Molecular Sensing with Protein and Solid-State Nanopores

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Abstract

In the past 15 years nanopore sensing has proven to be a successful method for probing a variety of molecules of biological interest, such as DNA, RNA and proteins. Of particular appeal is this technique’s ability to probe these molecules without the need for chemical modification or labeling, to do so at physiological conditions, and to probe single molecules at a time, allowing the possibility for results masked in bulk measurements to come to light. In this thesis these advantageous properties will be used in work on both a synthetic (solid-state) nanopore system and an engineered biological nanopore. I will describe the techniques for producing solid-state nanopores in thin membranes of silicon nitride and how these nanopores can be integrated into a fully functioning nanopore sensor system. I will then explore two applications of this system. First, a study of adsorption of bovine serum albumin (BSA), a protein found in blood serum, to the inorganic surface of nitride at the single molecule level. A simple physical model describing the behavior of this protein in the nanopore will be shown. Second, a study of the binding of the nucleocapsid protein of HIV-1 (NCp7) to three aptamers of different affinity, specifically three sequence 20mer mimics of the stem-loop 3 (SL3) RNA—the packaging domain of genomic RNA. Additionally, N-ethylmaleimide, which is known to inhibit the binding of NCp7 to a high-affinity SL3 RNA aptamer, will be used to demonstrate that the inhibition of the binding can be monitored in real time.

Following these applications of the solid-state nanopore system, I will explore the geometry of a newly engineered biological nanopore, FhuA ΔC/Δ4L, by using inert polymers to probe the nanopore interior.
Molecular Sensing with Protein and Solid-State Nanopores

by

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Chapter 1
Introduction
Introduction

A nanopore is a small hole in an insulating membrane. There are two types of nanopores: synthetic nanopores and biological nanopores. For synthetic nanopores, the hole is formed by the removal of material from the insulating membrane. The dimensions of this hole are dependent on the method of removal. Typical synthetic nanopores have diameters ranging from as small as a single nanometer to as large as several hundred nanometers. The thickness of synthetic nanopores is typically several tens of nanometers, though thicknesses as low as 0.3 nm have been achieved by creating nanopores in single sheets of graphene. Biological nanopores are membrane proteins that evolved to enable the transport of materials across cell membranes. The most widely used protein nanopores are the β-barrel pores, such as α-hemolysin from Staphylococcus aureus. The diameter of these pores is typically less than 2 nanometers, with a length near 5 nm.

In the past two decades nanopores have been used as highly sensitive detection systems for exploring the properties of small analytes, particularly molecules of biological interest. The small dimensions of a nanopore permit a single molecule to be confined within the nanopore, allowing for the extraction of information relating to that molecule’s physical properties. This is typically achieved by placing ionic solution on either side of the insulating membrane, applying of voltage across the membrane and measuring the resulting ionic current through the nanopore. When the analyte is in the nanopore interior, the current fluctuates and information can be extracted by monitoring current fluctuations. Some more modern techniques have looked at the possibility of
placing small electrodes on either side of the nanopore and using the tunneling of electrons to extract information.¹

That single molecules can be inspected at a time is significant. It means that nanopores are extremely sensitive. It also means that the nanopore system can extract information that might be masked in bulk measurements, which measure the aggregate properties of many molecules. There are several other advantageous properties of this system. For example, unlike fluorescence-based experiments, no labeling or chemical modification of the analyte is needed. Experiments may be carried out in salt solutions mimicking physiological conditions, allowing for analytes to be studied in biologically active states, unlike during electron microscopy. Another significant property of the nanopore system is the voltage drop across the nanopore. This sets up an electric field in the nanopore interior that can be controlled by applying different voltages. This property has been exploited to perform force microscopy experiments.

In this thesis we will make use of several of these advantages in work using both a synthetic (solid-state) nanopore system and an engineered biological nanopore. We will first describe the techniques for producing solid-state nanopores in thin membranes of silicon nitride and how these nanopores can be integrated into a fully functioning nanopore sensor system. We will then explore two applications of this system. First, we study the adsorption of bovine serum albumin (BSA), a protein found in blood serum, to the inorganic surface of nitride at the single molecule level.² We build a simple physical model describing the behavior of this protein in the nanopore. Second, we study the binding of the nucleocapsid protein of HIV-1 (NCp7) to three aptamers of different affinity, specifically three sequence 20mer mimics of the stem-loop 3 (SL3) RNA—the
packaging domain of genomic RNA. After this, we used N-ethylmaleimide, which is known to inhibit the binding of NCp7 to the high-affinity SL3 RNA aptamer, to test if inhibition of the binding can be monitored in real time. Following these applications of the solid-state nanopore system, we will explore the geometry of a newly engineered biological nanopore, FhuA ΔC/Δ4L, by using inert polymers to probe the nanopore interior.

We will explore the immediate future of these two systems, looking at preliminary results on the functionalization of solid-state nanopores and the possibility of bringing together the solid-state nanopore platform and the engineered FhuA ΔC/Δ4L to form a hybrid system that incorporates the advantages of both synthetic and biological nanopores. Finally, we will speculate on the future prospects of the nanopore field as a whole.

