Electrophoretic Capture of DNA into Nanopore-Zero-Mode Waveguides for Efficient Single-Molecule Sequencing

Joseph Larkin

B.S. in Physics, Stanford University
M.S. in Physics, Boston University

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Meni Wanunu
Professor of Physics and of Chemistry and Chemical Biology
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Abstract

Current single molecule DNA sequencing technologies require extensive biochemical sample amplification. These methods introduce biases and lose epigenetic information, preventing full exploitation of DNA sequencing technology. Single Molecule, Real-Time (SMRT) sequencing, the technique used in the work described in this thesis, requires DNA amplification due to inefficiency in the DNA loading process prior to sequencing. This inefficiency arises from an entropic barrier to DNA entrance into the confined sequencing volume, which is a \(~100\) nm cylindrical aperture, referred to as a zero-mode waveguide (ZMW). We propose overcoming this barrier by placing a nanopore at the waveguide base, creating a nanopore-zero-mode waveguide (NZMW). The nanopore is a \(~1-10\) nm hole in a \(~30\) nm-thick insulating film, which may be used to electrophoretically attract charged molecules like DNA. Nanopores may be made in a variety of dielectric materials, usually silicon nitride (SiN). When biased, this pore establishes a DC electric field that attracts DNAs. A molecule may traverse the pore itself, in a process called translocation, inducing a transient, discrete drop in nanopore conductance. Measuring the rate of these current spikes allows one to measure the rate of capture of polymers by the nanopore. This system allows efficient molecular capture at low concentrations. This implies that, with efficient molecular capture an NZMW could use significantly lower concentrations of sequencing sample molecules. This paves the way for low-amplification or amplification-free sequencing.

On the way to fabricating, characterizing, and utilizing the NZMW for sequencing, we investigate the capture of single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), and proteins by nanopores. We develop hafnium oxide as an alternative material for nanopore membranes and demonstrate its high stability and strong interaction with translocating DNA molecules. Further, using high bandwidth electronics, we detect fast, \(~\mu\)s-long protein translocation events, and establish solid-state nanopores as an excellent tool for measurements.
of single, unlabeled proteins. Furthermore, we show that, when confined in the nanopore, proteins interact with the nanopore surface, drastically reducing their diffusion compared to free solution. Finally we outline the fabrication and characterization of an NZMW device and show its ability to capture biomolecules. We close by demonstrating efficient capture and sequencing of template DNAs, including long, 20,000 base pair fragments.
# Table of Contents

Acknowledgements........................................................................................................ ii
Abstract........................................................................................................................... v
Table of Contents .............................................................................................................. vii
List of Figures .................................................................................................................. x

Chapter 1. Introduction and Background...................................................................... 1
  1.1 Single Molecule DNA Sequencing ...................................................................... 1
  1.2 Entropic Barriers to Single-Molecule Loading .................................................. 4
  1.3 Nanopores ............................................................................................................ 9
  1.4 The Nanopore-Zero-Mode Waveguide (NZMW) ............................................... 12
  1.5 Instrumentation .................................................................................................. 13

Chapter 2. DNA Transport through Nanopores in Hafnium Oxide Membranes .......... 19
  2.1 Introduction ......................................................................................................... 20
  2.2 HfO$_2$ Nanopore Fabrication .......................................................................... 22
  2.3 Double-stranded DNA transport ...................................................................... 26
  2.4 Single-stranded DNA transport ...................................................................... 30
  2.5 Discussion .......................................................................................................... 34
  2.6 Materials and Methods .................................................................................... 35

Chapter 3. High-Bandwidth Protein Analysis Using Solid-State Nanopores ............... 37
  3.1 Introduction ......................................................................................................... 38
  3.2 Materials and Methods .................................................................................... 42
  3.3 Data Collection and Processing ...................................................................... 43
  3.4 Protein Capture .................................................................................................. 43
  3.5 Protein Transport ................................................................................................ 46
  3.6 Protein Volume Measurements ......................................................................... 53
  3.7 Discussion .......................................................................................................... 56

Chapter 4. Reversible Positioning of Single Molecules inside Zero-Mode Waveguides . 59
  4.1 Introduction ......................................................................................................... 60
  4.2 Device Characterization .................................................................................... 62
4.3 Detecting DNA/Protein Complexes ................................................................. 66
4.4 Simultaneous Positioning of DNA-Protein Complexes Inside NZMWs .......... 68
4.5 Focusing Long DNA Fragments into ZMWs .................................................. 71
4.6 Discussion ...................................................................................................... 73
4.7 Materials and Methods .................................................................................. 74

Chapter 5. Capture and Sequencing .................................................................... 76
  1. Introduction .................................................................................................... 77
  2. DNA Capture ............................................................................................... 78
  3. Sequencing .................................................................................................... 82
  4. Materials and Methods .................................................................................. 84

Chapter 6. Conclusions and Future Directions ................................................... 86

Appendices .......................................................................................................... 90

Appendix 1: Chapter 2 Supplementary Material .................................................. 90
  A1.1 HfO$_2$ deposition characterization ......................................................... 90
  A1.2. Elemental analysis of freestanding HfO$_2$ membranes ......................... 91
  A1.3. HfO$_2$ membrane crystallization .......................................................... 93
  A1.4. Dependence of pore conductance on diameter and thickness ............... 95
  A1.5. Determination of peak dwell time .......................................................... 97
  A1.6. Comparison of dsDNA translocation in SiN and HfO$_2$ pores ............... 99
  A1.7. Sequence of the 89 nucleotide molecule .............................................. 101
  A1.8. a-hemolysin (a-HL) measurements of single-stranded DNA ............... 101
  A1.9. Determination of capture rate for ssDNA ............................................. 103

Appendix 2: Chapter 3 Supplementary Material .................................................. 104
  A2.1. Data analysis details ............................................................................... 104
  A2.2. Size, charge of proteins and effect of electro-osmosis ......................... 106
  A2.3. Dwell time distributions and estimation of $F_{\text{obs}}$ ............................ 108
  A2.4 Continuous electrical traces of protein translocations ........................... 109
  A2.5. Protein analysis using silicon nitride (SiN) pore ................................... 111
  A2.6. DNA Dwell Time Distributions ............................................................. 114

Appendix 3: Chapter 4 Supplementary Material .................................................. 116
  A3.1 NZMW Fabrication ............................................................................... 116
  A3.2. Piranha cleaning of ZMW chips ............................................................ 119
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3.3 YOYO-1-labeled DNA traces</td>
<td>121</td>
</tr>
<tr>
<td>A3.4 NZMW optical cell</td>
<td>121</td>
</tr>
<tr>
<td>A3.5 Determination of DNA on-time</td>
<td>122</td>
</tr>
<tr>
<td>A3.6 DNA-streptavidin gel</td>
<td>123</td>
</tr>
<tr>
<td>Appendix 4: Chapter 5 Supplementary Material</td>
<td>124</td>
</tr>
<tr>
<td>A4.1. RIE etching and photoluminescence</td>
<td>124</td>
</tr>
<tr>
<td>A4.2. Sequencing templates</td>
<td>125</td>
</tr>
<tr>
<td>Publications</td>
<td>127</td>
</tr>
<tr>
<td>Permissions</td>
<td>128</td>
</tr>
<tr>
<td>References</td>
<td>143</td>
</tr>
</tbody>
</table>
## List of Figures

<table>
<thead>
<tr>
<th>Figure Number</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Single Molecule, Real-Time (SMRT) sequencing</td>
<td>3</td>
</tr>
<tr>
<td>1.2</td>
<td>Loading efficiency of different length DNA templates</td>
<td>6</td>
</tr>
<tr>
<td>1.3</td>
<td>The nanopore zero-mode waveguide</td>
<td>13</td>
</tr>
<tr>
<td>1.4</td>
<td>Schematic of nanopore measurement and analysis</td>
<td>15</td>
</tr>
<tr>
<td>1.5</td>
<td>Fluorescence microscope</td>
<td>17</td>
</tr>
<tr>
<td>2.1</td>
<td>Hafnium oxide nanopores</td>
<td>22</td>
</tr>
<tr>
<td>2.2</td>
<td>Freestanding HfO2 membrane fabrication</td>
<td>24</td>
</tr>
<tr>
<td>2.3</td>
<td>Pore characterization</td>
<td>26</td>
</tr>
<tr>
<td>2.4</td>
<td>Transport of 150 nM 100bp double-stranded DNA (dsDNA) through a d = 3.6 nm HfO2 nanopore</td>
<td>28</td>
</tr>
<tr>
<td>2.5</td>
<td>Transport of 89-mer single-stranded DNA (ssDNA) through HfO2 pores</td>
<td>32</td>
</tr>
<tr>
<td>2.6</td>
<td>Time stability data for a 1.4 nm diameter HfO2 pore</td>
<td>34</td>
</tr>
<tr>
<td>3.1</td>
<td>Protein detection using small solid-state nanopores</td>
<td>41</td>
</tr>
<tr>
<td>3.2</td>
<td>Protein current data</td>
<td>44</td>
</tr>
<tr>
<td>3.3</td>
<td>Protein scatterplots</td>
<td>45</td>
</tr>
<tr>
<td>3.4</td>
<td>Protein capture rates</td>
<td>48</td>
</tr>
<tr>
<td>3.5</td>
<td>Protein dwell-time distributions</td>
<td>49</td>
</tr>
<tr>
<td>3.6</td>
<td>Protein transport parameters</td>
<td>50</td>
</tr>
<tr>
<td>3.7</td>
<td>Transport Scatterplots</td>
<td>53</td>
</tr>
<tr>
<td>3.8</td>
<td>Volumetric measurement of proteins using nanopores</td>
<td>56</td>
</tr>
<tr>
<td>4.1</td>
<td>NZMW device</td>
<td>63</td>
</tr>
<tr>
<td>4.2</td>
<td>Electrical properties of the NZMW</td>
<td>65</td>
</tr>
<tr>
<td>4.3</td>
<td>Reversible positioning of a single DNA–protein complex inside a NZMW</td>
<td>68</td>
</tr>
<tr>
<td>4.4</td>
<td>Immobilization of DNA–protein complexes in an array of NZMWs</td>
<td>70</td>
</tr>
<tr>
<td>4.5</td>
<td>DNA focusing into a NZMW</td>
<td>72</td>
</tr>
<tr>
<td>5.1</td>
<td>Capture and sequencing with NZMWs</td>
<td>78</td>
</tr>
<tr>
<td>5.2</td>
<td>NZMW DNA capture</td>
<td>81</td>
</tr>
<tr>
<td>5.3</td>
<td>NZMW DNA sequencing</td>
<td>84</td>
</tr>
<tr>
<td>A1.1</td>
<td>Thickness and roughness characterization of ALD HfO2</td>
<td>90</td>
</tr>
<tr>
<td>A1.2</td>
<td>EDS characterization of the HfO2 membrane</td>
<td>92</td>
</tr>
<tr>
<td>A1.3</td>
<td>Demonstration of crystallization of the HfO2 membrane</td>
<td>93</td>
</tr>
<tr>
<td>A1.4</td>
<td>Dependence of pore conductance on pore diameter</td>
<td>95</td>
</tr>
<tr>
<td>A1.5</td>
<td>Dwell time analysis</td>
<td>97</td>
</tr>
<tr>
<td>A1.6</td>
<td>Comparison of dsDNA/pore interaction in HfO2 and SiN pores</td>
<td>99</td>
</tr>
</tbody>
</table>
Figure A1.7. Translocations of the 89 nucleotide molecule in α-hemolysin ......................................................... 101
Figure A1.8. ssDNA capture rate analysis .......................................................... 103
Figure A2.1. Protein Data Analysis ........................................................................ 104
Figure A2.2. Frequency content of nanopore current signals .................................. 106
Figure A2.3. Dynamic light scattering measurements ........................................... 107
Figure A2.4. Fitted $td$ distributions .................................................................. 108
Figure A2.5. Estimated fraction of observed events .............................................. 109
Figure A2.6. Continuous Protein Data ................................................................. 110
Figure A2.7. SiN Scatterplots .............................................................................. 111
Figure A2.8. Dwell time distributions for the 4.8 nm SiN nanopore ......................... 112
Figure A2.9. SiN Transport Parameters ............................................................... 113
Figure A2.10. Protein Mixture Data .................................................................... 114
Figure A2.11. Distributions of dwell times for 100 base pair DNA in a 5nm HfO2 pore .......................................................... 115
Figure A3.1. Schematic illustration of the steps of the NZMW fabrication process .......... 119
Figure A3.2. Piranha Photos .............................................................................. 120
Figure A3.3. Time traces of ZMW fluorescence with YOYO-labeled 6000 base pair DNA .... 121
Figure A3.4. NZMW optical cell ....................................................................... 122
Figure A3.5. Agarose gel .................................................................................... 124
Figure A4.1. SiN Photoluminescence .................................................................. 125
Figure A4.2. SMRTbell ....................................................................................... 126
Chapter 1. Introduction and Background

1.1 Single Molecule DNA Sequencing

DNA sequencing has revolutionized medicine and biomedical research, and promises to continue the revolution. Many proponents have celebrated the application of sequencing to diagnostics, but remarkable applications of sequencing lie all over biomedical research, from evolutionary studies of ancient genomes to the cell biological effects of small molecules and protein-nucleic acid interactions.

Single-molecule methods promise to push the DNA sequencing enterprise forward by dramatically increasing the length of DNA molecules that can be sequenced. This value is called the read length. In bulk approaches, which read one base at a time on many copies of the same DNA sample, molecules become out of phase with each other as more bases are sequenced. Phasing limits molecular read length to a few hundred bases. This limit prevents sequencing of long repeat regions, makes construction of de novo genomes difficult, and lowers consensus accuracy for studies that require repetitive reading, such as those in epigenetics.

The growing feasibility of single-molecule sequencing thus offers improved reference genomes and opens up entirely new experimental studies on previously unsequenceable parts of genomes.

In this thesis, we focus on Single Molecule, Real-Time (SMRT) sequencing, a technology developed by Pacific Biosciences. In this approach, one observes a DNA polymerase incorporating fluorescently labeled deoxynucleotide triphosphate (dNTP) analogs into a growing DNA strand while it sits at the base of a sub-wavelength-sized aperture in a 100 nm-thick aluminum film on top of a glass coverslip (see Figure 1.1a). The metal aperture is called a zero-mode waveguide (ZMW). Because the ZMW cross section is too small to support a travelling
mode of visible light, it creates an exponentially decaying field at its base when illuminated.

Figure 1.1b shows a computational solution to Maxwell’s Equations for this system illustrating the ZMW’s light confinement. This evanescent field volume is so small (~zeptoliter) that at analyte concentrations up to µM, there will still only be one or a few molecules in the excitation zone. This makes the ZMW ideal for single molecule fluorescence study of enzymes requiring µM reagent concentrations, such as a DNA polymerase synthesizing DNA from dNTPs.
Figure 1.1. Single Molecule, Real-Time (SMRT) sequencing. (a) Cartoon schematic of the technique. Template DNA is bound to a polymerase and immobilized at the base of a zero-mode waveguide for detection of fluorescent bursts each time a fluorescently labeled dNTP analogs is incorporated by the enzyme. (b) Computational solution to Maxwell’s Equations in the ZMW illustrates the light confinement, with color logarithmically illustrating the electric potential, which achieves single molecule detection at high concentration. This image is reused with permission (see Permissions section).¹ (c) Sample traces (right) show raw SMRT sequencing fluorescence data. The two traces illustrate epigenetics with SMRT technology: when the polymerase sits on a methylated nucleotide (top left), it pauses for longer than for an unmodified base. This image is reused with permission (see Permissions section).² Images are at highest resolution offered by the publishers.
In SMRT sequencing, one monitors a polymerase in a ZMW as it incorporates bases from special fluorescently labeled dNTP analogs. These analogs have a fluorophore attached to an extra-long, five-phosphate chain on each nucleotide. Each base (A, T, C, or G) receives a different, color-coded fluorescent tag. The long phosphate chain accomplishes two things: the extra length keeps the bulky fluorophore away from the active site of the enzyme, enabling near native incorporation kinetics,\(^{10}\) and the label being on the end of the phosphate causes the enzyme to cleave off the dye after each base incorporation. This way one collects fluorescence from the labeled dNTP analog only while the enzyme makes the new phosphodiester bond. After bond formation the dye will diffuse back into the bulk, away from the excitation volume. With this method one observes a single, discrete burst of fluorescence for every single base event with no overlap (see Figure 1.1c).\(^2\) From each ZMW one detects a series of these single-base events. The color of each burst identifies which base was incorporated by the enzyme, enabling parallel, single molecule sequencing.\(^{11}\)

1.2 Entropic Barriers to Single-Molecule Loading

How do the DNA molecules get to the bottom of these ZMWs for sequencing? Traditionally, experimenters have simply relied on diffusion. The ZMWs are first treated with a phosphonic acid that binds to the native oxide layer of the aluminum, preventing molecules sticking to the sides of the ZMWs.\(^{12}\) The silica base of the ZMW is then treated with PEG-biotin. To load DNA samples, one pre-binds them to a DNA polymerase-streptavidin fusion protein, and then incubates them with the chemically treated ZMW device. Many of these DNAs will diffuse into the ZMW volume, and the streptavidin on the polymerase will bind to a biotin at the base of a ZMW. One may now add the proper reagents for sequencing to the bulk solution, and observe fluorescence bursts from active polymerases.
One might intuitively suspect that single molecule approaches also enable lower input concentrations than bulk sequencing. Indeed, single-molecule biophysics experiments have detected molecules in the attomole regime.\textsuperscript{13} However, the two most prominent single molecule techniques, SMRT sequencing and nanopore sequencing, both require input DNA in the 100s of nanograms to microgram range.\textsuperscript{14} This compares unfavorably to the mass of a single human genome, ~ 6 picogram. In each case, this requirements comes from the entropic barrier encountered by DNA molecules as they attempt to enter a nanoscale constriction: in the case of SMRT sequencing, a pre-bound DNA-DNA polymerase complex must enter a cylindrical waveguide of ~100 nm diameter; in nanopore sequencing, a single-stranded DNA must thread into the ~1 nm opening of a nanochannel membrane protein.\textsuperscript{15} This confinement drastically reduces the entropy of the DNAs, making the process thermodynamically unfavorable. This barrier results in highly inefficient DNA loading for both techniques. Figure 1.2 illustrates the practical consequences of this for SMRT sequencing. It shows the results of a simple sequencing experiment with equimolar concentration of ten different sized DNA fragments. The plot’s y-axis indicates the fraction of ZMWs that contained a single molecule of each size. If the molecular loading process were not entropically limited and large DNA molecules did not encounter a barrier to ZMW entry, then the longer molecules would be just as likely to load as the small ones. The plot of Figure 1.2 would be a flat spectrum in this case. Instead, beyond a DNA length of 500 base pairs, the fraction of sequencing ZMWs drops off sharply due to the inefficiency of loading large molecules. The practical consequence of this is that, to get any data, one must use a drastically larger amount of input DNA than is actually sequenced. This necessitates extensive amplification of DNA samples.
Amplification reactions such as polymerase chain reaction (PCR), while traditionally necessary, present several barriers for sequencing. PCR is the most common amplification approach. In this reaction, one first defines a region on a small quantity of sample DNA to be amplified. Two small, complementary single-stranded molecules called primers bookend the region of interest. One amplifies the region of interest by incubating the sample DNA, primers, and necessary synthesis molecules (including DNA polymerase), and cycling the bath from hot to low temperatures and back. On each cycle, the polymerases produce new DNA in the region between the primers, and this product serves as more template for the next cycle.

