, we soon overcame that fundamental limitation with a striking experimental result, which we describe next.

In 1988, Krasilnikov and colleagues reported a method to estimate the limiting diameter of the alpha-hemolysin ion channel using differently-sized non-electrolyte polymers.¹¹ His results were particularly intriguing to us because the dependence of that pore's conductance on polymer size did not agree with theory. When we repeated those experiments in 1990, the very first experiment revealed that the mean residence time of a polymer in the pore was some 500 to 1,000 times greater than expected!⁸ One of us (JK) had described those interesting results to Deamer and others during a workshop at NASA Ames (1991) and to Deamer personally at a Biophysical Society meeting soon thereafter. Deamer, et al. summarized this work as "*Kasianowicz was also collaborating with Bezrukov to investigate the effect of polyethylene glycol on pore conductance and, consistent with earlier reports, found that a pore radius of ~1.1 nm accounted for their results.*" However, that summary completely misses the fundamental nature of our study, which would not be surprising 22 years ago (it took us several years to convince others that the work should be published), but the significance of our findings should not be lost today¹⁰. Nevertheless, the results gave one of us (JK) experience-based confidence to detect individual molecules of single-stranded nucleic acids with the alpha-hemolysin nanopore¹² and had planned to start on that project when Deamer suggested a collaboration.

The possibility of passing DNA through nanopores was also plausible to others studying ion channels. For example, Zoratti and colleagues used PCR to show that DNA could be transported through membranes containing the *Bacillus subtilis* or VDAC channels.^{13,14}

The authors also did not mention that Bayley and Oxford Nanopore Technology's abandoned a particular nanopore-based DNA sequencing method. Specifically, in the early 2000s, they aggressively proposed the use of an exonuclease (attached adjacent to one of the pore's entrances) to cleave mononucleotides that would be "read" serially by the nanopore, and published a paper implying that the technique would be viable.¹⁵ However, one of us subsequently demonstrated that this method would not work unless the substantial diffusion of cleaved mononucleotides away from the pore could be eliminated.¹⁶ We point out this and our own setbacks to illustrate how the seemingly smooth arrow of time in science, which is implied in Deamer, et al.'s perspective, is often not the case. In our perspective, a more frank discussion of what actually happens is more helpful to those preparing for careers in science and technology.

Finally, Deamer, et al. left unanswered the important question of whether the alpha-hemolysin nanopore was sufficient to sequence DNA. Specifically, it is not at all clear whether the ONT MinION device uses alpha-hemolysin, genetically engineered MspA^{17,18} or alpha-hemolysin with lessons learned from MspA or Electronic BioSciences alpha-hemolysin mutagenesis experiments. This information would obviously prove useful to scientists in the field and those who invest in the technology.

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