Before moving on to these subjects, it will be necessary to describe some of the basic physics behind nanopores, first looking at the physical properties of nanopores themselves, then moving on to nanopore interaction with analytes. With this knowledge, it will be possible to understand the basic principles behind nanopore detection, the resistive-pulse technique. It will also be useful to look at some of the seminal contributions to the nanopore field, so that this thesis can be put in historical perspective.

**The basic properties of nanopores**

To begin, consider a simple conical nanopore in a perfectly insulating membrane separating baths containing ionic solution of resistivity $\rho$. Ohm’s law will give the resistance of the nanopore,
\[ R_{\text{pore}} = \rho \frac{l}{\pi a^2} \]  
(1)

Where \( l \) is the pore length and \( a \) is the pore radius.\(^7\) In addition to this term, there will be an access resistance contribution due to the resistance along the convergent paths from the bulk solution to the opening of the pore. The contribution on each side is given by,\(^7,8\)

\[ R_{\text{access}} = \frac{\rho}{4a} \]  
(2)

Finally, if the nanopore sidewalls are highly charged, there will be an effect on resistance. This is attributed to the attraction of counterions to the nanopore walls

\[ R_{\text{side}} = \frac{l}{2\mu\sigma \pi a \sigma} \]  
(3)

Where \( \mu\sigma \) is the solution mobility of the adsorbed counterions and \( \sigma \) is the surface charge density of the pore sidewalls.\(^9,10\) The total resistance is then,

\[ R_{\text{total}} = \frac{\rho}{\pi a^2} \left( l + \frac{\pi a}{2} + \frac{la}{2\mu\sigma \rho \sigma} \right) \]  
(4)

We can re-write the above to find the expected current, \( I \), of ions through a nanopore at a given voltage \( V \),

\[ I = \frac{V}{\rho \left( \frac{l}{\pi a^2} + \frac{1}{2a} \right)^{-1} + \frac{2V \mu\sigma \pi a \sigma}{l}} \]  
(5)

Note that as the pore length becomes shorter, the current of the nanopore increases. This will be important later when discussing the used of ultra-thin nanopores, whose greater conductance improves the current-to-noise ration of the nanopore system.

Since their geometry is relatively simple and their dimensions relatively large, the cylindrical pore calculation using Ohm’s law serves as a good estimate of
nanopore behavior for solid-state nanopores. In biological pores the story is subtler, and smaller more complex geometries mean that other effects, such as electro-osmotic flow and the diffusion-limiting translocation rate of ions, come into play. However, as is discussed in chapter 6, Ohm’s law can still give order of magnitude estimates of biological nanopore conductance.

As was mentioned earlier, information about analytes in nanopore sensing is extracted from fluctuations of nanopore current. What sort of current change will occur when an analyte enters a nanopore? Consider the \( R_{\text{pore}} \) given above. We may generalize this term to

\[
R = \rho \int \frac{dz}{A(z)} \quad (6)
\]

Where \( A(z) \) is the pore’s effective cross-sectional area perpendicular to a point on the axis \( z \), which passes through the nanopore. The integral is along the length of the pore.

Now consider what happens when a small, non-conducting, spherical obstruction of diameter \( d_m \) is introduced into a nanopore of diameter \( d_p \). The resistance can be expressed as,

\[
R_b = \rho \int_0^{L-d_m} \frac{dz}{\pi \left( \frac{d_p}{2} \right)^2} + \rho \int_0^{d_m} \frac{dz}{\pi \left( \frac{d_p}{2} \right)^2 - \left( \frac{d_p}{2} - \left( \frac{d_p}{2} \right)^2 - \left( \frac{d_p}{2} \right)^2 \right) \right)^2} \]

\[
R_b = \frac{4\rho}{\pi} \left[ L-d_m \frac{d_m}{d_p^2} + \frac{\text{ArcTan} \left( \frac{d_m}{\sqrt{d_m^2+d_p^2}} \right)}{\pi \sqrt{d_m^2+d_p^2}} \right] \quad (7)
\]

The current of a nanopore with the obstruction is then,
\[
I_b = \frac{V}{R_b} = \frac{\pi V}{4 \rho} \left[ \frac{L-d_m}{d_p^2} \right]^{1} + \frac{\text{ArcTan} \left[ \frac{d_m}{\sqrt{-d_m^2 + d_p^2}} \right]}{\pi \sqrt{-d_m^2 + d_p^2}} \] (8)

The change in current due to this obstruction is,

\[
\Delta I_b = I_0 - I_b = \frac{\pi V d_p^2}{4 \rho L} - \frac{\pi V}{4 \rho} \left[ \frac{L-d_m}{d_p^2} \right]^{1} + \frac{\text{ArcTan} \left[ \frac{d_m}{\sqrt{-d_m^2 + d_p^2}} \right]}{\pi \sqrt{-d_m^2 + d_p^2}} \] (9)

Expanding in series and retaining up to cubic terms gives

\[
\Delta I_b \cong \frac{\pi V d_m^3}{6 \rho L^2} \] (10)

Note is that the current blockade scales with the volume of the obstruction.