Figure 1.2. Loading efficiency of different length DNA templates. SMRT sequencing was carried out on ten different lengths of SMRT DNA inserts, which are double-stranded, each at an equimolar ratio. The y-axis shows the fraction of sequencing ZMWs with each sized DNA (x-axis). If the longer DNAs loaded as efficiently as the short DNAs, the graph would be a flat line, with an equal percentage of each insert size. However, a sharp dropoff in percentage above 500 bp is indicative of an entropic barrier for DNA loading, and hence inefficient sequencing for longer inserts.
resulting in exponential growth of the genomic region of interest. PCR can introduce errors, but critically, it does not amplify all sequences equally. Specifically, PCR is biased against regions of extreme base content (~60% or more only GC or AT content). Moreover, degree of bias and error increases with increasing number of PCR cycles. This hinders sequencing of high- or low-GC content sections of the genome. Many medically and scientifically important genomes have extreme base content, including tumor suppressor genes and malaria genomes. These errors and biases are a major reason why we seek to develop approaches for mitigating the necessity and degree of sample amplification.

One of the most powerful and promising capabilities of SMRT sequencing is its ability to sequence native epigenetic modifications. In addition to the normal bases, A, C, G, and T, organisms contain numerous chemical modifications to those bases, including methyl-cytosine and hydroxyl-methyl cytosine. These chemical modifications are called epigenetic modifications. They play a role in gene expression, and development. Traditionally, to sequence methyl-C sites, one must first sequence the sample, then treat it with bisulfite. This chemical reacts with C, converting it to uracil. Methyl-C, however, remains unaffected by the bisulfite. After a second round of sequencing the bisulfite treated genome, any base that remained a C was a site of methylation. SMRT sequencing, however, offers a method of detecting methyl-C, and other modifications, with no chemical treatment. In a SMRT sequencing template, the polymerase may go around and around in a circle, allowing repetitive sequencing of a sample until the polymerase becomes inactive. For each site in the sequence, one examines the fluorescent bursts from every time the polymerase incorporated a new base at that particular spot and collects two data parameters: the duration of fluorescence during incorporation and the length of time the polymerase paused before incorporating the next base in the sequence. Figure 1.1c illustrates the method. Performing principle component analysis on this data easily separates the populations of C, methyl-C, and hydroxymethyl-C. However, amplification processes removes all epigenetic content: an
amplifying enzyme will simply incorporate a C at a site where the original genome had a hydroxymethyl-C, for example. As a result, it has been tremendously difficult to perform epigenetic, single-molecule sequencing with mammalian genomes, where DNA is generally available in such small quantities that it must be amplified to be sequenced.

SMRT sequencing, then, has tremendous single molecule potential, offering exceptionally long read lengths, and gives geneticists a method a directly detecting epigenetic modifications. However, the entropy of the long DNA coil prevents full manifestation of this potential. Longer molecules load inefficiently. Epigenetic information is lost in amplification. All of this demands a way to reduce or eliminate amplification from the SMRT sequencing protocol.

In order to execute SMRT sequencing with no amplification, we must introduce a method to reduce the entropic barrier to DNA loading. Several methods have been proposed and/or developed for molecular loading into ZMWs. The group of Phil Tinnefeld has developed a DNA origami approach. In this study, they create a DNA origami template for molecular loading. It is a rectangular scaffold made out of DNA with a single binding site at the center for a DNA polymerase-streptavidin complex. In principle, one could load an array of ZMWs with these origami templates, and each ZMW would only have the ability to bind a single sequencing template. This approach potentially overcomes the problem of loading more than one template DNA in a ZMW, and pushes the fraction of single-molecule loaded ZMWs beyond the Poisson limit. However, it offers no mechanism to overcome the entropic barrier to DNA loading in the first place. That means that extensive sample amplification would still be needed. Second, the technique would require massive quantities of DNA origami constructs.

A more practical and well-established approach to loading DNA into ZMWs is with magnetic beads. First one prepares SMRT sequencing DNA templates which have a poly-A overhang on their sequencing primers. One then binds them to paramagnetic beads functionalized with poly-T oligos. Next one lowers the beads with a magnet onto the ZMW
surface. They then roll around and when a sequencing template attached to a bead lands inside a ZMW, it will bind to a biotin at the base. As the bead continues to move, it will sever its connection to the DNA bound to the base of the ZMW because the biotin-streptavidin bond is stronger than the poly-A/poly-T bond holding it onto the bead. This loading method accomplishes two things. It lowers the amount of input DNA for sequencing by a factor of six to eight and it prevents the sequencing of extremely short DNA templates, thus increasing one’s potential read lengths. The beads mitigate binding of small templates because the small templates, due to their short length, do not hang far enough of the side of the bead to reach the bottom of the ZMWs and bind. They thus remain bound to the bead.

Even though origami scaffolds enable super-Poissonian loading and beads reduce input DNA by roughly an order of magnitude, neither approach has the potential to increase loading efficiency by the several orders of magnitude necessary to enter the regime of amplification-free sequencing. For this, we need yet a more powerful, active method of localizing large DNA molecules in the confined volume of the ZMW. For this, we turn to solid-state nanopores.

1.3 Nanopores

Nanopores have established themselves as a technique to localize molecules from bulk solution to a location that can be specified with nanometer precision. The principle of nanopore sensing is that when an insulating membrane separates two electrolyte reservoirs with a nanometer-scale channel in the membrane, a transmembrane voltage will drop almost entirely across the nanopore. This creates an electric field at the nanopore. Far from the pore, the field looks just like that due to a point charge, except the field lines point in different directions on each side of the membrane. When a charged molecule, for example a nucleic acid, diffuses into the vicinity of the pore, it will feel the effects of this field and its trajectory will be directed toward the pore. In this way, molecules may be “focused” at the nanopore’s location.
The nanopore field has two separate experimental paradigms: biological or protein pores, which are membrane channels in a lipid bilayer, and solid-state pores, which are nanoscale holes milled in synthetic insulating membranes. Biological pores have included the proteins alpha hemolysin (aHL) and Mycobacterium smegmatis porin A (MspA). These pores offer the advantage that all pores have precisely the same folded geometry, allowing meaningful comparisons of data from different experiments. Additionally, aHL and MspA have, respectively, 5 and 0.5 nm-thick constrictions, which are as small or smaller than those currently achievable with solid-state pores. Solid-state pores offer the advantage that their size and shape may be customized, synthetic membranes are more robust than lipid bilayers, and they may be more easily incorporated into nanofabricated devices.

Nearly every nanopore study has focused on the translocation of molecules through the pore. A translocation event is when a molecule passes from one side of the membrane to the other by going through the pore. To measure this one first monitors the ionic current through a nanopore in the presence of an electrolyte solution. Depending on salt concentration, applied voltage, and pore size, this current will usually be in the 100s of picoamp to nanoamp range. This current is called the open-pore current. When a molecule enters the pore, it excludes ions from the pore, resulting in a drop in pore current. After the molecule has passed all the way through the pore, the current will return to the open-pore level. Experimenters collect many of these current spikes, each corresponding to one molecule. The number of molecules captured by the pore in a given period of time follows a Poisson distribution. As a result, the times between translocation events are exponentially distributed.

The capture rate is the inverse of the average time between events. Intuitively, it is the rate, under given conditions, at which a nanopore captures a molecule. This rate depends on voltage. Specifically, experimenters generally observe two different regimes of capture rate as a function of voltage, exponential and linear. The exponential regime arises when an entropic barrier dominates the translocation process. This happens, for example, when a polymer like
DNA must translocate a pore whose diameter is similar to that of the local polymer diameter. In this case, the end of the polymer must find the pore entrance, and the chain must partially linearize as it translocates (see Figures 2.4d and 2.5c). This corresponds to a decrease in chain entropy as it transits the nanopore. The linear regime arises when drift dominates the translocation process, for example at very high voltages or for polymers that do not require significant linearization to cross the pore (see Figure 3.3a).

Analyzing the depth, duration, frequency, and even noise spectrum of molecular translocation data has enabled numerous single-molecule measurements.\textsuperscript{24b} We illustrate this analysis process in Figure 1.4b. Experiments have probed the force necessary to dissociate binding proteins from DNA\textsuperscript{31} and the kinetics of unzipping double-stranded DNA,\textsuperscript{32} by comparing translocation/unzipping time scales; they have differentiated DNAs from microRNAs\textsuperscript{33} and DNA homopolymers from one another\textsuperscript{28} via analysis of current blockage. Nanopores have also emerged as useful tools for unlabeled, single protein detection. Experiments on protein translocation have examined, for example, protein unfolding,\textsuperscript{34} protein charge,\textsuperscript{35} protein size,\textsuperscript{36} and the fast time scales of protein translocation.\textsuperscript{37}

Importantly, by mediating DNA translocation speed with a DNA processing enzyme, often φ29 DNA polymerase, the groups of Mark Akeson and Jens Gundlach have demonstrated the utility of protein nanopores for DNA sequencing.\textsuperscript{15} In this technique, the enzyme moves along the DNA one base at a time, discretely shuttling the DNA through the nanopore. The nanopore conductance depends on the sequence of bases in the constriction. Each time the enzyme moves one base along the DNA, the current jumps to a different discrete state. By studying known sequences, one may correlate these different discrete current levels with DNA sequence in the pore.

All of the above studies have focused on biomolecules passing through nanopores. However, for the purposes of localizing DNAs in ZMWs for sequencing, we need only the
nanopore’s ability to focus molecules at a particular spot. This process was analyzed in detail in a 2010 publication from Amit Meller’s group.\textsuperscript{13a} In this paper, the authors introduce the idea of a nanopore capture radius. Because the nanopore’s resistance is orders of magnitude greater than the conducting solutions on either side of the membrane, almost all of the voltage drops across the nanopore, resulting a relatively strong electric field in the pore. However, the field decays away from the pore with a Coulombic $1/r^2$ dependence, much like a point charge. The capture radius is the distance from the nanopore at which a molecule’s motion becomes dominated by electrophoretic drift due to the nanopore field instead of diffusive motion in bulk solution.

1.4 The Nanopore-Zero-Mode Waveguide (NZMW)

In the experiments presented in this thesis, we take advantage of the nanopore’s ability to localize DNA molecules in a nanoscale volume to actively loading DNAs into the sequencing volume of a ZMW. We present a hybrid nanopore-ZMW (NZMW) device. Instead of sitting atop a glass coverslip, the ZMW sits on a ~30-thick insulating membrane with a nanopore at its base. Figure 1.3a schematically illustrates the concept. In this case, when we bias the membrane, the nanopore creates an electric field, which penetrates beyond the ZMW opening (see Figure 1.3b). If a DNA diffuses into the vicinity of the ZMW opening, it will feel this field, and be pulled into the ZMW. We thus enable large DNAs to cross the thermodynamic barrier they encounter when attempting to enter the ZMW.
1.5 Instrumentation

The experimental setup used to collect the data in this thesis combines two common biophysics instruments: a patch clamp amplifier for electrical measurements and a fluorescence microscope for optical detection.

We measure nanopore current and apply transmembrane bias with the same instrument, a patch clamp amplifier. The key to patch clamp amplifier measurement of small nanopore currents is clamping the transmembrane voltage to a prescribed value with feedback electronics. This voltage is offset by any current flowing through the nanopore. The current the electrodes must inject into the solution to maintain the clamped voltage is equal and opposite to the current flowing through the nanopore, allowing measurement of the miniscule pore current. This explanation simplifies the patch clamp system, but communicates the key ideas.
Figure 1.4a schematically illustrates the system used in the experiments presented in this thesis. AgCl electrodes contact experimental solutions containing KCl. The electrochemistry at the interface of the AgCl electrodes and the KCl solution allows flow of current and application of transmembrane voltage. As current flows between the electrodes and solution, one of the electrodes slowly converts to Ag in a chemical reaction. The terminal at which the transmembrane voltage is applied is referred to as the headstage. It connects to the amplifier itself. The output from the amplifier feeds into a computer via a DAQ card, which also communicates external commands (apply voltage, change gain, etc) to the amplifier. Custom software (National Instruments LabView and Mathworks MATLAB) controls the amplifier and collects data. Depending on the experiment, we used two different amplifiers: the Molecular Devices Axopatch 200B and the Chimera Instruments VC100. The VC100 was developed specifically for nanopore experiments. By minimizing parasitic capacitance, the makers of the VC100 reduced the electrical noise enough to expand sampling frequency to 4 MHz from the Axopatch’s 250 kHz. With this high bandwidth, one may measure, in principle, translocation events down to 1 μs. Chapter 3 explores the application of these fast electronics to detection of native, folded proteins translocating solid-state nanopores.
We constructed an epi-fluorescence microscope with three lines of laser illumination (488 nm, 532 nm, and 650 nm) and an EMCCD camera for optical measurements. Figure 1.5a depicts the light path. We combined the three lasers with dichroic mirrors and focused them to a coincident spot on the face of an optical fiber. An output lens then feeds the lasers into the back port of an inverted microscope. Figure 1.5c shows a photo. The tube lens focuses the laser light onto the back aperture of a water immersion, 60X, 1.2 NA objective, causing it to emerge collimated onto the sample, which is enclosed in a Faraday Cage for electrical measurement. The same objective then focuses the fluorescence image through a filter set onto a confocal

Figure 1.4. Schematic of nanopore measurement and analysis. (a) Ag/AgCl electrodes contact a chloride solution (usually 0.1 – 2 M) on either side of the nanopore membrane. The patch clamp amplifier clamps the transmembrane voltage and measures nanopore current by observing the magnitude of current electrodes must inject to maintain the voltage clamp. Amplifier output is read into a computer via a DAQ card, which also enables external software control. (b) The three most commonly analyzed parameters of nanopore translocation data are current blockage (ΔI), the magnitude of pore current excluded by a molecule, dwell time (t_d), the length of time a molecule resides in the pore, and inter-event time (δt), the time between the beginnings of successive events.
pinhole array. This array matches the pitch of our ZMW array, rejecting off-axis background for each ZMW. We show the multiband fluorescence emission filter window along with the spectrum of the four SMRT sequencing analogs in Figure 1.5b. Prior to detection with a camera, we reimaged the ZMW array through a prism. The prism deflects light by a different angle depending on its wavelength. The final lens is one focal length removed from both the prism and the camera. A lens projects a Fourier transform of an image one focal length behind to a spot one focal length in front of it. Consequently, the lens converts the wavelength-dependent angle generated by the prism to a wavelength dependent position on the camera plane: the prism spreads the light from each ZMW into an emission spectrum. One may then detect the spectrum of emission light from each ZMW. In SMRT sequencing, we identify bases by matching the spectrum of each single base incorporation photon burst to the appropriate dNTP analog spectrum.
Figure 1.5. Fluorescence microscope. (a) Optical path: three lasers are focused onto the face of an optical fiber and refocused onto the back aperture of an Olympus 60X, 1.2 NA, water immersion objective, such that laser light emerges collimated onto the sample. Fluorescence from ZMWs is imaged through a confocal pinhole array, and through a prism in order to spread fluorescence over ~20 pixels on a CCD (Andor Ixon) in order to tell apart different emission wavelengths for sequencing. For sequencing, four different dyes are used for the different dNTPs, whose spectra are given in (b). The spectra have good overlap with our multiband filter (gray regions). (c) In a photo of the detection part of the setup, CCD, prism, pinhole array, and microscope are
The prism system was only present for the data in Chapter 5 of this thesis, in which we perform sequencing. For all other fluorescence data, in which we used one color at once, we simply imaged the ZMW array onto the EMCCD without a prism.
Chapter 2. DNA Transport through Nanopores in Hafnium Oxide Membranes

Abstract

We present a study of double- and single-stranded DNA transport through nanopores fabricated in ultra-thin (2-7 nm) freestanding hafnium oxide (HfO$_2$) membranes. The high chemical stability of ultrathin HfO$_2$ enables long-lived experiments with <2 nm diameter pores, which last several hours, and in which we observe >50,000 DNA translocations with no detectable pore expansion. Mean DNA velocities are slower than velocities through comparable silicon nitride pores, providing evidence that HfO$_2$ nanopores have favorable physicochemical interactions with nucleic acids that can be leveraged to slow down DNA in a nanopore.

2.1 Introduction

Natural and synthetic nanopores are increasingly popular tools for characterizing various biomolecules and their complexes at the single-molecule level. Pioneered by the demonstration of voltage-driven, single-file transport of DNA molecules through a lipid-embedded α-hemolysin protein channel, nanopore research has been fueled by new potential applications for genomic analysis and DNA sequencing. Nanopores are attractive apparatuses for mapping and quantifying interactions within biomolecular complexes. Fabrication of synthetic nanopores by irradiation using electron-beams, ion-beams, and He-beams has gained popularity due to a more flexible pore geometry that accommodates various-sized biopolymers, as well as the intriguing potential to explore various biopolymer/materials interfaces.

A significant hurdle for nanopore-based analysis of DNA, RNA, and protein molecules has been that the reported translocation speeds are too fast relative to the speed at which current is detected using conventional patch-clamp amplifiers. Various systematic explorations of biomolecular transport through synthetic nanopores suggest that biopolymer detection requires a combination of nanopores with optimal geometry (diameter and thickness), surface properties and improved temporal resolutions. While nanopores in silicon oxide and silicon nitride (SiN) membranes with various geometries have been thoroughly studied, their physical stability is compromised by unavoidable chemical damage during and after pore fabrication. This material instability has set a practical limit on membrane thicknesses that can be used for SiN membranes (~5-10 nm). This limitation compromises the durability and performance of ultra-thin and ultra-small solid-state nanopores, which invites the exploration of biomolecular transport through other membrane materials, for example, aluminum oxide, graphene, boron nitride, and DNA origami. While each of these alternative materials presents unique advantages, none have the combined benefits of hydrophilicity, low-leakage,
chemical resistance to strong cleaning acids, robust mechanical stability, and a simple means of fabrication.

Hafnium oxide (HfO$_2$) is a wide band gap, high-dielectric insulator with excellent chemical resistance and comparable strength to SiN. While SiN is as strong as HfO$_2$, it is plagued by a problem of stability at the nanoscale: the oxide of silicon is chemically favored over its nitride. This tendency of nitrides to oxidize is exemplified by the standard enthalpy of formations of Si$_3$N$_4$ (-198 kcal/mol), SiO$_2$ (-217 kcal/mol), HfN (88.2 kcal/mol), and HfO$_2$ (-266 kcal/mol). Therefore, while SiN is normally a robust material, in an oxygen-rich environment the nitride surface is an evolving mixture of nitrogen and oxygen, the proportion of which can vary during nanopore fabrication and following cleaning using oxygen-rich agents (e.g., O$_2$ plasma and hot piranha solution). In contrast, the chemical form of HfO$_2$ is stable, which can improve reliability and reproducibility during nanopore experiments, and in principle offer a well-regulated interaction of the pore walls with biomolecules. Finally, the isoelectric point of ~7 for HfO$_2$ renders its surface near-neutral under physiological pH, which suggests compatibility of solid-state nanopores with studying transport of negatively-charged biomolecules such as nucleic acids.

In this chapter we investigate single-stranded and double-stranded DNA transport through nanopores in ultrathin HfO$_2$ membranes at high temporal resolution. Figure 2.1a shows the scheme of our nanopore setup, as well as typical traces during experiments with (b) double-stranded and (c) single-stranded DNA. First, we present the fabrication details of ultrathin HfO$_2$ membranes and nanopores in such membranes. Next, we show that transport speeds of single-stranded and double-stranded DNA are slower than for SiN pores of equivalent geometries, and we argue that this slowing down is due to coordinative interaction of the DNA backbone phosphates with the HfO$_2$ surface. Finally, we show for the first time that HfO$_2$ pores with diameters as small as 1.4 nm are stable in size for several hours of continuous DNA translocation experiments, during which an estimated 50,000 DNA molecules are “flossed” through the pore.
without any detectable erosion of the pore walls. These results suggest that HfO$_2$ is a superior material to SiN for nanopore biosensors.