The equations for the current just discussed depend on a potential, \(V\), placed across the nanopore. This potential is applied via electrodes placed in the bath solution on both sides of the nanopore. Since the resistance of the solution is very low with respect to the nanopore, we may expect that the potential drop between the two electrodes occurs almost entirely within the nanopore itself. Simulation and experiment suggest that this is the case.\(^{1,12}\)

Molecular dynamics simulations of solid-state nanopores suggest that the potential gradient within the interior of the nanopore is nearly constant.\(^1\) This allows for a simple case estimate of the magnitude of a force experienced by a charged analyte, such as a protein, when it is in the nanopore.

Consider a protein with a net charge \(n\). The net force it will experience in the interior of a nanopore with a constant gradient will be \(F = n \frac{V}{L}\), where \(V\) is the applied potential and \(L\) is the nanopore length. Realistic values of \(n = -10e\), \(V = 200\) mV, and \(L = 20\) nm return a net force of \(\sim 15\) pico Newton (pN).
Experiments with optical tweezers holding DNA in a nanopore, suggest that the forces exerted by a nanopore are indeed of this magnitude.\textsuperscript{12}

The precise control of the electric field in the nanopore, allowing for forces to be exerted at picoNewton levels, suggests that it might be possible to actively manipulate bio-molecular systems of interest, as many such systems, like protein unfolding,\textsuperscript{13,14} are sensitive to forces in this range. One example is the adaptation of nanopores to force spectroscopy. Several studies have used nanopores to pull apart systems, such as DNA hairpins,\textsuperscript{15-17} EcoR1 from DNA,\textsuperscript{18} and exo1 from DNA.\textsuperscript{19}

\textbf{Figure 1.1:} Force spectroscopy measurements using a nanopore. (A) Simple cartoon representing the process of the electric field within a nanopore pulling apart a complex of DNA from a protein. The electric field acts on the highly charged DNA strand. (B) Free energy diagram showing the reduction of the free energy barrier, $\Delta G$, after a force, $F$, is applied. The reaction coordinate, $x$, is taken to be the distance along the axis passing through the nanopore.
Figure 1.1a shows a simple schematic of a force spectroscopy experiment, in which DNA is pulled from a binding protein. The residence time of the DNA in the pore will depend on how long it takes for the DNA protein complex to disassociate. In the simplest case, a single barrier in a free-energy landscape (Figure 1.1b) can model this dissociation. The free-energy barrier, $\Delta G(x)$, will decrease by $Fx$, where $x$ is the reaction coordinate, and the applied force is $F$. According to Bell’s formula\textsuperscript{20} the force dependent dissociation rate $k(F)$ will scale exponentially with the applied force: 

$$
 k(F) = k_0 \exp \left( \frac{Fx}{k_BT} \right), \nonumber
$$

where $k_0$ is the kinetic dissociation at $F=0$, $k_B$ is the Boltzmann constant, and $T$ is the temperature.

The above considerations assume that the voltage drop occurs entirely within the nanopore, yet the extension of the electric field beyond the nanopore, while very small in comparison to the field within its interior, does exist and has important implications in driving charged analytes to the nanopore entrance. It has been demonstrated\textsuperscript{21} that the potential experienced outside the nanopore can be expressed as,

$$
 V(r) = \frac{d^2}{8\pi r} \Delta V, \nonumber
$$

where $V(r)$ is the potential at a distance $r$ from the nanopore opening, $d$ is the nanopore diameter, $l$ is the nanopore length, and $\Delta V$ is the applied voltage difference across the electrodes. An analyte with an electrophoretic mobility $\mu$ will have a drift velocity $v(r) = \mu \nabla V(r)$.\textsuperscript{21}

The implications for analyte capture are immediate. It is typical to break the capture process of charged polymers into three separate regimes: the diffusion regime, the drift regime, and the barrier regime. In the diffusion
regime, the motion of the polymer is dominated by diffusion. At some distance from the pore the electrophoretic drift will begin to dominate. The distance at which this occurs is taken to be $r^*$, where the potential $V(r^*) = D/\mu$, with $D$ an diffusion coefficient of the polymer. We have,

$$r^* = \frac{d^2 \mu}{8lD} \Delta V$$  \hspace{1cm} (12)

This defines a half-sphere surrounding the nanopore. Analytes of the proper charge in this half-sphere will drift to the nanopore entrance. Theoretically, the entrance of analytes into this half-sphere is diffusion limited and sets a limit on the capture rate of analytes into the nanopore; however, under experimental conditions, it is the third regime that sets the true limit on the analyte capture rate.

The third, barrier, regime occurs near the entrance of the nanopore and is not well understood, though there has been extensive experimental inspection. It is typically modeled as an energy barrier near the pore entrance, such that entrance of analytes into the pore depends exponentially on the applied voltage, according to Kramer’s theory,

$$Rc = \omega \exp\left((q\Delta V - U)/k_B T\right)$$  \hspace{1cm} (13)

where $R$ is the capture rate, $c$ is the concentration, $\omega$ is a prefactor, usually interpreted as the number of attempts made at entrance, $q$ is the analyte charge, $U$ in the height of the barrier and $T$ is the temperature. While this theory works well for polymers such as DNA it is far less successful in predicting the capture rate of globular proteins into large nanopores.

The question of how an analyte behaves once it enters a nanopore is a subject of much theoretical debate. At the simplest level, scaling models have
been applied to the question of how neutral, hydrophilic polymers behave in a nanopore. These models are discussed more thoroughly in chapter 5 of this thesis. Yet, as we shall see later, even for this simplest case, the theory diverges from experiment.

Typically, full molecular dynamics simulations are performed to arrive at a better understanding of molecular transport through nanopores.\textsuperscript{28-30} Though in some cases modeling analyte passage through the pore as crossing a complex energy landscape can explain the qualitative features of observed transport.\textsuperscript{31}

For analytes of biological interest, the main confounding factor seems to be the interaction of the pore walls with the analyte, a process described in chapter 3 of this thesis. The magnitude of the interaction may be conveyed by asking how a analyte might move through a nanopore when no interaction occurs. Talaga and Li have modeled the passage of a protein though a nanopore as a one-dimensional biased diffusion,\textsuperscript{13} where the drift speed is given by $v_{\text{drift}} = \mu V/L$. Their results indicate the expected passage time is on the order of nanoseconds, whereas the observed passage time is three orders of magnitude larger.

While nanopore-analyte interaction is a theoretical complication, it is vitally important for the nanopore-sensing field. Without the increase in residence times from the nanosecond to the microsecond scale, sufficiently long data collection with modern amplifiers would be impossible. We will now briefly discuss some of the major achievements in nanopore sensing.
Historical Background

**Figure 1.2:** Coulter counter concept. The translocation of a protein through a nanopore creates a current blockade. (A) before the protein enters the pore an open current, \( I \), is observed. The partitioning of the protein into the nanopore (B) causes a drop in current, \( \Delta I \). (C) The exit of the protein from the pore returns the current to its original value after a time \( \Delta t \).

As can be seen from equation (10), the introduction of an analyte into a pore creates a current blockade proportional to the analyte volume. From equation (13) we see that the rate of analytes entering a pore is proportional to its concentration in bulk solution. While we do not have an equation relating to the residence time of an analyte in a nanopore, we have discussed that, for analytes of interest, this is related to its interactions with the pore walls. Figure 1.2 describes the concept behind a device capable of measuring the current blockade caused by the partitioning of an analyte into a nanopore. The event frequency and event duration are also measured. This method of detection is referred to as the resistive-pulse technique. Historically, this device was pioneered by Coulter, who designed it to count red blood cells.\(^9,32\)
Highly sensitive techniques, such as the patch clamp, were developed to measure small ionic currents across biological membrane channels in the second half of the twentieth century. Most membrane channels gate, making resistive-pulse sensing impossible. However, with the discovery of non-gating pores, in particular α-hemolysin from Staphylococcus aureus, a new era of resistive-pulse sensing was born in the early 1990s.

Early nanopore studies performed on α-hemolysin explored how polyethylene glycols (PEG) partitioned into the pore demonstrating the resistive pulse technique. Later, PEGs were used to estimate the size of α-hemolysin. However, the most significant pioneering work was the detection of RNA and DNA by Kasianowicz, et al. in 1996. In that work, three states of RNA were distinguished and attributed to different orientations of RNA entering the nanopore. The power to inspect nucleic acids was to dominate the field for the next decade. Several early studies inspected the physics behind DNA interaction with these biological pores. Of great appeal was the idea that DNA might be sequenced with a nanopore, with each base causing a different amplitude drop in the ionic current, allowing for the sequential reading of long strands of DNA without the need for further chemical modification. Before this could be achieved a great deal of development was needed.

Another important development in the sensing applications of nanopores was made by Gu et al in 1999, with the placement of “adaptors” in the interior of the pore. In particular, cyclodextrins were non-covalently bound to the interior of α-hemolysin and used to discriminate the binding of adamantane
derivatives. The significance of this work was the chemical modification of the nanopore interior to be selective for certain compounds.

Another body of experiments explored the use of nanopore force spectroscopy, as discussed earlier, to actively manipulate analytes using the electric field in the nanopore interior. Hornblower, et al. developed a protocol for this technique, studying the binding of DNA to exonuclease I as a test case. The probing of the energy barriers affecting complex dissociation was explored.

The redesign of other biological nanopores for sensing applications has also been developed. A modified phi29 motor protein was able to translocate double-stranded DNA. The FhuA ΔC/Δ4L nanopore described in this thesis was shown to have superior stability in low-pH and low-salt conditions. An engineered ClyA nanopore was also adapted for nanopore sensing.