Figure 2.1. Hafnium oxide nanopores. (a) Cartoon schematic of the experiment. A sample of DNA is placed on the negatively-charged electrode side and ion current through the pore is monitored. Electrophoretic transport of a DNA molecule produces a single spike. Inset shows a transmission electron microscope (TEM) image of a 3.6 nm diameter HfO$_2$ nanopore (scale bar = 2 nm). (b) Continuous 3-second current traces of 100 bp dsDNA (top) and 89-mer ssDNA (bottom) translocating through HfO$_2$ pores at respective biases of $V = +175$ mV and $+150$ mV (pore diameters $d$ indicated in Figure).

2.2 HfO$_2$ Nanopore Fabrication
We present a three-step fabrication process for HfO$_2$ pores in Figure 2.2a. First, atomic-layer deposition (ALD) was used to deposit a 4.5 nm thickness of HfO$_2$ film onto a free-standing low-stress SiN window (see Appendix 1). Next, electron-beam resist was spun on the membrane, and a $< 2 \mu m$ square portion of the SiN window was irradiated using e-beam lithography and subsequently developed, after which the entire thickness of the exposed SiN was etched using an SF$_6$ reactive ion etch plasma. We have found that RIE overetching of the SiN layer did not remove the HfO$_2$ film. The membrane’s elemental composition was investigated using energy dispersive x-ray spectroscopy (EDS) with a transmission electron microscope (TEM). Figure 2.2b shows a dark-field scanning TEM (STEM) image in which stark contrast between the thick SiN support and the freestanding HfO$_2$ membrane is visible. In addition, an atomic force microscope (AFM) scan of the same area is shown, in which the removed thickness of the SiN layer is confirmed. Hafnium and oxygen were present throughout the image in similar amounts, while the signals for silicon and nitrogen were virtually absent in the etched area. By combining a map of the integrated EDS spectra (see Appendix 1) with AFM topography data, a reconstructed thickness map of the membrane layers is presented in Figure 2.2c. We note that noise of the signal in the height map arises from instrumental noise and actual roughness of the deposited SiN and HfO$_2$ films. Finally, since both the ALD and lithography steps are scalable to a whole wafer, these steps were carried out in parallel to produce a large number of HfO$_2$ membranes for experiments.
Figure 2.2. Freestanding HfO2 membrane fabrication. (a) 1. Atomic-layer deposition is used to deposit a 3-8 nm-thick HfO2 layer onto the trench side of a freestanding silicon nitride (SiN) window. 2. Reactive-ion etching of a pre-defined window to expose the freestanding HfO2. (b) Atomic force microscopy (AFM) topograph (left) and dark-field scanning transmission electron micrograph (right) of a freestanding HfO2 region. Dashed red line represents line scan that confirms the 50 nm etch step height. (c) Energy dispersive spectroscopy (EDS)-based thickness map of SiN and HfO2 (thickness estimated from AFM).
The third and final fabrication step was nanopore drilling using a transmission electron microscope (TEM). Hard irradiation \( (2.5 \times 10^8 \text{ e/nm}^2) \) of a ~2×2 nm\(^2\) region of the membrane resulted in slow formation of a nanopore, the kinetics of which are ~10x slower than for similar thickness silicon nitride membranes.\(^{63}\) In contrast to SiN, soft electron-beam irradiation of freestanding HfO\(_2\) using 200 kV electrons \( (10^6 \text{ e/nm}^2) \) for 40-60 seconds leads to a phase transition from an amorphous to a polycrystalline state (see Appendix 1), as previously observed for Al\(_2\)O\(_3\) nanopores.\(^{52}\) While we were able to produce pores in these crystallized HfO\(_2\) nanodomains, their ionic conductance was always larger than anticipated. We hypothesize that these pores are unstable as a result of mechanical failure of the crystalline domain due to strain mismatch with the amorphous membrane.

Example bright-field TEM images of nanopores in the diameter range of 1.4 – 6.5 nm are shown in Figure 2.3a. Contrasting patches in the image correspond to thickness variations in the semi-crystallized HfO\(_2\) film, which are clearly induced by extended e-beam irradiation. Following nanopore fabrication, the devices were cleaned in hot piranha and then rinsed copiously in hot de-ionized water, and after vacuum drying they were immediately assembled in a custom PTFE cell. Current-voltage curves were used to measure the pore conductance, as shown in Figure 2.3b for a 5.9 nm and a 2.0 nm pores fabricated in 4.5-nm-thick HfO\(_2\) membranes. Linearity of the current-voltage curves was consistent with a symmetric and/or weakly charged pore surface. For a given batch of HfO\(_2\) membranes we found a good agreement between pore conductance and diameter, although the pore thickness varied by as much as 50%, as determined by sizing from DNA translocation experiments (see Appendix 1). The pores exhibited low noise, as shown in Figure 2.3c by the power spectral density (PSD) and integrated current noise for a 4.0 nm diameter pore at an applied voltage of 250 mV. By painting most of the chip surface with an elastomer gasket\(^{64}\) we were able to reduce the capacitance of our chips to the range 60-150 pF, which is sufficient to enable measurements at wide signal bandwidths (≥200 kHz).
2.3 Double-stranded DNA transport

We first characterize the voltage-driven transport of double-stranded DNA (dsDNA) through our HfO$_2$ pores. While a good correspondence is found between the TEM-measured pore size and the observed conductance, quoted pore sizes throughout the chapter were independently...
assessed from the blocked current level during DNA translocation experiments. In Figure 2.4a, a representative two-second current trace of a 3.6 nm diameter pore at $V = 100$ mV following the addition of 150 nM of 100 bp dsDNA to the negatively biased cis chamber. For each experiment, >60 seconds of data similar to shown in Figure 2.4a was analyzed offline using OpenNanopore, an open source translocation data analysis package from the Radenovic Lab at EPFL. OpenNanopore fits all detected single-level spikes from the trace with rectangular pulses, as illustrated in Figure 2.4b (multi-level events were rare and as such they were ignored). The duration of the pulse corresponds to the dwell time ($t_d$), while the amount of reduction in the baseline current from the open-pore level ($I_o$) is referred to as $\Delta I$. The molecule’s arrival time, $\delta t$, is the wait time between consecutive event beginnings.
Figure 2.4. Transport of 150 nM 100bp double-stranded DNA (dsDNA) through a $d = 3.6$ nm HfO2 nanopore. (a) Continuous two-second current trace at $V = 100$ mV. (b) Representative concatenated events following analysis identifying mean current amplitude ($\Delta I$) and dwell time ($t_d$) (c) Histograms of $\Delta I$ at voltages in the range $V = 100 – 250$ mV, showing a regular increase in $\Delta I$ with voltage. Inset shows the fractional blockage, $\Delta I/I_0$, which is found to be independent of voltage. (d) Distributions of inter-event arrival times ($\delta t$) across voltages, with exponential fits, from which capture rates ($R_c$) are extracted. (e) Scatterplots of $\Delta I$ vs. $t_d$ for three voltages. The decrease in spread of $t_d$ with increasing voltages exemplifies the transition from diffusion-dominated to drift-dominated transport. (f) Peak $t_d$ (left axis) and $R_c$ (right axis) values as a function of voltage (see Appendix 1), showing exponential dependence for both parameters (see text).
Figure 2.4c plots histograms of ΔI for each experimental voltage. We see that ΔI increases linearly with voltage (as does I₀, not shown). However, as shown in the inset to the figure, the fractional blockade \(\langle ΔI/I₀\rangle\) is independent of voltage in the range 100-250 mV. As mentioned above, the pore diameter can be characterized based on the fractional blockade value, assuming a dsDNA cross-sectional diameter of 2.2 nm.\(^{33}\) Assuming that the current blocked is entirely due to a fractional excluded volume of DNA from the pore, we use measured \(I₀\) and ΔI values to determine the pore diameter, \(d\), and its effective height, \(h_{\text{eff}}\):\(^{33, 46c}\)

\[
\text{Eq. 2.1} \quad I₀ = V \sigma \left( \frac{4h_{\text{eff}}}{\pi d^2} + \frac{1}{d} \right)^{-1}
\]

\[
\text{Eq. 2.2} \quad \Delta I = V \sigma \left( \frac{4h_{\text{eff}}}{\pi (2.2 \text{ nm})^2} + \frac{1}{2.2 \text{ nm}} \right)^{-1}
\]

where \(V\) is the applied voltage and \(\sigma = 0.096 \text{ S/cm}\) is the measured specific conductance of the buffer at 25°C. For the pore in the experiment shown in Figure 2.4, we find \(h_{\text{eff}} = 7 \text{ nm}\) (the highest pore thickness we observed) and \(d = 3.6 \text{ nm}\). Further, we determine the capture rate \(R_C\) from each experiment by fitting the arrival time distributions to a first arrival time process \(P(t) = A \cdot \exp(-R_Ct)\), as seen in Figure 2.4d.\(^{29}\) The inverse time constant of each fit corresponds to the event rate. In small pores, DNA capture rates are limited by an energetic penalty of DNA confinement within the pore, and the event rate is expected to depend exponentially on voltage,\(^{29}\) whereas in large pores capture is limited by arrival time to the pore mouth (Smoluchowski limit), in which case capture rate is linearly dependent on voltage.\(^{66}\) We indeed observe exponential capture rate dependence with voltage (plotted on the left axis of Figure 2.4f), as previously observed for similar-sized pores.\(^{46b}\) We find that the capture rates in our HfO₂ pores are higher than in prior studies: using a 25-nm-thick silicon nitride nanopore, a previous study found a capture rate for 400 bp DNA of 0.35 s\(^{-1}\)nM\(^{-1}\), whereas in our case obtaining this capture rate was higher for 100 bp DNA by a factor of \(~4\) at the same applied voltage.\(^{13a}\) The increased observed capture rates reduce the minimum practical voltage for experiments, which allows measurements of DNA transport through small pores at lower voltages.
Scatter plots of $\Delta I$ vs. $t_d$ for selected voltages in the range 100 - 250 mV are displayed in Figure 2.4e. Upon increasing the applied voltage, a noticeable decrease of the spread in $t_d$ distributions is observed, coupled to an increase in $\Delta I$ spread. We attribute this reduction in the spread of $t_d$ to a transition from diffusion-dominated transport to drift-dominated transport: at low bias values (100-150 mV), DNA transport is hindered by interactions with the pore walls and hydrodynamic interactions that present a barrier for transport, whereas at high bias values (200 – 250 mV) electrophoretic forces dominate DNA transport. In order to obtain the most likely dwell times we have plotted log-normal distributions (see Appendix 1) and extracted the peak positions, which are plotted in Figure 2.4f (left y-axis). As seen in the plot, the characteristic dwell times in the voltage range 100 – 250 mV correspond to average DNA velocities of 5.5 – 1 µs/bp, respectively. The minimum characteristic velocity we obtained $V = 100$ mV, 181 bp/ms) compares favorably with other works, as seen in a recent compilation by Venkatesan et al. Comparison of our measured DNA average velocity with a similar-diameter SiN pore shows a higher dependence on voltage for HfO$_2$, which may be due to the stronger interactions of DNA with the HfO$_2$ pore walls (see Appendix 1). Although the exact mechanism of this interaction is not clear, prior studies of a series of M(IV) oxides such as ZrO$_2$, TiO$_2$, and HfO$_2$ indicate a reasonable affinity towards phosphate groups, which may mediate DNA slowing by increasing frictional forces with the pore walls during the translocation process.

2.4 Single-stranded DNA transport

Nanopore detection of single-stranded DNA (ssDNA) has been studied extensively as a potential technology for DNA sequencing. To show that HfO$_2$ nanopores are compatible with single-stranded DNA experiments we evaluated the transport kinetics of an 89-mer single-stranded DNA molecule through two different HfO$_2$ pores with diameters of 1.4 and 1.7 nm. Representative one-second snapshots of the traces collected for both pores at different
voltages are shown in Figure 2.5a. The traces show events with very deep blockages for both pores. Specifically, for the 1.4 nm pore the mean fractional blockade is 83%, while for the 1.7 nm pore the mean fractional blockade is 70%. These fractional blockades are close in magnitude to α-hemolysin (αHL), for which we have independently measured a $\Delta I/I_o$ value of 80% (see Appendix 1). It is noteworthy to remark that while αHL nanopores do not exhibit efficient capture from the β-barrel (‘trans’) side of the membrane without a vestibule, capture of molecules into both HfO$_2$ pore sizes was at least as efficient as capture into the vestibule (‘cis’) side of αHL (see Appendix 1). (~20 s$^{-1}$µm$^{-1}$ for HfO$_2$ @ 200 mV vs. ~5 s$^{-1}$µm$^{-1}$ for αHL @ 120 mV). However, it would be more accurate to compare the normalized capture rates under identical applied bias values.
Figure 2.5. Transport of 89-mer single-stranded DNA (ssDNA) through HfO2 pores. (a) Continuous current traces at various voltages (white text). Fractional blockades are observed, $\Delta I/I_0 = 83\%$ for $d = 1.4$ nm and $70\%$ for $d = 1.7$ nm pore. (b) Scatterplots of $\Delta I$ vs. $t_d$ show the impact of a 0.3 nm pore diameter reduction on the spread of dwell times. Strong dependence is evidence of interactions between the ssDNA molecule and the HfO2 pore. (c) Normalized capture rates as a function of voltage show exponential dependence of $R_c$ on voltage, owing to an energetic barrier for capture into the pores (error bars smaller than markers). (d) Orders of magnitude difference in mean dwell times for 1.7 nm pore (left axis) and 1.4 nm pore (right axis) evidence strong pore-molecule interactions (see Appendix 1), as well as the super-exponential dependence of dwell times on voltage for the 1.4 nm pore.
Similar to dsDNA, a ssDNA molecule experiences strong interactions with the HfO₂ pore walls which causes an enormous distribution of dwell times. In Figure 2.5b, scatter plots of fractional current blockades vs. dwell times are shown for the same 89-mer ssDNA sample transported through 1.7 nm and 1.4 nm diameter pores. It is striking that a ∼0.3 nm reduction in pore diameter increases the most-likely dwell times by ∼30 and the spread in dwell times by ∼100. This large variance in dwell time can be attributed to strong interactions between the ssDNA molecule and the walls of the HfO₂ pore, which may cause stick-slip motion of the ssDNA through the pore. In addition to phosphate backbone interactions, we cannot rule out interactions of the HfO₂ surface with specific nucleobases. While not dynamically controllable as in other proposed devices, this enhanced interaction of DNA with HfO₂ pores can be useful for slowing DNA motion through the pore. Upon fitting the dwell time data to log-normal distributions (see Appendix 1), the dependence of the most-likely dwell time (\(t_d\)) on voltage is shown in Figure 2.5d. In the figure, one-sided error bars represent the long-tail variance of the distributions. While exponential dependence is observed for the 1.7 nm diameter pore (left axis), we find super-exponential behavior of \(t_d\) for the 1.4 nm pore (right axis). Recent Langevin dynamics simulations of a strongly interacting pore find a super-exponential relationship between driving force and dwell time, which produce remarkably similar behavior to our experiments. Though further investigation is required, two non-exclusive mechanisms can explain this behavior: 1) the pore we have used is too small to allow unhindered passage of ssDNA nucleobases, resulting in steric-dominated stick-slip motion through the pore, and 2) chemical interactions between ssDNA and the HfO₂ surface are responsible for this observed friction.

Finally, in the regime where the pore diameter is close to the DNA cross-section, exceedingly small changes in pore dimensions can critically impact the transport kinetics of molecules through the pore. Since the properties of synthetic nanopores are more susceptible to change over the course of an experiment than those of protein pores, we investigated the abrasion-resistance of a sub-2 nm HfO₂ pore during a multi-hour experiment. In Figure 2.6, we
plot the fractional current blockade ($\Delta I/I_o$) as a function of experiment time for a 1.4 nm diameter pore at $V = 350$ mV (closed circles, left axis). The pore conductance $\Delta G$ over time is also plotted (open circles, right axis). During this experiment, >50,000 ssDNA molecules have been passed through the pore, and yet the unchanged $\Delta I/I_o$ indicates that the pore diameter remains constant; the minor <10% conductance change is merely the result of water evaporation from the buffer. Given the strong interactions of ssDNA with the 1.4 nm pore, this result exemplifies the strong chemical and mechanical stability of the HfO$_2$ membrane.

![Diagram](image)

Figure 2.6. Time stability data for a 1.4 nm diameter HfO$_2$ pore. Plot shows the fractional current blockade $\Delta I/I_o$ (left axis) as a function of the ~2.5 hours experiment time. The pore conductance as a function of time is shown on the right axis. Insets show current traces at different times of the experiment ($V = 350$ mV). Top axis shows the estimated number of molecules passed through the pore.

2.5 Discussion

In conclusion, we have demonstrated that HfO$_2$ is a viable alternative to SiN for solid-state nanopore sensors. Fabrication of a wafer-full of HfO$_2$ membranes and nanopore fabrication in
these membranes using a TEM is straightforward. HfO\textsubscript{2} pores are hydrophilic, stable, and they have nearly neutral surface charge in physiological conditions. By studying voltage-driven transport of DNA molecules, we have shown that 3.6 nm diameter HfO\textsubscript{2} pores efficiently admit dsDNA molecules at lower bias voltages than SiN pores, while transport is slower than for SiN pores of similar geometry. Likewise, with 1.4 nm diameter pores we have measured much longer and more spread out ssDNA transport time statistics than with a 1.7 nm diameter pore, suggesting very strong interactions between the material and the nucleic acid molecules. The combined experiments point to interaction between the DNA backbone and HfO\textsubscript{2}, which we posit comes from phosphate/ HfO\textsubscript{2} interactions. Finally, the pores exhibit a remarkable stability over time, which enables the fabrication of small pores in thin membranes that are usable for hours of continuous measurements. Further studies of the interactions between DNA and HfO\textsubscript{2} in the context of voltage-driven or enzyme-driven DNA translocation\textsuperscript{[15a]} may improve the detection of DNA polymers through solid-state nanopores,\textsuperscript{[28]} enable a more controlled transport through nanopores equipped with transverse electrodes,\textsuperscript{[73]} enable high-resolution studies of DNA/protein interactions via rupturing forces,\textsuperscript{[72, 74]} be used in conjunction with small graphene pores for a further reduction of DNA velocity,\textsuperscript{[46d, 75]} as well as for other nanopore-based applications.

2.6 Materials and Methods

Substrates for nanopore fabrication were 5 x 5 mm\textsuperscript{2} Si chips with a 50-nm-thick SiN film deposited on a 2.5-µm-thick thermal SiO\textsubscript{2} layer, which helps to reduce electrical noise. HfO\textsubscript{2} films were deposited at 150°C using a GEMSTAR benchtop ALD system (Arradiance), with tetrakis(ethylmethylamino)hafnium and H\textsubscript{2}O used as a precursor and oxidizer, respectively.\textsuperscript{[62]} AFM- and ellipsometry-calibrated thicknesses of SiN were etched in a Technics Micro-RIE Series 800 etcher using sulfur hexafluoride (SF\textsubscript{6}) at 300 mTorr and 150 W. SiN was protected with a 950 PMMA etch mask, and a small region was exposed using Nabity NPGS e-beam writing software
on a Hitachi S-4800 scanning electron microscope. Exposed PMMA was developed with 3:1 isopropyl alcohol and methyl isobutylketone, and following SiN thinning PMMA was removed using acetone. Nanopores were fabricated and imaged at Northeastern University using a JEOL 2010FEG transmission electron microscope at 200 kV.