Recently, the discrimination of DNA nucleotides has been achieved using both a modified α–hemolysin channel, and the MspA protein nanopore. Additionally, the coupling of DNA polymerases to the nanopore system has allowed for DNA to pass though nanopores at slow rates, allowing for enough time for continuous base pair discrimination. Very recent developments suggest that DNA sequencing has been achieved by the Oxford Nanopore company, which announced the desktop sequencing device will go on sale in the near future.

While α–hemolysin was making great strides, artificial nanopores also came onto the scene, though their development has been slower. Li et al. were responsible for the first publication detailing the interaction of double-stranded DNA with a nanometer scale pore. They pioneered the technique of ion beam
sculpting to create such nanopores in silicon nitride. Shortly after this development, Storm et al. developed a technique for the manufacture of nanopores in silicon nitride with a TEM,\textsuperscript{46} which has been widely adopted and is detailed in Chapter 2 of this thesis. Other techniques of pore manufacture, such as track-etched pores, and pore shrinking by laser heating and electron beam exposure have also been developed.\textsuperscript{1,47}

Early papers with solid-state nanopores focused on the characterization of the nanopore itself. For example, Smeets et al.\textsuperscript{48,49} investigated the properties of noise in silicon oxide based nanopores. The charge of nanopore walls was examined also examined by Smeets,\textsuperscript{10} showing that, at low ionic salt concentrations, flow of ions along the side of the nanopore increased the conductance of the pore beyond what would be expected from Ohm’s law. Keyser et al. demonstrated the linear increase of force on a DNA molecule with increased potential across a nanopore by using optical tweezers to hold DNA near a nanopore.\textsuperscript{12}

While these studies were necessary, it is only in the past 5 years that solid-state nanopores have increased their usefulness. One development of major importance has been the ability to functionally coat these nanopores. In 2007, Wannunu and Meller developed a protocol for the chemical modification of nitride nanopores with different monolayer coatings.\textsuperscript{50} In the same year Iqbal et al. functionally coated a silicon-based nanopore with hair-pin loop DNA, allowing for the selective transport of short single stranded complementary DNA fragments.\textsuperscript{39} In 2010, Hall et al. demonstrated the formation of a hybrid nanopore by dragging α-hemolysin into a silicon nitride pore.\textsuperscript{51} This development
promises to couple the robustness and the ability to integrate synthetic nanopores into devices with the precise atomic structure of protein nanopores. In 2011 Yusko et al. coated the sidewalls of a silicon nitride nanopore with a lipid bilayer via liposome fusion. This allowed for the time-resolved measurement of lipid-tethered proteins passing through the pore. It also significantly decreased adsorption of proteins to the pore walls. Also in 2011, Kowalczyk et al. reverse engineered a nuclear pore complex by coating a silicon based nanopore with the active phenylalanine-glycine (FG) nucleoporins demonstrating the selective uptake of Impβ transport receptor. In 2012, Wei et al. demonstrated the placement of a single functional group within the interior of a nanopore for the first time, a development that will enable the study of nanopore-analyte binding directly within the nanopore itself.

The development of solid-state nanopores is still underway. The studies in this thesis represent early applications of this technology to biological problems.
Chapter 2
Methods
Introduction

This chapter details the methods used in this thesis. It will describe the nanopore sensing setup, first giving a general description of the system and its components. This will be followed by a more comprehensive look at each system component, discussing their purpose and properties. Details of the manufacture of ultra-thin solid-state nanopores will be given, as well as the procedure for preparing them for ionic channel experiments. A section outlining the use of biological nanopores will also be given.

![Diagram of nanopore sensor setup](image)

**Figure 2.1:** Schematic outline of the nanopore sensor setup.

The function of a nanopore sensing setup is to measure the picoAmpere-scale ionic current through a single nanopore. To achieve this, several components are necessary. Figure 2.1 outlines a schematic of the system. The first component is the nanopore itself, which must be filled with ionic fluid. Next is a chamber to hold this nanopore between two separate baths of ionic solution. The chamber is connected to an amplifier by silver chloride electrodes, which convert the ionic signal to an electronic signal. The amplifier measures and amplifies this
current signal, after which the signal is fed into an 8-pole Bessel filter. After filtering, this analog signal is converted by the digitizer into a digital readout that can be processed by a desktop computer.

Nanopores

Two types of nanopores are used in this thesis: solid-state nanopores in silicon nitride and the FhuA ΔC/Δ4L biological nanopore.

Solid-state nanopore manufacture

Figure 2.2: Description of solid-state nanopore chip. Nanopore is drilled into a thinned film of silicon nitride (yellow). This nitride rests upon a thicker layer of silicon oxide (blue). The entire membrane is supported on a silicon substrate (gray).

Figure 2.2 describes the solid-state nanopores used in this thesis. The base of the nanopore chip is a square silicon piece of 2.7 mm per side and a thickness of 0.2 mm. On top of this silicon is a 1500 nm thick layer of silicon oxide. A 30 nm thick layer of silicon nitride sits on top of this oxide. In the center of the nanopore chip, a section of the silicon base and oxide is removed, leaving a freestanding “window” of silicon nitride. A small (50 nm square) section of this window is
thinned (to 8-20 nm thickness). The nanopore is created by removal of nitride in this thinned section. A layer of Polydimethylsiloxane (PDMS) is painted around the nanopore. Both the oxide and PDMS function to decrease the capacitance of the insulating membrane. The importance of this decrease to the system noise will be discussed later.

The patterning of nanopore chips is achieved by basic contact lithography. The design of the pattern was created using the L-Edit layout software. This pattern is transferred to a “photomask”. The photomask consists of a piece of glass coated with a Chromium film and photoresist. A Heidelberg Instruments DWL 66 mask-making tool was used to optically expose small sections of the photoresist in the desired pattern. The photoresist of the photomask was developed and the Chromium removed by an acid etch.
**Figure 2.3**: Outline of the manufacture process in creating a solid-state nanopore chip. (A) silicon oxide layer is grown with LPCVD. (B) silicon nitride layer is grown with LPCVD. (C) photoresist is spun onto one side of the wafer. (D) photoresist is patterned using contact lithography and developed to remove small window section. (E) plasma etching is used to remove the silicon oxide and silicon nitride layer. (F) KOH etching is used to etch silicon. (G) BOE is used to remove silicon oxide. (H) PMMA is coated on wafer. (I) E-beam lithography is performed. (J) nitride layer is thinned by plasma etching. (K) PMMA is removed.

**Figure 2.4**: Description of LPCVD deposition process. LPCVD is used to grow a layer of silicon oxide. It is then measured for thickness before a silicon nitride layer is grown.

Figure 2.3 gives an overall outline of the steps involved in creating the nanopore chip. The process of creating nanopore chips started with a 100 mm silicon <100> wafer of 200-300 micron thickness. A layer of 1500 nm thick film of silicon oxide was grown on this wafer using a Wet HCl oxidation process in a Low Pressure Chemical Vapor Deposition (LPCVD) furnace. The thickness of this oxide film was checked using a FileMetrics F40 optical measurement system. Low-stress silicon nitride was then grown to a thickness of 40 nm on the wafer.
using a LPCVD furnace. This thickness was checked, again with the FileMetrics F40. These steps are outlined in Figure 2.4.

Figure 2.5: Patterning with contact lithography. A layer of S1813 is coated on the wafer. A photomask is used to expose the desired pattern. Silicon nitride is removed using plasma etching. Silicon oxide is removed by plasma etching.

The wafer was coated on one side with Shipley S1813 photoresist using a spin-coater and baked at 90 degrees Celsius in an oven for 30 minutes. Contact lithography was performed using an ABM contact aligner. The aligner transferred the pattern from the photomask to the photoresist on the wafer. The photomask was placed on top of the photoresist with the chromium side facing upwards. Light in the Near-UV (405-365 nm) wavelength was shown at the photomask, exposing the sections of photoresist where chromium was absent.
Photoresist undergoes a chemical change with the exposure to UV light that enables its removal by a 60 second development with MIF 726 developer.

After development, the photoresist had a pattern identical to that of the photomask, with silicon nitride exposed. The silicon nitride was removed by an Oxford 81 Etcher using a CF3/O2 chemistry. This exposed the silicon oxide below the silicon nitride. The silicon oxide was also removed using the Oxford 81, this time using CHF3/CF4/Ar chemistry.

The silicon base was exposed in the desired pattern. These steps are outlined in Figure 2.5. Oxygen Plasma cleaning for 10 minutes removed residual photoresist. At that point, the wafer had one side coated completely with silicon nitride and the other containing exposed silicon in the desired pattern.

![Diagram](image)

**Figure 2.6:** KOH etching of silicon to expose a nitride window. Heated KOH is used to etch silicon along its crystal lattice to expose a freestanding layer of silicon oxide and nitride. Oxide is removed either by buffered oxide etch (BOE 1:6) or by longer KOH etching.
The silicon was etched using heated potassium hydroxide (KOH), which etches along the silicon base, but does not etch silicon nitride. The etching formed freestanding windows of silicon oxide and nitride on the opposite side of the wafer. The silicon oxide was removed using Buffered Oxide Etch (BOE), leaving a freestanding layer of silicon nitride.

The thinning of this freestanding nitride can be achieved in several ways. Here we discuss two: the use of E-beam lithography and the use of a Focused Ion Beam (FIB). Each of these techniques has advantages and disadvantages. These are discussed below.

E-beam thinning

![Diagram](image)

**Figure 2.7**: E-beam thinning of a small silicon nitride region. PMMA is coated over the silicon wafer and baked. A JEOL 9300 is used to pattern a 50 by 50 nm region into the PMMA. The small section of PMMA is removed and plasma etching is used to thin the region to the desired thickness. The remaining PMMA is removed.