Nanopore chips were cleaned using hot piranha followed by hot water. After vacuum drying, the chips were mounted in a PTFE cell using a quick-curing elastomer gasket (Smooth-On Ecoflex 5). Cell chambers were filled with 1 M KCl buffer solution (pH 8.3, 10 mM Tris, 1 mM EDTA), and Ag/AgCl electrodes were inserted into each chamber. Current data was collected at 4 MS/s and digitally low-pass filtered using a Chimera Instruments VC100 amplifier system unless otherwise indicated. Before addition of a DNA sample, a current-voltage curve and a several second current trace at constant bias were collected to ensure a steady open pore current. Sample molecules were then thoroughly mixed with the buffer in the chamber using a pipette to achieve a final desired concentration. Molecules and concentrations were as follows: for ssDNA experiments, a 30-100 nM 89-mer solution was used (see Appendix 1). For dsDNA experiments, a 150 nM solution of 100 bp Fermentas NoLimits DNA fragment was used (Thermo Scientific).

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Chapter 3. High-Bandwidth Protein Analysis Using Solid-State Nanopores

Abstract

High-bandwidth measurements of the ion current through hafnium oxide and silicon nitride nanopores allow the analysis of sub-30 kD protein molecules with unprecedented time resolution and detection efficiency. Measured capture rates suggest that at moderate transmembrane bias values, a substantial fraction of protein translocation events are detected. Our 2.5 µs dwell time resolution enables translocation time distributions to be fit to a first-passage time distribution derived from a 1-D diffusion-drift model. The fits yield drift velocities that scale linearly with voltage, consistent with an electrophoretic process. Further, protein diffusion constants (D) are lower than the bulk diffusion constants (D₀) by a factor of ~50, and are voltage independent in the regime tested. We reason that deviations of D from D₀ are a result of confinement-driven pore/protein interactions, previously observed in porous systems. A straightforward Kramers model for this inhibited diffusion points to 9-12 kJ/mol interactions of the proteins with the nanopore. Reduction of µ and D are found to be material-dependent. Comparison of current blockage levels of each protein yields volumetric information for the two proteins in good agreement with dynamic light scattering measurements. Finally, detection of a protein-protein complex is achieved.

3.1 Introduction

Nanopore sensors have recently emerged as popular tools for structural analysis of proteins and their complexes at the single-molecule level. While a wide palette of established techniques is available for protein analysis, many analytical techniques require chemical modifications that can alter a protein’s structure and/or properties. In contrast, chemical modification is not a prerequisite for nanopore-based analysis. In this method, individual proteins and their complexes are analyzed in solution by monitoring stochastic blockades in the ionic current signal through the pore. Stochastic nanopore sensing has been developed by Bayley et al. for measurements of the interaction kinetics and thermodynamics of protein complexes using alpha-hemolysin pores. Stochastic sensing of proteins has also been demonstrated by Martin et al. using synthetic pores. Other studies using biological and solid-state nanopores have probed polypeptide structure and kinetics, protein folding/unfolding, protein charge, protein size, protein sequence, prion structure, and protein-aptamer interactions. These studies clearly suggest that nanopores are a valuable tool for protein studies at single-molecule levels.

Numerous protein detection studies using synthetic nanopores in the diameter range 8-55 nm have been reported recently. A general problem has precipitated from these works: protein molecules translocate far too quickly to be detected. A recent survey of protein translocation through synthetic nanopores by Plesa, et al. has concluded that mean protein translocation times (~1 µs) are faster than detection time limits, which leaves the vast majority of protein translocation events undetected. In order to provide a rationale for these missed
events, Plesa and co-workers simulated protein translocation time distributions derived from 1-D drift-diffusion models that were previously used in DNA and protein translocation studies.

\[ P(t) = \frac{(h_{\text{eff}} / \sqrt{4\pi D t^3}) e^{-(h_{\text{eff}} - vt)^2 / 4Dt}}{e} \]

In Eq. 3.1, \( h_{\text{eff}} \) is the pore’s effective height, \( D \) is the protein’s diffusion constant, and \( v \) is the protein’s drift velocity. While it can be argued that this 1D model is oversimplified, the model is attractive because it is analytically solvable, allowing biophysical parameters, namely \( D \) and \( v \), to be extracted from a particular experiment. Using bulk \( D \) and \( v \) values, Plesa estimated that only the slowest ~0.1% of translocations of sub-100 kD proteins are observed using >10 nm diameter synthetic pores and current amplifiers with 10 kHz bandwidths. Indeed, in the first report of protein translocations by Talaga et al. both the \( D \) and \( v \) values obtained from their fits were three orders of magnitude smaller than their bulk values (i.e., in free solution), which the authors point out. Clearly, insufficient measurement time resolutions present a severe technical obstacle in nanopore-based protein analysis by greatly abberating the obtained experimental values of \( D \) and \( v \). Several studies using solid-state nanopores and glass capillaries have since confirmed the importance of increasing time resolution by demonstrating measurements at increasing filter bandwidths up to 100 kHz.

Further evidence for insufficient time resolution is borne by inspection of the observed capture rates of the proteins. The flux of protein arrival into an absorbing pore, \( J \), can be approximated by a diffusion-drift model:

\[ J = \frac{R_C}{C_0} = \frac{-D_0 \nabla C}{C_0} + \mu E(r) \]

where \( R_C \) is the measured capture rate, \( C_0 \) is the bulk protein concentration in the chamber, \( D_0 \) is the bulk diffusion coefficient of the protein, \( \nabla C \) is the concentration gradient profile, \( \mu \) is the electrophoretic mobility of the protein, and \( E(r) \) is the electric field as a function of distance \( r \) from the pore. The first and second flux terms in Eq. 3.2 characterize protein diffusion down a concentration gradient and protein drift under bias, respectively. While the diffusive term
dominates under low-field conditions (i.e., small applied bias), the drift term dominates under nanopore experimental conditions, and should linearly depend on the applied bias. Another approach to describe protein capture is the Smoluchowski rate equation, $J = R C / C_0 = 2\pi D_0 r$, where $r$ is effectively the radius of an absorbing hemisphere that extends from the pore mouth. For DNA capture into pores, values of $r$ much greater than the pore radius were found. Using a similar argument for proteins, a recent compilation of experimental capture rates in the molecular weight range of 10-50 kD has concluded that observed fluxes are factors of $10^3 - 10^4$ lower than the Smoluchowski rate prediction. This points to an overwhelming fraction of missed events in nanopore-based protein analysis experiments, issues which can in principle be resolved by increasing the measurement time resolution and protein dwell times. Modifying the experimental parameters, i.e., the pore surface charge, and increasing the solution viscosity can increase molecular transport times, although these approaches could potentially compromise the resolution and sensitivity to protein molecules by inhibiting the protein flux into the pore.

Recently, new amplifiers for high-bandwidth current measurements through nanopores have been developed, and their utility for detecting small nucleic acid biomolecules at >100 kHz bandwidths has been demonstrated. The ability to detect small biomolecules in these works owes to a combination of high-bandwidth current detection, the use of small pores which enhances molecule/pore interactions by increasing molecular confinement within the pore during its transport, and the use of pores in sub-10 nm thick membranes which enhances the signal-to-noise of the measurement.
In this chapter, we show that high-bandwidth current measurements through nanopores in ultrathin hafnium oxide (HfO₂) and silicon nitride (SiN) membranes can be used to efficiently detect sub-30 kD proteins. We compare the transport of two positively charged proteins,
Proteinase K (ProtK, 28.9 kD, pl = 8.9) and RNase A (RNase, 13.7 kD, pl = 9.6), through HfO$_2$ and SiN nanopores with similar geometries: A diameter of $d = 5.2$ nm and effective pore height of $h_{\text{eff}} = 7$ nm for the HfO$_2$ pore, and $d = 4.8$ nm and $h_{\text{eff}} = 6.2$ nm for the SiN pore (see Appendix 2). Figure 3.1a shows an idealized ProtK molecule in flight through a 5 nm diameter nanopore drawn approximately to scale (electrodes not to scale). To maximize confinement of the proteins that reduce protein mobility within the pore, pore diameters were chosen such that $d_p/d_h < 1.5$, where $d_h$ is the protein's hydrodynamic diameter. Upon the addition of ProtK molecules to the cis chamber of the nanopore cell (to which the ground electrode is connected), application of -125 mV to the trans chamber results in spikes that indicate stochastic protein passage through the pore, as seen in the current trace in Figure 3.1b. Analysis of the current vs. time trace yields a set of events, a concatenated set of which is shown in the Figure. Using a 250 kHz bandwidth, 2-2.5 µs minimum dwell times are detectable.

3.2 Materials and Methods

Experimental Setup Ultrathin HfO$_2$ membranes and nanopores in these membranes were fabricated as outlined in a prior study that demonstrated exceptional pore stability over many hours of experiments. Similar-sized ultrathin silicon nitride (SiN) pores$^{33}$ were fabricated for comparison using previously reported methods. Prior to experiments, pores were treated with heated piranha (3:1 H$_2$SO$_4$:H$_2$O$_2$), followed by hot deionized water. Nanopore chips were then dried under vacuum, assembled in a PTFE cell, and sealed by painting with a quick-curing silicone elastomer gasket to reduce the capacitance to under 100 pF.$^{64}$ Electrolyte solution was then flowed using a syringe to hydrate both chambers (1 M KCl, 10 mM Tris, buffered to pH 8.1). Proteins were added to the cis chamber and thoroughly mixed to the indicated final concentrations. Proteinase K and RNase A samples were purchased from New England BioLabs or from Thermo Scientific. All experiments were carried out at ambient room temperatures (23-
25°C). For control experiments with DNA, 100 bp DNA (Fermentas NoLimits, Thermo Scientific) was added to the cis chamber to a final concentration of 500 nM.

3.3 Data Collection and Processing

Nanopore current signal was acquired continuously and digitally at a rate of 4.19 MS/s using a Chimera Instruments amplifier.\(^{28}\) Before the introduction of a protein sample, several seconds of current data were collected at multiple voltages (\(\Delta V\)) to verify the pore’s stability by checking that no spikes are observed. Data was digitally low-pass filtered at 250 kHz unless otherwise indicated, and subsequently analyzed using OpenNanopore, an open source translocation analysis software package developed by the Radenovic Group at EPFL.\(^6\) The code was modified to allow data import from the Chimera Instruments format. OpenNanopore fits each translocation event with a rectangular pulse (see Figure 3.1b and Appendix 2). Three key parameters are extracted from the data: the dwell time \(t_d\) is the spike duration which corresponds to the protein’s residence time in the pore, the current blockage \(\Delta I\) is the mean current excluded from the pore by the analyte, and the inter-event waiting time, \(\delta t\), from which protein capture rates are extracted.

3.4 Protein Capture

As shown in Figure 3.1b, the addition of protein molecules to the cis chamber results in a stochastic set of current spikes that correspond to protein stochastic presence in the pore. In Figure 3.2a-b representative snapshot current traces in the voltage range +/-200 mV are shown for both ProtK and RNase, respectively. For both proteins spikes appear only for negative voltages, apart from rare spikes at positive voltages which are due to contamination. Another observation from the snapshot traces is that the spike rate increases with voltage magnitude, after which rates decrease for increasing voltages.
While our pore/amplifier combination can detect shorter current pulses from proteins than prior studies, our minimum time resolution of \(~2.5~\mu\text{s}\) also proves insufficient for detecting all protein translocation events. This is illustrated in Figure 3.3a-b, which shows dwell-time vs. fractional current \((\Delta I/I_0)\) scatterplots for both proteins at indicated voltages in range 75 – 200 mV. Each data point on the scatterplot represents \(\Delta I/I_0\) and \(t_d\) for a single protein event. The scatterplots indicate a transition to being sharply bandwidth-limited for \(|\Delta V| > 150\,\text{mV}\). This is indicated by the straight line cutoff at \(t_d \sim 2.5\,\mu\text{s}\). At this voltage, a majority of events have dwell times that are too short for our time resolution. For \(|\Delta V| < 150\,\text{mV}\), however, the data appear significantly less bandwidth-limited, as we observe a bounded distribution instead of a strict cutoff (see arrows I and II in scatterplots for comparison). We also see that, being the smaller of the two proteins, RNase events are faster than ProtK events for similar voltages.

Figure 3.2. Protein current data. Snapshot current traces for (a) RNase and (b) ProtK at various voltages in the range +/-200 mV ([KCl] = 1 M, pH 8.1, \(T = 25^\circ\text{C}\), low-pass filtered at 125 kHz for presentation only). Inset shows current spikes for \(V<0\), indicating positive charges for both proteins.
A quantitative representation is provided in Figure 3.4a, where normalized capture rates $R_C/C_0$ are plotted for both proteins at various applied voltages. As shown in Eq. 3.2, flux is expected to be diffusion-dominated at a low bias and drift-dominated at increasing bias. As common for nanopore experiments, we extracted $R_C$ values from single exponential decay fits to our first arrival time distributions. Due to signal-to-noise limitations, we are not able to access the diffusion-dominated regime for protein capture that dominates at $|\Delta V| \sim 0$ (first term in Eq. 3.2). However, for both proteins we find that $R_C/C_0$ increases linearly with voltage, then saturating and
gradually declining at voltages above $|\Delta V| > 125$ mV. While this linear increase is expected in the drift-dominated regime (second term in Eq. 3.2), declines in capture rates for higher voltages were not expected. However, judging from the shapes of the scatterplots in Figure 3.3 for high voltages, these declines are clearly due to experimental limitations: as voltage increases, proteins traverse the pore with higher velocities, and more and more fast events are missed by our detector, resulting in smaller apparent capture rates.

To check whether our obtained protein capture rates are reasonable, we calculate the effective radius of an absorbing hemisphere outside the nanopore from Smoluchowski-based protein arrival rates. Rearranging the Smoluchowski rate equation we calculate the effective radius $r^*_C$ of a perfectly absorbing pore, i.e., $r^*_C = R_C/(2\pi D_C \delta)$. As shown in the bottom of Figure 3.4b, we find that $r^*_C > r_p$ for all voltage values. Rationale for this result comes from the fact that the applied voltage creates an electric field outside the pore that assists the capture of charged biomolecules. Using a similar argument as for DNA, as a protein molecule undergoes Brownian diffusion near the pore, at some distance $r$ from the pore the electrophoretic drift velocity term overweighs the diffusional velocity term, i.e., $\mu_0 E > 2D_0/r$ (where $2D_0/r$ is the diffusional velocity term in the 1D direction opposite to the pore direction). This is depicted by the cartoon inset of Figure 3.4b, which labels $r_C$ as the hemisphere around which diffusional and electrophoretic terms are equal. Remarkably, our result of $r^*_C > r_p$ is the first among reported protein detection works. While $r^*_C$ increases for increasing voltages for $|\Delta V| < 125$ mV because of an increasing drift component outside the pore, it decreases with increasing voltage because of increasingly fast events. For comparison, in the work by Plesa et al., for proteins of similar size to our study capture radii were found to be 3-4 orders of magnitude smaller than the pore radius. These infinitesimal effective radii are a combined result of insufficient bandwidth and the use of larger pore sizes than in our study, as discussed in the introduction.

3.5 Protein Transport
Our investigation of the capture process revealed efficient protein detection. However, it remains to be seen whether our obtained dwell time distributions are sufficient to distinguish differences in translocation patterns in two proteins. Following earlier work\textsuperscript{80d, 86b} with a minor correction,\textsuperscript{86c, 87} we fit dwell time distributions to a first-passage time distribution derived from such a 1-D drift-diffusion model of Eq. 3.1, where the pore’s effective height, $h_{\text{eff}} = 7$ nm in this case (see Appendix 2). $D$ is the protein’s diffusion coefficient during its transport through the pore, and $v$ is its drift velocity. A naive value to use for $D$ is its bulk solution diffusion constant $D_0$, which can be estimated using available models to be 87.5 nm$^2$/µs and 124 nm$^2$/µs for ProtK and RNase, respectively.\textsuperscript{96} However, prior studies of protein diffusion through porous media have observed diffusion constants that are reduced significantly from bulk values. An empirical equation developed by Renkin for protein diffusion in a porous medium is given by:\textsuperscript{97}

$$\text{Eq. 3.3 } D = D_0(1 - \frac{r_s}{r_p})^2 \left[ 1 - 2.104 \left( \frac{r_s}{r_p} \right) + 2.09 \left( \frac{r_s}{r_p} \right)^3 - 0.95 \left( \frac{r_s}{r_p} \right)^5 \right]$$

where $D$ is the protein’s diffusion constant in the pore, $D_0$ is its diffusion constant in free solution, $r_s$ is the protein’s Stokes radius, and $r_p$ is the pore radius. We stress that Eq. 3.3 is only valid in the regime $r_s/r_p < 0.4$, i.e., for pores that are at least 2.5 times larger than the proteins.\textsuperscript{98} While the Renkin equation is clearly not valid for estimating $D$ in our experiments ($r_s/r_p \approx 0.8 - 0.9$), a gross estimation of $D$ using measured values for $r_s$ (see Appendix 2) reveals that $D$ can be reduced in our pores by as much as 2-3 orders of magnitude for our proteins.
We point to the Renkin equation, which has been experimentally validated, to illustrate that there is no reason to expect $D$ to be equal to its bulk value in the nanopore. In addition, our data shown in Figure 3.3 and its summary in Figure 3.4 point to efficient protein detection, indicating that we are capturing a substantial portion of the dwell time distributions. Therefore, rather than using bulk $D$ values for fitting dwell time distributions to Eq. 3.1, we have allowed both $D$ and $v$ to be free parameters. Example dwell time distributions for both ProtK and RNase are shown in Figure 3.5a-b, respectively, along with fits to the distributions for both free (calculated, $D$) and fixed (least-squares optimized, $D_0$) values (see Appendix 2 for complete sets of distributions). We note that the dwell time distributions show that the durations of some events faster than 2.5 µs may be overestimated, resulting in ‘pile-up’ in the briefest histogram time bins.

Figure 3.4. Protein capture rates. (a) Normalized mean capture rates (top) into a 5.2 nm diameter HfO$_2$ pore as a function of $V$. (b) Corresponding Smoluchowski-based capture radii (bottom) for both proteins (see text).
Upon integration of the fits to Eq. 3.1 we can compute the estimated fraction of detected events ($F_{\text{obs}}$) in each experiment, which ranged from 70-90% for ProtK and 30-80% for RNase (see Appendix 2). Our optimized fits yielded $D = 2.0 \pm 0.2$ nm$^2$/µs for ProtK at all voltages in range 75-200 mV, a factor of ~44 smaller than its bulk $D_0$ value, 87.5 nm$^2$/µs. For RNase, we obtained a consistent value of $D = 2.5 \pm 1.3$ nm$^2$/µs, a factor of ~50 smaller than its bulk value, $D_0 = 124$ nm$^2$/µs.\textsuperscript{96}

Figure 3.5. Protein dwell-time distributions. (a) ProtK and (b) RNase dwell-times at selected voltages, along with fits to Eq. 3.1 with D and v as free parameters (black curve) and constrained fits for bulk $D_0$ values (dashed red curve). Missed regimes shaded red in distributions).
In addition to extracting the values of D, our fits yield values for the drift velocity v. Figures 3.6a and 3.6b shows extracted diffusion coefficients (D) and drift velocities (v) with voltage for both proteins through the same HfO$_2$ pore, respectively. Linear dependence was found for both proteins, as expected for an electrophoretic transport process where v = \mu E. Assuming that the applied voltage drops entirely across the length of the pore, i.e., E = |\Delta V| / h$_{\text{eff}}$, multiplying the slopes of the lines in Figure 3.6b by h$_{\text{eff}}$ yields in-pore electrophoretic mobilities of \( \mu_{\text{RNase}} = 80 \pm 2 \text{ nm}^2\mu\text{s}^{-1}\text{V}^{-1} \) and \( \mu_{\text{ProtK}} = 60 \pm 3 \text{ nm}^2\mu\text{s}^{-1}\text{V}^{-1} \). Finally, when these mobilities are compared to a 100 bp double-stranded DNA molecule tested through a HfO$_2$ pore with nearly the same pore
geometry as the nanopore used in this study (d = 5 nm, \( h_{\text{eff}} = 7 \text{ nm} \), see Appendix 2), DNA mobility was 350 ± 14 nm\(^2\)\(\mu\text{s}^{-1}\)V\(^{-1}\), ~4 times larger than for the proteins (see Figure 3.6b). In addition, D values for the DNA molecule were in the range 4 - 7 nm\(^2\)/\(\mu\text{s}\). While this value for DNA mobility is larger than for the proteins due to its increased charge density, it is still ~100 times smaller than the free solution drift velocity for 100 bp DNA (3.6-4.5 x 10\(^4\) nm\(^2\)\(\mu\text{s}^{-1}\)V\(^{-1}\)), indicating restricted mobility through the pore due to confinement and wall interactions. Finally, determination of the drift velocity for DNA at voltages above 200mV proved challenging due to our system’s insufficient time resolution: with a contour length of ~30 nm for 100 bp DNA and a 2.5 \(\mu\text{s}\) time resolution, detection of drift velocities upwards of 12 nm/\(\mu\text{s}\) is difficult (see red dashed line in Figure).