Electron-Beam Lithography allows for the creation of very small features, down to 20 nm, in electron beam resist. It achieves this by patterning the resist
with electrons, overcoming the limits imposed by light wavelength in photolithography. The JEOL 9300 electron beam lithography system can easily pattern sections of 50 by 50 nm for thinning. To create thin regions of silicon nitride using E-beam lithography, a coating of Poly(methyl methacrylate) (PMMA) was spun on top of the nitride layer then baked for 60 seconds at 115°C. This wafer was then loaded into the JEOL 9300. A pattern of small squares 50 by 50 nm per side was made using L-Edit and transferred to the wafer using the JEOL 9300. Alignments using of the pattern were made by selecting three nitride windows. After removal and development the pattern was thinned using the Oxford 81.

**FIB thinning**

A Focused Ion beam allows for the creation of nanometer scale features in silicon nitride. In this thesis we used a FEI Strata 400 STEM FIB to thin out square regions of silicon nitride 50 nm per side. Ions of Gallium were accelerated and focused at the nitride, directly ablating the material. The thickness of the silicon nitride was determined by using the Dark Field mode of a Scanning Transmission Electron Microscope (STEM). The relative thickness of the thinned region of nitride to that of the unthinned region was obtained by comparing intensity of transmitted electrons through three regions: the unthinned region, the thinned region, and a region where there is no silicon nitride.

Electron beam lithography holds two advantages over FIB thinning. First, it has the advantage of consistency; each thinned region will be of the same thickness, so that different nanopore chips will all have nanopores of the same thickness. Second, unlike FIB thinning, E-beam thinning does not implant
charged gallium ions in the nitride. The major disadvantage of E-beam thinning is its initial cost and the inability to further thin films that have already been processed. In this respect FIB is more versatile, allowing each pore to be tailored to a specific thickness.

**Creating a nanopore**

Nanopores inspected in this thesis were drilled directly by ablation using the electron beam of a FEI Tecnai F20 S/TEM. The nanopore chip was loaded into the S/TEM and the thin region found. The acceleration voltage of the TEM was set to 200 kV and the monochrometer set to a low value to allow a greater current of electrons. In STEM mode, magnification of the thinned region was increased to 1.3 Million times. The STEM electron probe was placed in the thinned region and electrons created a nanopore. The spot size of the probe for nanopores less than 5 nm in diameter was 1-2 nm. For larger pores, spot sizes of up to 5 nm were used. Monitoring the Ronchigram allowed for the determination of when the nanopore was formed. After nanopore formation, the S/TEM was put in Bright Field TEM mode and the pore was imaged to determine its diameter.

After creation of a nanopore, ionic solution must be introduced into its interior before ionic current measurements can be made. For nanopores thicker than 15 nm, the following protocol aids in this wetting process.

First, place the nanopore chip into a 10 ml Pyrex beaker. Place the beaker on a hotplate in a fume hood and set the temperature to 90°C. Next, clean the nanopore chip with piranha solution for 10 minutes. Add 3 ml sulfuric acid to
the container using a glass pipette, and then add 1 ml hydrogen peroxide to the sulfuric acid to make piranha solution. After 10 minutes, dispose of the solution and fill the beaker with the de-gassed, de-ionized water using a clean glass pipette. Empty beaker of water and repeat water flushing at least 5 times. Remove the nanopore chip with clean tweezers and dry it by light suction. Coat the nanopore using fast sealing PDMS and let it dry for 10 minutes. Seal the nanopore chip into the chamber and add solution to both baths.

**Biological nanopores**

Biological nanopores consist of a single nanopore protein in an insulating lipid bilayer. A bilayer is formed by two monolayers of phospholipids and is approximately 5nm thick. The lipids contain hydrophilic and hydrophobic ends and the two monolayers align such that the hydrophobic ends face one another. In the experiments described in this thesis, the bilayer was formed across a 100 μm aperture in 25 μm thick Teflon. The aperture was pre-treated with hexadecane and allowed to dry for 3 minutes. 1,2-diphytanoyl-sn-glycero-3-phosphocholine lipids were added to an ionic solution below the level of the aperture. The solution was raised on both sides of the chamber, bringing the lipids into contact with the aperture, forming a bilayer.

The biological pore used was FhuA ΔC/Δ4L, an engineered version of the bacterial ferric hydroxamate uptake component A (FhuA) from the outer membrane of E. coli. FhuA ΔC/Δ4L inserted into a bilayer of 1,2-diphytanoyl-sn-glycero-3-phosphocholine after the addition of ~0.02 ng/ml to the bath solution of the chamber. The insertion can be monitored by application of a voltage bias.
across the bilayer. When the channel inserts into the bilayer, an increase in current is observed.

The chamber

![Diagram of solid-state nanopore chamber layout.](image)

**Figure 2.8:** Solid-state nanopore chamber layout. The solid-state nanopore chip is sandwiched between two wells containing electrolyte solution. A seal is made with either O-rings or PDMS.