Generally, the connection between a charged object’s mobility and diffusion constant is given by the Einstein-Smoluchowski relation (\(qD = \mu k_B T\)). Given this relationship and prior evidence of reduced D in porous systems, we suggest that a possible mechanism for the reduced D is pore-protein interaction. For a molecule to move a distance comparable to its diameter along the pore, we assume that it hops over a free energy barrier U. Furthermore, we take U as the net binding energy between the protein molecule and the pore. During a protein molecule’s transport through a small pore, interactions with the pore walls may effectively inhibit the diffusion coefficient D within the pore from its bulk value (\(D_0\)) as follows:

Eq. 3.4 \[ D = D_0 e^{-U/k_B T} \]

In this simplistic Kramers model D is reduced by interactions with the pore, yet confinement effects are assumed to be independent of applied voltage. This assumption is reasonable for a weakly charged protein in the low voltage regime. Solving for U using the reduced D values of 2.0 ± 0.2 nm\(^2\)/\(\mu\text{s}\) and 2.5 ± 1.3 nm\(^2\)/\(\mu\text{s}\) for ProtK and RNase, respectively, we obtain protein/pore interaction energies of 9 ± 0.25 kJ/mol for ProtK and 9.5 ± 1.3 kJ/mol for RNase. Similarly, for the SiN pore our obtained D values of 1.4 ± 0.5 nm\(^2\)/\(\mu\text{s}\) and 0.9 ± 0.3 nm\(^2\)/\(\mu\text{s}\) for ProtK and RNase yield
interaction energies of 10 ± 0.9 kJ/mol and 12 ± 0.8 kJ/mol, respectively (see Appendix 2). Remarkably, irrespective of the pore material, D values were nearly 50 times smaller than their bulk D₀ values (indicated earlier in the manuscript).

As stated above, our presented model of Eq. 3.4 assumes independence of D on applied voltage. Furthermore, in our fit of dwell-time distributions to Eq. 3.1, D and v were both free parameters and no assumptions or constraints were imposed on their limits or trend. To investigate this, we present in Figure 3.7 a scatter plot of calculated mobilities μ* for each experiment conducted (given by vh eff / ΔV) vs. obtained D values. Data are included from several nanopores of similar dimensions, as indicated in the legend. Apart from a single datapoint corresponding to RNase at -75 mV (circled in Figure), our data show robust D and μ* values to within 50% for each pore, which does not contradict our model in Eq. 3.4.
3.6 Protein Volume Measurements

Extracting volumetric information using resistive pulse measurements is a decades-old technique. In the 1970’s, DeBlois and Bean developed quantitative theory for obtaining volumetric particle information from resistive pulse data. Since then, Ito et al. applied this theory for measuring particle diameters that are a factor of 2-3 smaller than the pore diameter. Later, Han et al. applied this theory for measuring proteins that are a factor of 4-5 smaller than the pore diameter. Borrowing on this theory, protein diameters can be estimated from the current blockage data based on:

Figure 3.7. Transport Scatterplots. In-pore electrophoretic mobilities, calculated for each experiment as $\mu^* = v_{\text{eff}} / \Delta V$, vs. in-pore diffusion coefficients $D$ (where $D$, $v$, are extracted from 2-parameter fits to Eq 1., see text). Compiled results for experiments in different HfO$_2$ and SiN pores and different voltages are shown. Apart from an outlier for RNase at -75mV (circled), the data suggest voltage-independent values of $\mu$ and $D$ in the range tested.
Eq. 3.5 \( d_m \equiv \left[ \left( \frac{\Delta I/I_o}{I_o} \right) (h_{\text{eff}} + 0.8d_p) d_p^2 \right]^{1/3} \)

where \( d_m \) is the protein diameter, \( d_p \) is the pore diameter, and \( \Delta I/I_o \) is the ratio of the statistical mean blocked current for each experiment to the mean open pore current level, respectively. Note that the term \( (h_{\text{eff}} + 0.8d_p) \) is used as a correction factor because in our case \( d_p \approx h_{\text{eff}} \).  

Distributions of \( \Delta I/I_o \) for both ProtK and RNase at \( \Delta V = -125 \) mV are shown in Figure 3.8 for two different experiments in two pores of different compositions but similar geometry. For the HfO\(_2\) pore studied (Figure 3.8a) we observed 2 distinct populations, with a minor population (5-10\%) attributed to stuck or aggregated proteins occurring at higher \( \Delta I/I_o \) values. Therefore, double-Gaussian distributions were used to fit to the \( \Delta I/I_o \) distributions, although the mean \pm width of the \( \Delta I/I_o \) dominant distributions were used for our calculations. Volumes of the proteins based on a Vorlume solvent-accessible model are also indicated in the figure, along with to-scale PDB structures. Applying Eq. 3.5, our measurements yield 4.8 \pm 0.45 nm and 4.1 \pm 0.45 nm for ProtK and RNase, respectively. For our comparison with the SiN pore, our measurements yield 4.6 \pm 0.35 nm and 4.2 \pm 0.35 nm for ProtK and RNase, respectively. Error bars for these calculations were based on half-widths at half-maxima (HWHM) for the distributions.

Independent measurements of the proteins’ hydrodynamic radii (see Appendix 2) yielded 4.76 \pm 0.05 nm and 3.68 \pm 0.03 nm for ProtK and RNase, respectively (see Appendix 2). Somewhat surprisingly, these values are within the error of our nanopore measurements. Moreover, the derived volumes deviate in the two pores by 5\% for both ProtK and RNase, which can be attributed to subtle differences in nanopore geometry. Further, considering the small differences in geometry between the two pores, it is evident that pore material composition does not significantly impact volumetric measurement.

Further confirmation of our volumetric measurements is given by our pore-based detection of a ProtK:RNase complex. To obtain the complex we have added to the cis chamber a pre-formed sample of ProtK and RNase with 1:1 stoichiometry. Although the two proteins are
both positively charged, the complex is known to form as RNase is a common substrate for ProtK-based RNase digestion. After confirmation using dynamic light scattering experiments that the complex forms at 1M KCl (see Supporting Material), the nanopore experiments shown in Figure 3.8b reveal a third ΔI/Io level that is deeper than the levels of each of the two individual proteins. Since complex formation is not quantitative, some uncomplexed proteins may be present in the sample, and the population fits well to a three-Gaussian distribution (see red curve in Figure 3.8b), although the dominant population represents the complex. For the complex we obtain D and v values of 0.04 ± 0.006 nm²/µs and 1 ± 0.9 nm²µs⁻¹V⁻¹, both parameters over an order of magnitude smaller than values for the isolated proteins (see Appendix 2). This can be explained by the fact that the complex is slightly larger than the pore dimensions, which sterically retards its transport through the pore constriction. While previous studies have observed translocation signatures of complexed or aggregated proteins, we believe that this is the first to observe a completely separate population due to a protein complex. 82, 103, 105
3.7 Discussion

We have demonstrated here that by combining a high-bandwidth current measurement platform with small and ultrathin solid-state nanopores, sub-30 kDa proteins and their complexes can be efficiently detected and analyzed. Proteinase K and RNase A, two proteins of different
molecular weights and isoelectric points, were compared in this study along with their complex. A comparison of our capture rate data to results summarized by Plesa et al reveals an “effective” capture radius that is larger than the pore diameter, indicating efficient detection that worsens above a critical voltage of -125 mV due to bandwidth limitations. Application of a 1D diffusion-drift model to the dwell time distributions revealed distinctly different in-pore electrophoretic mobilities and diffusion coefficients for the two proteins. Diffusion is reduced by nearly two orders of magnitude in the pore’s confinement, in qualitative agreement with Renkin theory. We have suggested a straightforward Kramers model that accounts for reduced diffusion via interaction of the protein with the pore. Our model yields similar energies of interactions on the order of 10 kJ/mol for similar-geometry pores fabricated in SiN and HfO$_2$. Further, in-pore electrophoretic mobilities extracted for the two proteins consistently reveal higher mobilities for RNase than for ProtK, as confirmed in SiN and HfO$_2$ pores. However, mobility values are reduced by 1-2 orders of magnitude for all biomolecules tested here, as also found in DNA transport through larger pores to be due to hydrodynamic forces.$^{106}$ Electro-osmotic flow (EOF) can indeed impact translocation, and it was previously observed to be the driving factor for protein translocation, even against electrophoresis.$^{35}$ However, similar to SiN pores, at the pH of our experiment HfO$_2$ has a weak negative surface charge density (<0.02 C/m$^2$),$^{61}$ which corresponds to <0.15 e/nm$^2$. We therefore expect convective effects from electro-osmosis to be insignificant as compared to electrophoretic forces on sample proteins. Further, while varying the pH can be used for assessing more quantitatively the protein’s charge properties,$^{103}$ these studies also affect the pore’s charge properties,$^{35}$ which needs to be independently investigated. Finally, volumetric measurements of the two proteins using Coulter theory developed by DeBlois and Bean show that measured protein diameters coincide well with hydrodynamic diameters obtained from dynamic light scattering measurements. While further experiments with various proteins are necessary to establish the robustness of this volumetric measurement, discrimination of isolated proteins from their RNase:ProtK complexes highlights the
protein size sensitivity of our measurements. Future experiments may focus on measurement of other proteins, detection of conformational changes in proteins, the effects of electrolyte strength and pH on the measurements, and strategies to reduce the capacitance-induced noise of our membranes to enable higher-bandwidth measurements.

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Chapter 4. Reversible Positioning of Single Molecules inside Zero-Mode Waveguides

Abstract

We have developed a hybrid nanopore/zero-mode waveguide (NZMW) device for single-molecule fluorescence and DNA sequencing applications. The device is a freestanding solid-state membrane with sub-5 nm nanopores that reversibly deliver individual biomolecules to the base of 70 nm diameter waveguides for interrogation. Rapid and reversible molecular loading is achieved by controlling the voltage across the device. Using this device we demonstrate protein and DNA loading with efficiency that is orders of magnitude higher than diffusion-based molecular loading.

4.1 Introduction

The ability to isolate and study the dynamics of individual biomolecules using fluorescence has revolutionized our understanding of basic mechanisms in biology. Single-molecule fluorescence relies on the detection of photon emission from individual labeled molecules, which is often complicated by various factors including interference from neighboring molecules, a limited fluorescence lifetime due to photobleaching, and background optical noise from other molecules in the bulk solution. Zero-mode waveguides (ZMWs), nanostructures that comprise sub-wavelength cylindrical wells in an opaque metallic film,\(^1\) alleviate most of the challenges of single-molecule fluorescence. When illuminated, an exponentially decaying electric field forms at the ZMW base. Combined with the sub-wavelength lateral field confinement of the aperture, ZMWs can achieve zeptoliter excitation volumes.\(^{107}\) This confined illumination has been exploited for single molecule measurements of lipid diffusion in a bilayer,\(^{108}\) reverse transcription,\(^{109}\) DNA methylation,\(^2\) and translation,\(^{110}\) among other biophysical processes.\(^9\) Notably, the ZMW is an essential element of the Single Molecule, Real-Time (SMRT) DNA sequencing platform, where single DNA polymerases inside the ZMWs incorporate phosphate-labeled, color-coded nucleotides.\(^{11}\)

A key requirement for ZMW-based studies is that a single biomolecular entity occupies each ZMW. In SMRT sequencing, the yield of singly occupied ZMWs determines the overall efficiency, accuracy, and cost of DNA sequencing. Immobilization of single polymerases inside ZMWs is currently achieved using either diffusion or magnetic beads. Both modes of loading result in a theoretical maximum of 37% of singly occupied ZMWs due to a Poissonian statistical limit.\(^{12}\) In addition to single molecular occupation inside ZMWs, the kinetics and efficiency of DNA template loading also suffer in diffusion-based approaches, which can be a major hurdle for sequencing applications in which only low DNA concentrations are available, e.g., epigenetic...
analysis of mammalian cellular DNA.\textsuperscript{2} Diffusion-based DNA loading for SMRT sequencing typically requires 30-60 min exposure at \textasciitilde 80 pM DNA concentrations, and input DNA requirements further increase for fragments longer than 1,000 base pairs.\textsuperscript{14a} This has limited most epigenetic SMRT sequencing applications to bacterial studies,\textsuperscript{2,111} or created the necessity to develop chemically sophisticated enrichment methods for applicability to mammalian DNA samples.\textsuperscript{112} For magnetic-bead-based loading, the efficiency of immobilizing long DNA molecules is increased, translating to \textasciitilde 3-30 pM input concentrations, although hour-long immobilization times are needed.

The need for single occupation and time-efficient molecular loading for ZMW-based studies are both compromised by the reliance on diffusion- or bead-assisted loading. While a recent report that employs DNA origami scaffolds has allowed super-Poissonian occupation of single biotin groups at ZMW bases,\textsuperscript{22} the authors note an occupation limit even using a high origami concentration, as well as a significant fraction of doubly-occupied ZMWs. In contrast to diffusion, active methods that focus and manipulate molecules in space have the potential to greatly enhance ZMW-based studies. The ability to actively draw single molecules from bulk and position them inside the illumination volumes of ZMWs would impact DNA sequencing and a wide array of immobilization-based ZMW applications by enhancing the efficiency, sensitivity, and accuracy of the devices. Vice versa, reversing the conditions under which molecules are focused inside the ZMWs can release a used molecular complex from the ZMW.

Solid-state nanopores have the ability to focus and trap single molecules at specific, nanometer-precise positions. These pores are milled using electron or ion beams\textsuperscript{43-45} to form nanometer-scale holes through synthetic insulating membranes. Applying a trans-membrane voltage across an electrolyte-immersed pore generates a steady-state ion current through the nanopore constriction, resulting in a highly localized electric field profile in the pore vicinity that can be used to focus and capture individual charged biomolecules.\textsuperscript{13a} These pores have been suggested for nanopore-based DNA sequencing technology,\textsuperscript{113} and for delivering biomolecules
to a variety of patterned nanostructures. Recent experiments have used large (>50 nm) metallized nanopores to detect metal nanoparticles and fluorescently-labeled DNA, although no mechanism is provided in this work for immobilizing and precisely positioning them at a desired position within the ZMW.

In this chapter, we demonstrate ZMW devices equipped with nanopore-based biomolecular positioners at their base. Specifically, we have developed an integrated nanopore-ZMW device (NZMW) in which a ZMW array is fabricated on top of an ultrathin silicon nitride (SiN) membrane, such that sub-5 nm diameter nanopores are present at the base of ZMWs on the membrane. Using these devices we demonstrate the controlled immobilization/ejection of DNA-protein complexes from the pore, as well as a vastly improved loading kinetics of large DNA molecules (6,000 base pairs) into the ZMWs over diffusion-based loading.

4.2 Device Characterization

A schematic illustration of our NZMW single-molecule positioners is shown in Figure 4.1a. The device consists of a silicon chip processed using established methods to contain an array of ZMWs on a ~100 x 100 µm² freestanding SiN membrane (see Appendix 3 for details). The ZMW arrays were passivated from reaction with piranha solution by depositing an 11-nm-thick SiO₂ layer using atomic-layer deposition. Nanopores were then drilled in pre-determined locations in the ZMW arrays using transmission electron microscopy (TEM), followed by treatment with piranha solution in order to hydrate the pores prior to experiments (see Appendix 3). The device was assembled in a custom cell that allows fluidic access to both sides of the membrane, as well as optical access to the bottom side of the chip as shown in Figure 4.1a (see Appendix 3).
Figure 4.1. NZMW device. (a) Scheme of the NZMW. An array of ZMWs is positioned on a 35 nm silicon nitride membrane with nanopores at the bases of waveguides (inset). A voltage bias actively draws complexes of biotinylated DNA and fluorescently labeled streptavidin to the pore, which places the fluorophore in the ZMW excitation volume. (b) An AFM scan of the ZMW membrane illustrates the topography of the surface. ZMWs are spaced 1.3 μm × 4 μm. Line scans of each ZMW demonstrate uniformity with an average top diameter of 86.2 ± 6.4 nm (N = 21). The scans have a pointed bottom profile because the AFM tip cannot penetrate the full depth of the waveguide. (c) Dark-field scanning transmission electron micrograph (inverted contrast) of four ZMWs in the array (scale bar = 1 μm). (d) TEM images of ZMWs with 3 to 3.5 nm nanopores drilled in their centers (scale bars = 20 nm). ZMWs have a measured base diameter of 64.9 ± 3.7 nm (N = 57).
Figure 4.1b shows an AFM scan of a 2 x 4 ZMW array on a SiN membrane (4 µm x 1.3 µm spacing). The ZMWs are seen as dark uniform circles in the image, for which height profiles through the ZMW centers are shown as insets to the Figure. Although the base of the ZMWs cannot be accessed in AFM due to the tip geometry, the height profiles reveal uniform top diameters measured to be 86.2 ± 6.4 nm (N = 21). The base diameters were measured by dark-field scanning TEM (Figure 4.1c) and bright-field TEM (Figure 4.1d) to be 64.9 ± 3.7 nm (N = 57). The dark-field images of Figure 4.1c, in which the contrast was inverted for clarity, display a polycrystalline structure with grains in the range of 50 - 150 nm, characteristic of a thermally evaporated metal film. The images in Figure 4.1d show four typical NZMWs that contain 3 - 3.5 nm diameter pores drilled at their center. We note that the TEM images shown in Figure 4.1 represent the first non-cross-sectional view (e.g., top-view) of ZMWs using the TEM, since prior ZMW devices have all been fabricated on ~100 µm-thick glass substrates that are too thick for TEM imaging. Based on these AFM and TEM measurements of the top and base diameters respectively we arrive at a funnel-like ZMW shape, as previously obtained with other ZMW devices fabricated on fused silica substrates.\textsuperscript{107}

Capture of charged molecules into the NZMWs can be greatly impacted by an electric field gradient present near the NZMW volume. It is established that DNA capture into a nanopore is strongly assisted by the residual electric field near the pore mouth,\textsuperscript{130} which generates a localized electromotive force that migrates the molecule and focuses it to the pore. To examine the impact of ZMW presence on the electric field profile near the pore, we used finite-element simulations to numerically compute the voltage profile in the vicinity of a 3 nm diameter nanopore in the absence (Figure 4.2a) and presence (Figure 4.2b) of a 60 nm diameter ZMW above it. As the simulations show, the addition of the ZMW constriction results in an electric field gradient with significant presence beyond the ZMW top. The dotted contour lines, which indicate the positions at which the voltage drop is one percent of the total trans-
membrane bias, highlight a four-fold extension of the field away from the pore. This extended field facilitates the migration of charged biomolecules towards the ZMW volume.

Figure 4.2. Electrical properties of the NZMW. Numerical solution for the voltage profile induced by applying 800 mV to (a) a 3 nm pore without a ZMW and (b) a 60 nm diameter ZMW with a 3 nm pore ([KCl] = 400 mM, T = 25 °C). Dotted lines i* and i indicate the equipotential contour line where the voltage drop is 1% of the total transmembrane voltage. (c) I–V curve for an array of three NZMWs in 400 mM KCl (blue curve) compared to that for a SiN pore under the same conditions (red curve). (d) Power spectral density of electrical noise for a NZMW membrane under different experimental conditions as indicated in the legend (λ = 488 nm, P = 20 mW, ~40 W/cm² sample intensity).
Figure 4.2c plots an I-V curve measured on an array of three NZMWs with 3 nm diameter pores in 400 mM KCl (blue), as well as an I-V curve of a single 3 nm diameter pore (red). In both cases the I-V curves are linear, indicating open pores are present in the devices. The minor hysteresis in the I-V curve of the NZMW array is an artifact of the additional capacitance of the ZMW structure, which does not adversely interfere with our ability to capture and observe molecules inside NZMWs. Power spectral densities (PSD) of the current noise for an NZMW device under various experimental conditions are shown in Figure 4.2d. The PSD plots show a typical shape for nanopore measurements, characterized by 1/f noise at low frequencies, thermal (Johnson) noise at intermediate frequencies, and capacitive-dominated noise at high frequencies.\textsuperscript{38, 64, 118} Laser illumination at zero bias (red curve) affects the thermal noise, while having little impact on the 1/f and capacitive regimes. In contrast, upon application of voltage (black) the 1/f noise dominates the PSD, as previously observed in nanopore experiments.\textsuperscript{119} Despite the presence of ZMWs on the membrane, the overall noise is comparable to that of conventional SiN nanopores.\textsuperscript{64}

4.3 Detecting DNA/Protein Complexes

Using a device that contains a single NZMW with a 2.5 nm diameter nanopore, we demonstrate the ability to capture a DNA/protein complex and dissociate its biotin-streptavidin bond in a ZMW under high bias. A solution that contains 1,003 bp 5'-biotinylated DNA complexed to Alexa Fluor 647-labeled streptavidin (see Materials and Methods) was added to the cis chamber, which resulted in voltage-driven electrophoretic focusing of the complexes into the ZMW volume. When the DNA threads into the pore, the force on the DNA against the streptavidin that is anchored to the ZMW base causes the eventual dissociation of the complex.\textsuperscript{42d} Mounting our custom cell on an inverted microscope equipped with 640 nm laser illumination (Coherent Cube®, Coherent, Inc.) and EMCCD detection (see Appendix 3), we simultaneously recorded
nanopore current and NZMW fluorescence. Upon application of 850 mV, a stable open pore baseline current was observed, followed by a stochastic series of spikes that correspond to DNA and/or DNA/streptavidin interactions with the nanopore in the NZMW. In addition, we observed occasional long-lived events (>1 s) that correspond to long-lived presence of the complex within the nanopore. These long-lived events were coincident with discrete increases in fluorescence from the NZMW (Figure 4.3, points 1-5). Notably, in events 1 and 2 of Figure 4.3 we observed relatively shallow current blockades, which may represent a complex present in the NZMW without one of its DNA molecules being fully threaded. This explanation is supported by events 1 and 2, which respectively show a complex temporarily adhering before diffusing away, and a complex remaining near the pore during which we observed other DNA translocation events. For events 3–5, we observed deeper blockade levels accompanied by increases in fluorescence, which indicate full DNA threading and streptavidin presence at the NZMW base. Dissociation of the biotin-streptavidin bond at high voltage has previously been observed in a solid-state nanopore under similar applied voltage values. In each of these events, the simultaneous reduction of the nanopore current and increase in fluorescence indicates the capture of individual DNA/protein complexes in the pore. Finally, reversal of the voltage results in immediate ejection of the complex from the NZMW (e.g., events 2, 4, and 5), as observed by a coincident decrease in fluorescence intensity.
Figure 4.3. Reversible positioning of a single DNA–protein complex inside a NZMW.
Simultaneous current (250 kHz sampling, 10 kHz filtering) and fluorescence (1 pixel region of interest, 10.02 ms exposure time, signal-averaged to 400 ms) traces from a single NZMW containing a 2.5 nm pore for a sample of biotinylated 1003-bp DNA conjugated to Alexa Fluor 647-labeled streptavidin. Brief translocation spikes are translocations of free DNA. Points 1–5 identify events where a fluorescently labeled DNA–protein complex entered the ZMW illumination volume and occluded the pore, resulting in simultaneous fluorescence from the NZMW and blockage of the nanopore current. The inset schematically depicts the experimental scheme. DNA is pulled into the pore but prevented from translocating by the streptavidin, giving long-lasting current blockage. While immobilized in the pore, the labeled streptavidin sits in the ZMW excitation volume, resulting in fluorescence.

4.4 Simultaneous Positioning of DNA-Protein Complexes Inside NZMWs
ZMW devices are ideal for high-throughput fluorescence-based biomolecular analysis, which
requires immobilization of the molecule inside the ZMW excitation volume for extended periods
of time. We have tested the principle of voltage-driven capture of multiple complexes in a 2 x 4
array of NZMWs that contain 3-4 nm diameter nanopores, as shown in Figure 4.4. We imaged the
membrane while applying alternating biases of +500 and -500 mV to trap and eject the same
DNA/streptavidin complex as used for the experiment in Figure 4.3. Figure 4.4a shows a
fluorescence image of the NZMW array (left), as well as a series of three images during different
time periods of the experiment. The bright spots in the images represent fluorescence that is due
to occupied NZMWs. We note that five of the eight NZMWs in the array were active during the
experiment, with the remaining NZMWs not displaying optical signal. This yield of ~60% is a
reasonable yield of active nanopores in this diameter range. In Figure 4.4b we plot time traces of
fluorescence from five NZMWs, identified as 1-5 in Figure 4.4a, as well as from a ZMW that does
not contain a nanopore, labeled as “N”. At the beginning of the trace a (+) voltage was
applied, during which molecules are clearly observed in the ZMW volume. With the exception of
pore 1 (indicated by *), application of (-) voltage resulted in ejection of complexes from the
NZMWs, as indicated by a return of the fluorescence signal to the baseline level. Upon restoring
the (+) voltage we observed fluorescence activity in all five NZMWs, indicating molecular
loading. This infrequent occurrence of non-correlated signals in NZMW 1 is a possible result of
protein sticking to the surface of the device.120

We note that while activity was seen in many of the NZMW devices, no activity was
observed in the remaining ZMWs that contain no nanopores (e.g., ZMW “N” in Figure 4.4). We
suggest two main reasons for this observation: First, the radius of gyration of a 1,003 bp DNA
molecule is 40 nm, which is slightly larger than the ZMW radius (35 nm). This mismatch presents an
energy barrier for diffusion of the DNA-streptavidin complex into the ZMW. Second, since we
have not applied chemistry to covalently link the diffusing DNA to the ZMW surface, there is no
mechanism to immobilize the complex in the ZMWs.
Figure 4.4. Immobilization of DNA–protein complexes in an array of NZMWs. (a) Fluorescence images of a 2 x 4 NZMW array (within dotted line) with immobilized complexes of 1003 bp biotinylated DNA and A647-labeled streptavidin. Five active NZMWs are identified in the leftmost image, a projection of all frames in the experiment. Point “N” is a ZMW with no pore. The next three images from different times illustrate molecules entering and leaving NZMWs. (b) Fluorescence traces from ZMWs 1–5 and N (1 pixel, 42.55 ms exposure time, averaged to 500 ms) with membrane bias (±500 mV). * identifies a protein adhered to the membrane, resulting in fluorescence persisting through negative voltage.
4.5 Focusing Long DNA Fragments into ZMWs

Finally, we investigate the efficiency of DNA capture into NZMW devices. A solution of 230 pM 6,000 bp DNA labeled with YOYO-1 intercalating dye (10:1 bp:dye ratio, 488 nm excitation, see Materials and Methods) was placed on the cis side of the membrane. To monitor DNA entry, we imaged a ZMW array that contained a single NZMW while the applied voltage was toggled between +850 mV and -850 mV. Figure 4.5 shows fluorescence traces from the NZMW, as well as traces from three representative ZMWs. The inset shows three fluorescence images of the device that correspond to a time-averaged stack of frames from the whole experiment (i), as well as time-integrated images at negative (ii) and positive (iii) voltages. The NZMW (red arrow) was clearly visible based on its notable fluorescence at positive voltage values, while the remaining three ZMWs did not exhibit a voltage-induced fluorescence enhancement (see Appendix 3). Similarly, the traces in Figure 4.5 clearly show distinct entry of individual DNA molecules into the NZMW volume, as indicated by a stochastic set of fluorescence enhancement spikes. We find DNA capture to be highly efficient: the on-time of DNA within the NZMW was 51% when the voltage was (+), whereas the off-time was >99% for negative voltages (see Appendix 3). Additionally, we find a prolonged 6.0 ± 5.5 s mean duration of fluorescence spikes, during which we observe a very dynamic fluorescence signal that points to stochastic DNA fluctuations within the ZMW that occur on a slow time scale.
To quantify the DNA loading we compared the on-time of the NZMW with on-times of other neighboring ZMWs in our experiment for times in which positive voltage was applied. For the random sample of 13 ZMWs we have analyzed the resulting ratio of on-times $t_{NZMW} / t_{ZMW}$ is
highlighting the utility of nanopores as biomolecular focusing elements for ZMW-based studies (see Appendix 3). From a SMRT-sequencing perspective, we also compare the input DNA requirements for NZMWs to those of ordinary ZMWs. A typical protocol for diffusive loading of a 2,000 bp template uses a 150 pM DNA concentration and 60 minutes of reaction time, yielding a concentration-normalized loading rate of $1.9 \times 10^{-6} \text{pM}^{-1}\text{s}^{-1}$. Magnetic bead loading results in improvements on the concentration requirement (3-30 pM), but still requires long incubation times (60 minutes) for optimal Poisson loading, translating to a concentration-normalized loading rate of $1.7 \times 10^{-5} \text{pM}^{-1}\text{s}^{-1}$. In contrast, based on the mean DNA arrival time in our NZMW experiment (3.5 s), the loading rate in NZMWs is $1.3 \times 10^{-3} \text{pM}^{-1}\text{s}^{-1}$, orders-of-magnitude more efficient than in the case of diffusive or magnetic bead loading.

4.6 Discussion

We have demonstrated a novel device that consists of nanopores at the base of ZMWs for efficient and versatile positioning of single molecules. The fabrication process for these devices involved a combination of electron-beam and photolithography methods, and resulted in the first demonstration of ZMWs on freestanding SiN membranes that contain nanopores at their bases. Using synchronous optical and electrical recordings, we have demonstrated the reversible, voltage-driven positioning and ejection of individual DNA-protein complexes, as well as a mechanism for greatly enhancing the entry of long DNA molecules into ZMWs. We note that loading of long DNA molecules into ZMWs for SMRT sequencing is inefficient because of the large DNA coil size with respect to the ZMW dimensions. This need to “package” DNA into ZMWs results in a conformationally restricted DNA that is unlikely to encounter a DNA polymerase at the ZMW base on short timescales. Current protocols for activating ZMWs for sequencing involve incubation of the ZMWs with a preformed complex of DNA and a streptavidin-polymerase fusion protein, which still results in a slow binding to the ZMW surface due to the
imposed conformational restriction. The need to pre-react DNA and polymerase in solution, as well as the need for higher DNA concentrations, has limited certain studies involving precious DNA samples using this method. Finally, we have demonstrated the first ZMW platform in which the ZMW can be reused by releasing a molecular complex from the ZMW volume at the click of a button. The combination of ZMWs and nanopores greatly increases the efficiency of DNA loading, which can aid in the development of future SMRT sequencing applications in genetics and epigenetics. In addition, this ability to focus, hold, and release biomolecules from the illumination volume of the ZMW should allow many biophysical studies at the molecular level.

4.7 Materials and Methods

Sample Molecule Preparation: DNA-protein complexes were prepared from PCR-synthesized biotinylated DNA and Alexa Fluor 647-labeled streptavidin (Life Technologies, Carlsbad, CA). Biotinylated DNA was incubated with labeled streptavidin at a 4:1 DNA:streptavidin ratio for 15 minutes (see gel image in Appendix 3). YOYO-1-labeled DNA was prepared from 6,000 bp DNA (Thermo Scientific, Tewksbury, MA) and YOYO-1 intercalating dye (Life Technologies, Carlsbad, CA). DNA and dye were incubated for 20 minutes at 50 °C with a 10:1 base pair:dye molar ratio.

Numerical Simulations: Voltage distributions near pores (Figure 4.2a and 4.2b) were computed with COMSOL Multiphysics (COMSOL, Burlington, MA). The Poisson-Nernst-Planck equations were numerically solved for a geometry consisting of two micron-scale cylindrical compartments (i.e., cis and trans) connected by a nanopore embedded in a perfectly insulating membrane. An element size as fine as 0.1 nm and additional boundary meshing layers inside the pore were used to ensure no edge effects skew the physical results. A positive bias voltage of 800 mV was enforced at the bottom surface of the trans chamber and ground to the top surface of the cis chamber.
Data Acquisition and Analysis: AFM scans were taken with a Bruker FastScan AFM in tapping mode. TEM imaging and pore fabrication were performed with a JEOL 2010FEG (Northeastern University). NZMW chips were cleaned for five minutes in heated piranha solution, rinsed thoroughly in de-ionized water, dried under vacuum, and immediately assembled for experiment in a PEEK flow cell (see Appendix 3). The cell was mounted in a Faraday cage on the stage of an Olympus IX81 inverted microscope with a 60X, 1.2 NA water immersion objective. The membrane was illuminated with a Coherent Cube 640 nm laser and a Coherent Sapphire 488 nm laser. An Axopatch 200B amplifier was used for current monitoring off Ag/AgCl electrodes. Electrical data was recorded using custom-made LabVIEW software (National Instruments, Woburn, MA). Images were taken with a Hamamatsu ImagEM EMCCD, recorded with HCImage Live software (Hamamatsu, Sewickley, PA), and analyzed with ImageJ.

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Chapter 5. Capture and Sequencing

Abstract

We demonstrate efficient, parallel capture and immobilization of 48, 500 base pair DNA molecules with biotinylated NZMWs. We demonstrate immobilization of biotinylated DNA-streptavidin complexes by observing DNAs remaining in ZMWs after the release of voltage, when one would expect entropy to force the molecule out of the waveguide. Furthermore, we show proof of principle for sequencing with NZMWs by capturing DNA-DNA polymerase complexes, activating the enzymes, and deactivating them with a high-salt spike. Lastly, we capture a 20,000 base pair SMRT sequencing template, and observe its sequencing.

The material in this chapter comes largely from a manuscript in preparation "Electrophoretic Capture of Nucleic Acids into Zero-mode Waveguides for Efficient Sequencing." Larkin J, Henley RY, Korlach J, and Wanunu M. RYH aided in experiments and data analysis. MW aided in preparation of figures. MW and JK directed research.
5.1. Introduction

SMRT sequencing enables average read lengths of more than 5,000 bases. In the method, one optically observes a DNA polymerase inside a nanoscale zero-mode waveguide (ZMW) as it synthesizes a new strand from the sample template using fluorescently labeled dNTP analogs. The mismatch between hydrodynamic diameter of >10,000 base pair DNAs (~170 nm) and the ZMW diameter (~100 nm) creates an entropic barrier to molecular loading into the sequencing volume under equilibrium conditions: the 10,000 bp DNA coil must compress to enter the confined ZMW volume. This barrier limits the efficiency of diffusive molecular loading approaches and thus necessitates large quantities of input DNA (~µgs) for sequencing. The input DNA requirements prevent sequencing of low-quantity, unamplified samples, such as single cells and needle biopsies. Overcoming the input barrier is critical to fully exploit SMRT technology’s potential, especially for sequencing base modifications, where amplification eliminates the epigenetic information.

Here we demonstrate the utility of a nanopore-ZMW (NZMW) for efficient capture and sequencing of DNAs 10,000 bp or longer. In the NZMW, a waveguide array sits atop a <50 nm insulating membrane with <10 nm nanopores at their bases (Figure 5.1a). Under membrane bias, an electric field forms, which draws charged molecules in the vicinity of an NZMW into the sequencing volume. This electrophoretic force allows molecules to easily cross the barrier for DNA entry into ZMW. This efficient loading process potentially allows less input DNA and lower incubation times for sequencing experiments. In this article, we observe voltage capture of DNA-streptavidin complexes into NZMWs and subsequent binding to the biotinylated NZMW bases. We demonstrate the feasibility of sequencing by electrophoretically loading pre-bound DNA-DNA polymerase complexes and activating the enzyme with magnesium ions. Finally, we load a 20,000 base pair template for sequencing and lastly investigate the influence of the nanopore’s electric field on polymerase kinetics in an NZMW.
5.2. DNA Capture

We fabricated NZMW devices using previously described methods. For electrophoretic capture and sequencing experiments, we imaged NZMW arrays in a combined electrical and epifluorescence setup equipped with a prism system for distinguishing different emission wavelengths (Figure 5.1b, Figure 1.5a). To reduce optical background from inherent membrane photoluminescence, we plasma-etched NZMW chips (see Appendix 4). The function of the nanopore in an NZMW is to draw DNAs into the ZMW. Once in the waveguide,
sample molecules must adhere to the base for sequencing using biotin-streptavidin binding, with sample DNA tethered to streptavidin and the ZMW surface biotinylated. We may estimate the length of time a molecule must reside in the waveguide in order to bind the biotinylated base using measured reaction rates and estimated concentrations. The biotin-streptavidin association rate has been measured to be $\sim 4 \times 10^7$ M$^{-1}$s$^{-1}$.\textsuperscript{126} Assuming one streptavidin in the ZMW volume and one biotin at the base (in reality there are many more), the bulk binding will proceed at a rate of $4 \times 10^{-5}$ M s$^{-1}$. We take this as an indication that the NZMW need hold the molecule in the ZMW for a very short time ($2.5 \times 10^{-2}$ s or less) to bind. To functionalize the chip surface we coated it with PEG-biotin (Figure 5.1b; see Materials and Methods). In order to verify capture and colocalization of DNA and streptavidin, we imaged the electrophoretic capture of biotinylated DNAs conjugated to streptavidin (Figure 5.2a). We labeled 1519 bp biotinylated DNA with YOYO-1 intercalating dye (peak emission 505 nm) and incubated it with an equimolar ratio of Alexa Fluor 647-labeled streptavidin (peak emission 665 nm, Life Technologies; see Materials and Methods). Under 640 nm illumination only, we imaged a 4 × 1 array of NZMWs under ten seconds of 250 mV bias. A discrete spike in red fluorescence from a ZMW indicates the arrival of a labeled streptavidin. During this period of time, we observed fluorescence spikes in each ZMW, with single-step photobleaching indicative of a single molecule (Figure 5.2a inset). After releasing the voltage, we imaged the same array under 488 nm illumination. Three of four NZMWs exhibited fluorescence signals with smooth photobleaching curves (Figure 5.2a inset). This indicated the presence of YOYO-1-labeled DNA immobilized in the NZMW under no electrophoretic force. We present an integrated image of this experiment in Figure 5.2a. The signals from each are coincident in the same NZMWs, showing that biotinylated DNAs conjugated to streptavidin entered the NZMWs under voltage and immobilized upon streptavidin binding to the surface. We know immobilization occurred because we detected the fluorescence from the YOYO-1-labeled DNA after releasing the membrane voltage. With no
mechanism of surface attachment, entropy and diffusion should drive the molecule out of the ZMW.

To verify this, we imaged the capture of YOYO-1-labeled λ-DNA (48,500 bp) into NZMWs with and without streptavidin. Figure 5.2b shows fluorescence traces from the experiment. In the top trace, with unbiotinylated lambda DNA with no conjugated streptavidin, we observe a discrete increase in fluorescence from the NZMW upon the voltage-induced capture of a single molecule. When we release the voltage (Figure 2b dotted line), the fluorescence discretely drops to background level as entropy forces the molecule out of the waveguide confines. In the bottom trace, however, where the DNA is biotinylated and conjugated to streptavidin, we see continuous photobleaching after releasing the voltage. The biotin-streptavidin bond immobilizes the sample on the ZMW base.
Figure 5.2. NZMW DNA capture. (a) 3 out of 4 NZMWs (outlined) in an array coated with PEG-biotin capture biotinylated 1519 bp DNAs conjugated to fluorescently labeled streptavidin. Coincidence of DNA and streptavidin is verified by the two different colors of fluorescence (red Alexa647 for streptavidin and green for YOYO-1-labeled DNA) and sample immobilization is confirmed by persistence of DNA fluorescence after release of voltage (inset). (b) 23 of 25 NZMWs exhibit YOYO-1 fluorescence from labeled 48, 500 bp DNA conjugated to streptavidin after 5 seconds of 1 V, indicating DNA capture. DNA immobilization is illustrated by continued photobleaching after release of voltage (inset), as compared to non-biotinylated DNA, whose fluorescence drops off discretely after voltage release (inset).
We next investigated the ability of NZMWs to capture long (radius of gyration ≥ twice ZMW radius) DNAs in parallel. Again we used streptavidin-conjugated biotinylated 48, 500 bp λ-DNA. This time, we captured molecules into a 5 x 5 NZMW array. Figure 5.2b presents integrated images of the ZMW array before and after a pulse of 750 mV. Upon voltage bias for 16.5 seconds, the nanopore field drew λ-DNAs into 23 of 25 NZMWs as indicated by fluorescence spikes at NZMWs (Figure 5.2b, right image). The molecules then adhered to the bottom, again illustrated by the persistence of fluorescence after release of voltage (see supplemental media for full movie of DNA capture). Throughout the experiment, we observed no fluorescence from ZMWs without nanopores. Because DNAs may enter these waveguides only via diffusion, they encounter the entropic barrier to entry and cannot load efficiently.

5.3. Sequencing

As a proof of principle for capture and sequencing we tested the capability of NZMWs to trap a DNA-DNA polymerase complex (Figure 5.2a). We used a 72-nucleotide circular molecule pre-bound to a primer and polymerase as a sample (see Materials and Methods). The NZMW cis chamber contained a 1 nM concentration of pre-bound template and 330 nM of fluorescently labeled dNTP analogs. We fabricated a 2 X 2 array of NZMWs for this experiment on a membrane with roughly 100 total ZMWs. The solution lacked magnesium ions, preventing polymerase activity. Upon application of voltage, both the template DNA and negatively charged dNTP analogs were drawn into the NZMWs. This was evidenced by an increase in fluorescence at each NZMW due to influx of analogs (Figure 5.3b, see supplementary media for full movie). We plot an abridged 640 nm-illumination fluorescence trace from one of the four NZMWs in Figure 5.3b. Three roughly second-long voltage pulses captured and immobilized template DNAs on the biotinylated NZMW bases. We verified capture by then adding magnesium to the solution, inducing polymerase activity. This resulted in discrete bursts of...
fluorescence from the NZMWs as the polymerase incorporated bases in the growing DNA strand (Figure 5.3b). The other ZMWs on the membrane exhibited no steady, stochastic fluorescence because they did not load any template DNA during the loading time. The NZMWs with their electric field, however, all captured DNA during this ~3 second window. To verify that the fluorescence bursts came from polymerase synthesis, and not flux of base analogs through the pore or sticking to the membrane, we spiked the system with KCl to an 850 mM concentration. This would inactivate the enzymes, but not hinder other potential sources of fluorescence events. As illustrated in Figure 5.3b, we detected no more fluorescence pulses after the KCl spike.

We show demonstrate NZMW capture and sequencing with a 20,000 base pair SMRT sequencing template (called a SMRTbell, see Appendix 4). With a ~1 second pulse of 600 mV, we captured a SMRTbell. Figure 5.3c shows the raw sequencing trace from this molecule. In this data, the position of each fluorescence burst along the vertical axis determines its wavelength as the prism disperses emission light. The right-hand side of Figure 5.3c illustrates the structure of the molecule, with part of the sequenced bases aligned over the reference. Alignment was performed by imaging the spectra of the four base analogs individually, determining their spectra on the CCD, and comparing the position of the centroid of each sequencing fluorescence burst to those of the individual bases. We then identified each base as the one associated with the spectrum it most closely resembled in our sequencing data.
5.4. Materials and Methods

Surface treatment
To biotinylate the NZMW membrane surface, NZMW chips were immersed in hot piranha solution (3:1 H₂SO₄:H₂O₂) for five minutes and thoroughly rinsed in DI water. They were then dried under vacuum and baked at 85 °C for ten minutes. After baking, chips were immediately immersed in a room temperature solution of 0.5 mg/ml biotin-PEG-silane in anhydrous ethanol for two hours or more.

Sample molecule preparation

Biotinylated 1519 bp DNA was prepared via PCR with a biotinylated primer. Biotinylated λ-DNA was prepared by extending the single-stranded overhangs of λ-DNA in with a Klenow fragment polymerase in the presence of biotinylated dNTPs. DNA molecules were incubated with intercalating dyes at a 10:1 bp:dye ratio at 65 °C for 30 minutes. To conjugate to streptavidins, biotinylated molecules were incubated with a 2x excess of streptavidin for 20 minutes at room temperature. Circular DNAs were ligated from a 5'-phosphorylated single-stranded molecule with CircLigase II (Epicentre) using a standard protocol.

Primer binding was performed by incubating primer with template at a 20:1 concentration ratio at 80 °C for two minutes, followed by cooling to 30 °C at 1 °C/s. Polymerase (Pacific Biosciences P6) was then incubated with primer-bound template at a 6:1 concentration ratio at 30 °C for four hours (proprietary buffer solutions), followed by 37 °C for 30 minutes. Samples were then put in 50% glycerol with dithiothreitol and placed at -20 °C for storage.

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Chapter 6. Conclusions and Future Directions

We have presented work in this thesis that paves the way toward practical applications of solid-state nanopores as biophysical tools that can bring polymers over the entropic barrier. By establishing HfO$_2$ as a potential membrane material for ssDNA capture and translocation, we expanded the palate of available materials for nanopore detection. The robustness and high signal exhibited by these pores suggests applications in DNA sequencing with nanopores themselves. We showed capture and translocation of single- and double-stranded DNA, with fractional pore blockage approaching that of protein nanopores.

In our experiments with protein translocation, we showed that high-bandwidth electronics could be used to elucidate the physics of molecules passing through pores of diameter close to the molecule’s own hydrodynamic diameter. The results showed that diffusive transport in the constricted pore environment is quite different from that in free solution due to interaction with the surface. This work serves as a starting point for many interesting studies with solid-state nanopores. The physics of this translocation process, which we can access with fast electronics and “small” pores, could serve as a window into protein conformational change, protein-protein interaction, and many other studies, all with no molecular labelling.

We fabricated a novel nanostructure, the NZMW, which delivers molecules to the optical detection volume of a waveguide. In our experiments capturing DNAs and sequencing templates, we demonstrated proof-of-principle for pictogram sequencing: with the active loading of the nanopore field bringing large DNA molecules over the entropic barrier to ZMW entry, sequencing the 6 pg of a single cell may be achievable. The two nanotechnologies combined here, the ZMW and the solid-state nanopore, complement each other nicely. We have focused on the importance of nanopores for bringing molecules into ZMWs, but the ZMW
enables a new level of optical signal-to-background for nanopore experiments. Due to high background from bulk sample and SiN membranes, optical nanopore experiments have been relatively few and far between. The low background of our NZMW arrays will enable parallel, multicolor nanopore detection.

Now that we’ve established the NZMW as an experimental system, there are many directions to go experimentally. An obvious and very worthwhile first step is to parallelize pore formation. This will enable massive NZMW arrays for real sequencing. Making small nanopores is an art: each one is done by hand. However, if we could fabricate an array of ZMWs on a surface that already has pores, the artisanal process becomes industrial. For this goal, we propose the porous ZMW, or PZMW. One first fabricates a nanoporous membrane: the material itself has nanoscale pinholes running through it. In this way, when one biases a ZMW on such a material, the porosity of the PZMW base will establish the electric field à la NZMW. Figure 6.1 illustrates this. For such a porous membrane, we can use molecular layer deposition (MLD), which creates thin insulating layers with nanometer-scale pores. This method pulses an organo-metallic precursor gas over a sample just as in ALD, the technique used for the HfO$_2$ membranes in Chapter 2. Unlike with ALD, the oxidizer in MLD is ethylene glycol, which oxidizes the precursor layer, but leaves organic groups. After many such layers, one has a film of metal oxide with organic contaminants throughout. By annealing or treating with oxygen plasma, one may remove these organic groups, leaving nanoporous voids throughout the film. The films have been shown to have dense enough 1-10 nm channels that if we were to fabricate ZMWs on top of such a layer, each ZMW would have at least one nanoporous channel at its base.
Another potential application of NZMWs is to study the diffusion limitations of single enzyme kinetics. Once one has captured a sample molecule into an NZMW for sequencing, the nanopore may still be biased, creating an electric field at the site of the polymerase. This electric field pulls charged dNTP analogs near the NZMW into the illumination region. If this rate is greater than the rate of dNTPs passing through the nanopore, this will create a buildup in dNTP concentration in the neighborhood of the enzyme. This will cause a discrete jump in polymerization rate. We present preliminary data on this in Figure 6.2.

Figure 6.1. Porous ZMWs. By first fabricating a statistically nanoporous membrane, and then putting NZMWs on top, one creates an array of porous NZMWs (PZMWs). A transmembrane voltage bias will drive ions through these porous channels, creating an electric field to draw molecules into PZMWs (dotted line arrows). Figure created by Meni Wanunu.
We must perform more control experiments to verify that these fluorescence spikes come from polymerase incorporation and not simply dNTPs being pulled into the sequencing volume and sticking. This may be verified by sequencing a known template and verifying the base content of the voltage-enhanced traces. Once verified, this may open up a new realm of experiments where NZMWs may be used to deliver charged substrates to enzymes to study their behavior under fast changes in concentration. One system which would be interesting to study is the RecBCD helicase as it processes ATP.

One last possibility is to use the NZMW to assemble nanostructures. Recent work on metal nanoantennae inside ZMW-like structures has reached unprecedented concentration for single-molecule detection. The NZMW, with its ability to load DNA into the ZMW, could assemble such structures on the fly: DNA could be tethered to nanoparticles and then drawn into the ZMW with the pore’s electric field. Now that we have a working NZMW experimental system, the sky is the limit for new experiments.

Figure 6.2. Voltage-dependent enzyme kinetics. (a) The raw data from the red channel of a polymerase inside an NZMW over four voltages is presented. This corresponds to two out of four DNA bases. The rate of events clearly increases with voltage, as presented in (b), where each circle corresponds to a single fluorescence burst.
Appendices

Appendix 1: Chapter 2 Supplementary Material

A1.1 HfO$_2$ deposition characterization

Figure A1.1. Thickness and roughness characterization of ALD HfO$_2$. a) Thickness measurements of HfO$_2$ deposited on a $<111>$ Si wafer in 7 different areas indicate a linear growth of HfO$_2$ with number of ALD cycles, with an average of 0.14 nm/cycle. b) AFM scans demonstrate increasing surface roughness of HfO$_2$ with number of ALD cycles.
Thickness of HfO$_2$ films was calibrated using ellipsometry to determine the mean thickness of HfO$_2$ per ALD cycle. We have assumed a literature refractive index of $n = 2.0$ for our measurements, which agreed with our measured trajectories for $\Delta$ and $\Psi$. Sixteen ALD cycles of HfO$_2$ were deposited on a bare <111> silicon wafer, and the thickness of the resulting layer was measured with ellipsometry in several spots on the wafer. This process was repeated several times on the same wafer. HfO$_2$ thickness was found to increase linearly with the number of ALD cycles. Figure A1.1a shows this measured HfO$_2$ thickness after various numbers of ALD cycles, yielding a mean deposition rate of 1.4 Å/cycle.

Surface roughness of deposited HfO$_2$ surfaces was determined using an AFM. AFM scans of the HfO$_2$-coated Si wafers described above were performed after each series of ALD cycles. Figure A1.1b displays the relationship between number of ALD cycles and measured RMS or average surface roughness.

A1.2. Elemental analysis of freestanding HfO$_2$ membranes

Energy dispersive x-ray spectroscopy (EDS) maps of different elements were obtained at Harvard University’s Center for Nanoscale Systems with a Zeiss Libra 120 TEM at 80 kV equipped with and EDAX Genesis EDS system. For each elemental map, the peak signals from K- and L-series X-rays were added up at each pixel. Figure A1.2a shows the clear hafnium signal from the ALD-deposited membrane, with silicon, nitrogen, oxygen, and fluorine (present from SF$_6$ etching) peaks identified in A1.2b. In Figure A1.2c, we show the results of elemental maps of Si and N. These are EDS scans where the signals from the peaks identified in 2b have been added up at each pixel. In the dark square, where the SiN has been etched, we see the Si and N signal drop to the noise level, as expected from fabrication. The Hf and O scans contain little information (see 2c), as HfO$_2$ is present at the same thickness throughout the whole image.
Figure A1.2. EDS characterization of the HfO$_2$ membrane. a) EDS spectrum of various hafnium lines taken from the free-standing HfO$_2$ region of the membrane indicating presence of hafnium. b) EDS spectra showing carbon, nitrogen, oxygen, fluorine, and silicon also present in the film. c) EDS elemental map from an area of membrane that has been etched down to free-standing HfO$_2$. The brightness of each pixel represents the signal from the corresponding peak in b). We clearly see silicon and nitrogen removed in square region where we etch. As expected, the hafnium signal is uniform, as it was deposited uniformly over the whole area.
A1.3. HfO$_2$ membrane crystallization

When the HfO$_2$ membrane is exposed to a slightly condensed electron beam in the JEOL 2010FEG transmission electron microscope (TEM), it slowly changes from an amorphous HfO$_2$ layer to a polycrystalline one. This process occurs at an electron dose of $10^6$ e/nm$^2$, which is not enough to drill a pore. The HfO$_2$ membrane in the TEM image in Figure A1.3a has been exposed to the partially condensed beam. The central area shows crystalline atomic arrangement and the surrounding membrane exhibits greater thickness variation than the rest of the HfO$_2$ layer. This variation is evidenced by the changing contrast around the crystallized region, which correspond to sample thickness variations in a TEM image. Figure A1.3c illustrates this crystallization by showing reduced FFT’s of the amorphous and crystalline regions. The region that has been exposed to the condensed beam exhibits peaks in its FFT characteristic of crystallization, while the amorphous region, which has not experienced condensed electron
beam irradiation, has an FFT with no clear structure. Figure A1.3b shows this area after a pore has been drilled with the fully condensed electron beam. The pore may be made fully crystalline, but after piranha cleaning, the crystalline sections of the membrane are completely removed, perhaps due to a strain mismatch between it and the surrounding amorphous material.
A1.4. Dependence of pore conductance on diameter and thickness

We determine the diameter and thickness of the pore using a geometrical model of conductance, as described in the main text.\cite{Wanunu, 2010 #105} In the Figure A1.4, we plot the measured conductance of pores of several different diameters. On the same axes, we plot the conductance curves from the geometrical model for three different pore heights. The height of $h = 4.5$ nm agrees best across all pores, with one 2 nm pore and some as thick as 7 nm. However, based on the ALD calibration presented in A1.1, we expect that each pore is in the thickness...
range of 2 – 3 nm. As discussed in A1.3, the drilling process crystallizes membrane in the area of the pore, and generally causes local membrane thickness variation. We attribute the pores’ unexpectedly high thicknesses to an increase in membrane height due to this e-beam induced change in HfO$_2$ structure. It seems that pores drilled in the smallest amount of time possible show the least increase in membrane thickness.
A1.5. Determination of peak dwell time

To determine the peak dwell time, \( \langle t_d \rangle \), we first plot a histogram of the logarithm of every \( t_d \) in a given data set. For the double-stranded DNA (dsDNA) data, this histogram is normally Figure A1.5. Dwell time analysis. a) The histogram of dwell times for a dsDNA experiment fits to a single Gaussian. The position of the Gaussian’s mean is quoted as \( \langle t_d \rangle \). b) For ssDNA, the dwell times fit to two Gaussians, with the longer-time peak representing translocations.

To determine the peak dwell time, \( \langle t_d \rangle \), we first plot a histogram of the logarithm of every \( t_d \) in a given data set. For the double-stranded DNA (dsDNA) data, this histogram is normally
distributed. We perform a least squares fit of the histogram to a Gaussian. The position of this Gaussian’s mean is quoted as \( \langle t_d \rangle \), and represents the characteristic dwell time for the experiment. This analysis is illustrated in Figure A1.5a for the dsDNA data at 250 mV. For ssDNA experiments, the log histogram has two peaks—one representing collision events and the other translocations. We then fit the data to two Gaussians. The position of the mean for the longer dwell time peak is quoted as \( \langle t_d \rangle \). The width of the corresponding Gaussian is the spread given in Figure 1.5d in the main text. This analysis process is illustrated in Figure A1.5b for data from the 1.4 nm pore at 500 mV.
A1.6. Comparison of dsDNA translocation in SiN and HfO$_2$ pores

In Figure A1.6, we present a comparison the 3.6 nm HfO$_2$ pore studied in the main text with a SiN pore of similar diameter from a previous study. We compare the interactions between dsDNA molecules and these pores by examining molecular velocity in the pores. The y-axis in Figure A1.6 represents the average velocity of a molecule in the pore obtained by dividing the length of the sample dsDNA polymer by the mean dwell time. If the translocation process were simply electrophoretic, we would expect a linear dependence of this velocity on applied voltage. However, we see an exponential dependence for both pores. This shows that there is an

![Graph showing exponential dependence of average molecular velocity on applied voltage for HfO$_2$ and SiN pores.](image)

Figure A1.6. Comparison of dsDNA/pore interaction in HfO$_2$ and SiN pores. Exponential dependence of average molecular velocity on applied voltage is evidence of strong interaction between the molecule and the pore walls. The steeper exponential dependence for HfO$_2$ than for SiN indicates stronger interaction with dsDNA.
energetic barrier to the translocation process. The origin of this barrier is the interaction between the DNA molecules and the walls of the pore.\textsuperscript{46b} By comparing the constants for each exponential, we see a stronger exponential dependence for the HfO\textsubscript{2} pore than for the SiN pore. Given that the pores have nearly the same diameter, we interpret this finding as evidence that the HfO\textsubscript{2} pore walls interact more strongly with dsDNA than those of the SiN pore.
A1.7. Sequence of the 89 nucleotide molecule

The 89 nucleotide molecule was purchased from Stanford Protein and Nucleic Acid Facility. Its sequence is

CTCACCTATCCCATCATACTATCATATCTACATCTACCATTCTACGATCTCACTATCGCATTTCTCATGCAGGTCGTAGCCXZ in the 5' → 3' direction. X is an abasic residue and Z is a 3 spacer C3 CPG modification.

A1.8. α-hemolysin (α-HL) measurements of single-stranded DNA

![Graph showing translocations of the 89 nucleotide molecule in α-hemolysin.](image)

Figure A1.7. Translocations of the 89 nucleotide molecule in α-hemolysin.

The fractional blockage of the HfO₂ nanopores in this study is quite close to that of α-hemolysin.
To compare the HfO$_2$ nanopores in our study to α-HL, we performed an α-HL translocation experiment with the same 89-nucleotide molecule used in this thesis (n ~ 300). In Figure A1.7, we plot the fractional blockades ($\Delta I/I_0$) for an experiment carried out at $V = 120$ mV. We interpret the peak near $\Delta I/I_0 \approx 0.4$ as collision events and the peak at $\Delta I/I_0 \approx 0.8$ as translocations. The fractional blockage for the 1.4 nm HfO$_2$ pore in our study is 0.83, which is quite close to that of the α-HL.
A1.9. Determination of capture rate for ssDNA

As with dsDNA, we expect the capture of ssDNA into our pore to be Poissonian, and for the distribution of inter-event times to be exponential. We then perform a least squares fit of our $\delta t$ histograms to an exponential. In Figure A1.8, we show these histograms and their fits at each experimental voltage. The inverse time constant of each exponential fit is the capture rate at the given voltage.

Figure A1.8. ssDNA capture rate analysis. a) The histograms of inter-event times for ssDNA in a 1.7 nm HfO$_2$ pore with exponential fits. b) The histograms of inter-event times for ssDNA in a 1.4 nm HfO$_2$ pore with exponential fits.
Appendix 2: Chapter 3 Supplementary Material

A2.1. Data analysis details

Current traces were analyzed using OpenNanopore, an open source translocation software package developed by the Radenovic Group at EPFL. The code was modified to allow data importing from Chimera Instruments format. OpenNanopore fits each translocation event with a rectangular pulse, as shown in Figure A2.1, and extracts three parameters for each event. The dwell time, \( t_d \), is the residence time of a molecule in the pore. The current blockage, \( \Delta I \), is the amount of current the molecule excludes from the pore. The inter-event time, \( \delta t \), is the length of time between the beginning of one event and the beginning of the next. Since multi-level blockade pulses were extremely rare, the code was modified to bypass multi-level events.

Current signals from nanopores are usually considered in the time domain, while random noise is best described in the frequency domain. Nanopore traces are invariably low-pass.

Figure A2.1. Protein Data Analysis. (left) Close-up trace of two successive events for protK at \( V = -100 \) mV. OpenNanopore, the analysis software used, fits each translocation even to a rectangular pulse, and extracts the \( \Delta I, t_d, \) and \( \delta t \) parameters, displayed in blue. Raw data is in black and the fit from OpenNanopore appears in red. (right) Series of analyzed events for ProtK. All experiments were carried out in the dilute regime, i.e., \( \delta t \gg t_d \).
filtered to reduce noise, but this can filter out some of the signal as well. In thinking about the detection efficiency of brief protein translocations, it can be useful to consider a signal representation which includes both time- and frequency-domain information. Fig. A2.2 shows a spectrogram produced from a recording of protK translocations through an HfO$_2$ nanopore. (A spectrogram plots the Fourier transform of a short segment of the data as a function of time; Fig. A2.2 was produced using a 64-microsecond window computed every 16 microseconds.) The spectral content of longer-duration events is concentrated at lower frequencies, and thus the features of these events are impacted less by the low-pass filter. Faster events’ energy is spread more widely across the spectrum, and thus low-pass filtering can remove significant fractions of the signal. It is clear, for example, that 10 kHz bandwidth would be inadequate to capture the full energy of the signals in Fig. A2.2. In this work we used a digital low-pass filter with a 250 kHz cutoff frequency.

A range of different algorithms can be used to identify and characterize nanopore events. The simplest is a low-pass filter followed by a threshold-crossing level detector. This approach is not ideal for datasets with appreciable noise, and more advanced techniques can be utilized which incorporate statistical models for both the signal and noise. The OpenNanopore software package used in this work implements a level detector that is optimized for fitting abrupt stepwise signals in the presence of Gaussian noise.
A2.2. Size, charge of proteins and effect of electro-osmosis

ProtK and RNAse volumes were independently determined using two methods. ZetaPALS analysis was performed at Brookhaven Instruments (Holtsville, NY), which measured effective diameters of $4.76 \pm 0.05$ nm for ProtK and $3.68 \pm 0.03$ nm for RNAse A (see results tabulated in Figure A2.3). Similarly, the effective diameter of the RNase:ProtK complex was measured at 1M KCl buffer to be $8.00 \pm 0.42$ nm. Additionally, the volume of each protein was computed with the Vorlume algorithm. In this computation, a solvent-accessible model was used, where each residue’s radius was expanded by a 1.4 Å van der Waals shell. The algorithm returned a solvent-accessible volume of $45.9 \text{ nm}^3$ for ProtK and $32.7 \text{ nm}^3$ for RNAse A.

Figure A2.2. Frequency content of nanopore current signals. (a) A segment of raw data recorded with a bandwidth of 1 MHz. The signals are obscured by noise. (b) A spectrogram of the trace, showing the frequency content as a function of time (rainbow color scheme, intensity increases from blue to red). (c) The same signal after a digital low-pass filter with a cutoff of 200 kHz reveals the events.
Both Proteinase K and RNAse A are positively charged in the 1 M KCl, 10mM Tris, 1mM EDTA, pH ~7.7 experimental buffer. Support for this comes from early measurements of the pI for each protein, which obtained pI = 9.6 for RNAse A and pI = 8.9 for Proteinase K. In our experimental pH of 8.1, we would expect these molecules to be positively charged.

Figure A2.3. Dynamic light scattering measurements. Hydrodynamic diameters of proteinase K, RNAse A, and a 1:1 mixture of the two proteins at 1M KCl. Note that for the complex the report inadvertently displayed the effective radius, while for the isolated proteins measurements displayed are the effective diameters.
A2.3. Dwell time distributions and estimation of $F_{\text{obs}}$

The $t_d$ distribution used in the main text comes from a first passage calculation using a drift-diffusion model outlined in previous studies.\textsuperscript{86c, 87} Figure A2.4 displays the fits to this distribution for ProtK and RNase at several voltages in the 5.2 nm HfO\textsubscript{2} pore. Once we have fit a given set of $t_d$ values to this distribution, we may use the distribution equation to estimate the fraction of detected events. Our time resolution limits detection to events of ~2.5 $\mu$s or more. Shorter events are either severely distorted, which makes them erroneously appear in the first 1-2 bins, or not detected at all. However, we can fit our measured dwell times to the distribution equation from the drift-diffusion model. Once we have this total distribution function of dwell times, we may estimate the fraction of translocation events that we detect. We do this by dividing the area under the distribution curve between $t = 2.5 \mu$s and $t = \infty$ (the temporal region we can detect) by the total area under the curve. This process is illustrated in Figures A2.4. Based on this, using

![Figure A2.4. Fitted $t_d$ distributions. RNAse A (left) and Proteinase K (right) distributions fitted using freely varying $D$ values. Fitted distributions are shown by the black curve. These curves are used to estimate the fraction of detected events ($F_{\text{obs}}$) at each voltage. This fraction is the area under the curve from $t = 2.5 \mu$s to $t = \infty$, represented by the green region, divided by the total area under the curve.](image-url)
bulk D values we miss 76-96% of the events for RNase and 62-89% of the events for ProtK, as shown in Figure A2.5.

Figure A2.5. Estimated fraction of observed events, $F_{\text{Obs}}$, for ProtK and RNase in the 5.2 nm HfO$_2$ pore discussed in the main text.

A2.4 Continuous electrical traces of protein translocations
Figure A2.6. Continuous Protein Data. (a) Continuous, 3-second traces current traces for RNase (1 nM) and ProtK (69 nM) at $\Delta V = -100$ mV, -125 mV, and -150 mV in a 5.2 nm HfO$_2$ pore. (b) Traces of RNase (~30 nM), ProtK (~40 nM), and a 1:1 mixture of the two through a 4.8 nm SiN nanopore. (c) Sample events from the RNase:ProtK complex (all traces low-pass filtered to 250 kHz).
Here we present supplementary data for the 4.8 nm diameter SiN pore discussed in the main text. Figure A2.7 shows scatterplots of translocation data for RNase and ProtK through this pore.

Figure A2.7. SiN Scatterplots. Scatterplots of a) RNase and b) ProtK translocations from -100 mV to -200 mV in a 4.8 nm SiN pore.
In Figure A2.8, we present the dwell time distributions for the SiN data, along with the corresponding fits to the 1-D first passage model (Eq. 3.1 in main text).

Figure A2.8. Dwell time distributions for the 4.8 nm SiN nanopore at $\Delta V = -75$ mV, -100 mV, -125 mV, and -150 mV. D and v values obtained from these fits are displayed in Figure 5 of the main manuscript text.
Mobilities of proteins in SiN pore: As with the 5.2 nm HfO$_2$ pore, from the distributions fitted in Figure S5b, we extract drift velocities. These velocities increase linearly with magnitude of applied voltage, as shown below in Figure A2.9. Although the RNase data somewhat deviates for this pore at low voltage, the general trend is expected for electrophoretic transport through the pore. Mobility values for the SiN pore are slightly smaller than for the HfO$_2$ pore, although the mobility difference relationship between the two proteins is maintained.

Figure A2.9. SiN Transport Parameters. Drift velocity ($v$) vs. applied voltage ($\Delta V$) for the SiN nanopore with $d = 4.8$ nm, $h_{\text{eff}} = 6.2$ nm. Results from these data are plotted in the scatter plot in Figure 3.5 of the main text.
Dwell-time distribution of 1:1 RNase:ProtK complex: The measured volume of the complex resulting from RNase/ProtK mixture is nearly the sum of the two proteins’ volumes (see above). However, we see in Figure A2.10 that the mixture displays dwell times that are orders of magnitude longer than either protein individually. Because the pore in this study has $d = 4.8$ nm, we believe the complex formed from the RNase/ProtK mixture is too large to fit through the pore unhindered. These long dwell times may be due to dissociation or unfolding of the complex.

A2.6. DNA Dwell Time Distributions
Figure A2.11. Distributions of dwell times for 100 base pair DNA in a 5nm HfO$_2$ pore. Insets provide continuous, 3-second example traces for 100 base pair at each voltage. As with the proteins, apparent event rate drops at the highest voltage as events become too fast for the displayed bandwidth.

Figure A2.11 displays the measured dwell time distributions and resulting fits for 100 base pair DNA translocating through a 5 nm HfO$_2$ pore. The drift velocities from these fits are displayed in Figure 3.4 in the main text.
Appendix 3: Chapter 4 Supplementary Material

A3.1 NZMW Fabrication

The starting substrate was a 175 µm-thick, 100 mm diameter, <100> silicon wafer. Prior to LPCVD, the wafers were cleaned with a standard RCA process, rinsed with deionized water, and spun dry. Next, they were immediately placed in a MRL Industries LPCVD furnace. 35 nm of SiN was deposited on either side of the wafers (Figure A3.1, step 1). One side will eventually be SiN membranes; the other will be used as a KOH etch mask to etch down to the membranes.

The next major step was e-beam lithography (EBL). The EBL process closely followed previously outlined protocols with negative e-beam resist. Wafers were first cleaned for ten minutes in the 400 W oxygen plasma of a Glenn 1000 plasma asher to remove any residual water. Subsequent priming with hexamethyldisilazane in a YES Vapor Prime Oven ensured proper adhesion of e-beam resist to the silicon nitride. Upon removal from the vapor priming oven, negative e-beam resist NEB-31 was spun onto the wafers at 4500 RPM for 60 seconds and baked at 115 °C for two minutes (Figure A3.1, step 2). The ZMW pattern, which consisted of arrays of circles of nominal 80 nm diameter, was exposed in a JEOL 6300FS e-beam writer. In addition to the circles, two alignment marks were exposed in the resist. These would line up with photolithography marks later in the process to ensure that each ZMW array is aligned with a SiN membrane. After exposure the resist was baked for two minutes at 90 °C. The wafers were then immersed in MF321 development solution for 30 seconds followed by two successive baths of deionized water. The samples were dried under a gentle stream of nitrogen. Before metallization, wafers were placed in a Glenn 1000 for one minute of descumming 100 W oxygen plasma (Figure A3.1, step 3). 100 nm of thermally evaporated aluminum was deposited immediately following the descum step (Figure A3.1, step 4). To lift off the e-beam resist and form ZMWs, the wafers were immersed for three hours in 1165 Remover solution heated to 68 °C, with
ultrasonication for the final fifteen minutes. Following a thorough rinse in deionized water, the wafers were descummed once more in a 100 W oxygen plasma (Figure A3.1, step 5). Prior to photolithography on the wafer’s reverse side, ZMWs were inspected in a scanning electron microscope (SEM) to verify metallization, liftoff, and uniformity.

Following fabrication of ZMWs with EBL, photolithography was used on the other side of the wafer to define the pattern for KOH etching down to membranes. First, Shipley Microposit S1818 positive photoresist was spun on the non-ZMW side of the wafers (Figure A3.1, step 6). Resist was exposed in a Suss MicroTech MA/BA 6 contact aligner. A photomask with alignment marks was used. These marks were identical to those written during the EBL step. While the wafer is being aligned, the MA/BA 6 projects a live image of the ZMW side of the wafer over an image of the front-side alignment marks in the photomask. The sample may then be translated and rotated until the marks overlap, ensuring alignment of ZMW arrays with membranes defined during photolithography. After this back side alignment, the resist was exposed and then developed in MicroDev for one minute (Figure A3.1, step 7). Next, the SiN was etched with SF6 plasma in a Technics Micro-RIE Series 800 to expose windows of bare silicon for KOH etching down to free-standing SiN ZMW membranes (Figure A3.1, step 8).

Before wet etching of silicon, it is essential to somehow protect the ZMW aluminum nanostructure from alkaline KOH etching solution. To accomplish this, the aluminum layer was covered with Brewer Scientific ProTEK B1-18. This polymer protects features from highly basic solutions, but may be removed with organic solvents after KOH etching. The ProTEK was spun onto the ZMWs at 2500 RPM for one minute, followed by successive bakes at 115 °C for two minutes and 205 °C for one minute (Figure A3.1, step 9). The wafer was then tightened between the o-rings of an Idonus PEEK backside protection wafer chuck to further protect the ZMWs from KOH. The sample was placed in 40% KOH at 60 °C for 8 hours to etch the exposed silicon squares down to silicon nitride membranes, each supporting an array of ZMWs (Figure A3.1, step 10). Following a thorough rinse in deionized water, the wafer was soaked for several hours in
successive baths of methyl isoamyl ketone, n-methyl pyrrolidone, and isopropyl alcohol to remove the ProTEK. This process results in a wafer of roughly 200 chips, each with a free-standing SiN membrane containing an array of ZMWs (Figure A3.1, step 11).

To use these chips for nanopore experiments, they will need to be treated with piranha solution (H$_2$O$_2$ and H$_2$SO$_4$) to hydrophilize the nanopore surface. As with the KOH, the ZMWs must be protected from this harsh chemical treatment. In order to do this, the ZMWs were coated with a 11 nm-thick layer of SiO$_2$ by atomic layer deposition (ALD) at 250 °C. The ALD layer coats the entire ZMW surface conformally, ensuring good chemical protection (SEE SI), but is still thin enough that a pore may easily be fabricated in it (Figure A3.1, step 12).
A3.2. Piranha cleaning of ZMW chips

Cleaning with piranha solution (1:4 H$_2$O$_2$:H$_2$SO$_4$) is essential to measuring stable open pore currents from small solid-state nanopores. Piranha, however strips metal from nanopore chips.
as shown in Figure A3.2a. Therefore, in order to successfully integrate ZMWs into a solid state nanopore system, the metal of the ZMWs must be somehow protected from the piranha treatment. To do this, we have coated the ZMW surface with 11 nm of ALD. ALD coats an entire surface conformally, so a thin layer will penetrate a ZMW and coat the inside walls. This relatively thin layer is enough to protect ZMWs from piranha, as shown by the photos in Figure A3.2a, and the TEM image of an ALD-coated ZMW in Figure A3.2b after 5 minutes of immersion in heated piranha.

![Figure A3.2. Piranha Photos. (a) Photograph of four NZMW chips. The chips in the left column have no SiO\textsubscript{2} coating, while the ones in the right column do. The chips in the top row have not had piranha treatment, while the chips in the bottom row have had five minutes of piranha treatment. The chip with no SiO\textsubscript{2} has had nearly all of its Aluminum removed during piranha. The chip with SiO\textsubscript{2} coating has no visible difference from the untreated NZMW chips. (b) A high resolution TEM image of a SiO\textsubscript{2}-coated ZMW after five minutes in piranha shows that the ZMW structure is intact.](image)
A3.3 YOYO-1-labeled DNA traces

In Figure A3.3, we show twelve ZMW traces from the experiment discussed in Figure 4.5 of the main text. The bottom trace is from a NZMW.

Figure A3.3. Time traces of ZMW fluorescence with YOYO-labeled 6000 base pair DNA. The bottom trace is from a NZMW; the others are from conventional ZMWs. Spikes in fluorescence correspond to DNA capture into a given ZMW.

A3.4 NZMW optical cell

The NZMW chips were assembled in a custom-made PEEK cell, shown in the photos of Figure A4.4. The chips were glued to the cell with a quick-curing silicone paste (Smooth-On Ecoflex 5). A No. 1 glass coverslip was then glued over the chip with the same silicone elastomer. Following
assembly, electrodes were screwed into the cell, as shown in Figure A4.4f. The cis and trans sides of the membrane were then filled with the desired buffer using syringes. The fully assembled cell was then screwed into a Faraday cage on an inverted fluorescence microscope with an integrated Axopatch 200 headstage for experiments.

Figure A3.4. NZMW optical cell. The chip is glued onto the circular well in the side of the cell shown in b. A No. 1 coverslip is then glued over the chip, as shown in f. The trans side of the membrane may be filled with buffer via the ports shown in a and d. The cis side buffer is filled through the top opening shown in c. After wetting the chips, the electrodes may be inserted to their ports on the side of the cell. f shows the fully-assembled cell. The trans side buffer may be swapped while the cell is assembled via the ports in a and d.

A3.5 Determination of DNA on-time
A ZMW was considered “on” if its fluorescence level exceeded three standard deviations above its mean baseline level, which was measured with no applied voltage. On-rates, $k_{on}$, were calculated for NZMW and non-pore ZMW devices using:

$$k_{on} = \frac{t_{on}}{t_{on} + t_{off}}.$$  

where $t_{on}$ is the total on time and $t_{off}$ is the total off time for a particular trace.

A3.6 DNA-streptavidin gel

To show that streptavidin binds to our biotinylated DNA we incubated our 5’-biotin- 1,003-bp DNA molecule with Alexafluor-647- labeled streptavidin (A647Stv) in a 4:1 DNA:Stv ratio for 15 minutes, followed by analysis using agarose gel electrophoresis (see Figure A3.5). We first imaged the red channel to show that A647Stv binds to the DNA (red-toned bands in the image), and then imaged the DNA after staining the gel with ethidium bromide. Even though a 4:1 DNA:Stv ratio was used, we found that the predominant complex formed had a 2:1 DNA:Stv stoichiometry, whereas much of the DNA remains unbound (labeled as 1:0 in the image). This excess of free DNA explains the presence of spikes in the electrical traces in Figure 3 for the DNA:Stv complex.
To reduce membrane photoluminescence without compromising physical integrity, we fabricated ZMWs on a 35 nm layer of SiN with 20 nm of SiO$_2$ on top, and etched through most of the SiN prior to pore drilling (see Figure A4.1a). SiN has innate photoluminescence in blue and green excitation wavelengths, overlapping with the spectra of SMRT sequencing analogs (Figure A4.1b, top). The bottom plot of Figure A4.1b illustrates the drastic reduction in photoluminescence with etching.
A4.2. Sequencing templates

The sequence of the 72-nucleotide circular template used was

CTC AGA CAC TGA CTG TGA CTG TGA CTG TGA CTG TGA CTG TGA CAC TGA CAC
TGA CTG AGA CTC ACA

The 20,000 bp template was a SMRTbell, which is a dumbbell-shaped template illustrated by the cartoon in Figure A4.2.
Figure A4.2. SMRTbell. The shape of the SMRTbell is illustrated by the above schematic.

Sample DNA is ligated to dumbbell adapters, then bound with primer and polymerase.
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