The chamber for solid-state nanopores consisted of three pieces, each made of Teflon. The middle piece was a flat platform approximately 1 mm thick with a small aperture in its middle. The nanopore chip was sealed over this aperture. The two other pieces were U-shaped halves that formed a cup when pressed flush against the first piece, creating two wells into which the ionic solution was added. These pieces contained several holes that allowed the introduction of new solution to the bottom of the wells. The chamber for biological nanopores was similar, only the first piece was 25 μm thick Teflon and contained a 100 μm. The second two parts were identical to those of the first, only made of Acetle instead of Teflon.
Electrodes

The electrodes used were silver/silver chloride electrodes. These were formed by soaking silver in bleach. When a positive voltage bias is placed on an electrode in a bath solution containing KCl or NaCl, the silver chloride undergoes an oxidative reaction $\text{Ag} + \text{Cl}^- \rightarrow \text{AgCl} + e^-$. The amplifier measures the electron on the right side of this equation. If a negative voltage bias is applied to the electrode an electron migrates through the wire to the amplifier where it is measured, producing current and generating a charge imbalance at the electrode, the reaction is reversed $\text{AgCl(s)} + e^- \rightarrow \text{Ag(s)} + \text{Cl}^-$.\textsuperscript{54}

Amplifier

The Axon 200B patch-clamp amplifier was used for these studies. For all experiments it was used in the voltage-clamp mode. The intrinsic noise of the amplifier was low compared to the noise caused by the effective capacitance of the nanopore membrane. In the case of solid-state nanopores, this effective capacitance is caused by the nitride membrane and its effect can be reduced by the introduction of an oxide layer and PDMS. For the biological pores, the effective capacitance is due to the bilayer.

Filter and Digitizer

A Frequency Devices 900D 8-pole Bessel filter was used to filter out higher bandwidth noise of our signal. The filtering of this noise has a consequence for the time resolution of the nanopore system. If a rapid change in current occurs, such as when an analyte enters a nanopore, the rise time of the filter is
characterized by the amount of time it takes for the current signal being filtered to rise from 10% of previous value to 90% of its current value. This time is given by $0.3/f_{3r}$, where $f_{3r}$ is the cutoff frequency of the filter. The implication is that Events shorter than rise time will be heavily distorted in nanopore experiments.

After filtering, the signal is sent to an Axon 1440A Digidata, which takes the analog signal into a digital signal. The acquisition rate of the 1440 Digidata should generally be 5 times shorter than the cutoff frequency of the filter; the Nyquist Sampling Theorem states the bare minimum sampling rate of 2 times $f_{3r}$ is necessary.\textsuperscript{54}
Chapter 3

Single-molecule observation of protein adsorption onto an inorganic surface
Introduction

Spontaneous adsorption of proteins onto solid-state surfaces is at the heart of a broad spectrum of areas, including biochip applications, nanomedical devices, and design of a new class of functional hybrid biomaterials. Despite many experimental studies on protein adsorption at the liquid-solid interface, this phenomenon is still not comprehensively understood. In general, protein adsorption is considered an irreversible nonspecific process, where the occupied area remains excluded for other proteins in the aqueous phase, because proteins attached to the solid surface do not show lateral mobility or significant desorption rates. The complexity of protein adsorption on solid surfaces results from the multitude of electrostatic and hydrophobic forces among the side chains of the proteins and the reactive groups at the solid-liquid interface.

In this chapter, we probe protein adsorption on a low-stress silicon nitride (Si$_x$N$_y$) surface at single-molecule resolution using the resistive-pulse technique. In this technique, single-channel current measurements are employed to detect, explore and characterize an analyte by measuring the fluctuations in a current signature produced by ions passing through a single nanopore. These fluctuations occur when the analyte partitions into the nanopore, excluding the volume available for ion passage, thus causing a decrease in the current. We employed solid-state nanopores that feature an array of advantages, such as the robustness of the membrane and the ability to easily tune the diameter of the nanopore. Below, we describe time-resolved,
long-lived captures of single bovine serum albumin (BSA), a 66.4 kDa-molecular mass protein, into a Si$_x$N$_y$-based nanopore. While such long-lived captures have been observed before, to our knowledge, this is the first time they have been studied systematically.

**Figure 3.1**: Representative Si$_x$N$_y$ nanopores imaged by a Technia F-20 S/TEM in TEM mode. The diameters of the nanopores were 5 nm (A), 10 nm (B), and 20 nm (C).

The nanopores were drilled into a 20 nm-thin amorphous Si$_x$N$_y$ film using a concentrated electron beam (Fig. 3.1). Over 40 different nanopores were used, with diameters ranging from 3 to 25 nm. BSA, the most abundant protein in the bovine blood stream, is folded in a globular conformation with the approximate dimensions of 4 x 4 x 14 nm, giving it an excluded volume of $\sim$224 nm$^3$. When using nanopores of diameter greater than 8 nm, the addition of low nanomolar concentrations of BSA to the chamber produced transient short-lived current blockades in the range of 20 $\mu$s or shorter.

We show experimental evidence that the long-lived captures of single BSA proteins, in a broad range from tens of milliseconds to several minutes, are
caused by nonspecific, random and spontaneous attachment of single proteins to the Si\textsubscript{x}N\textsubscript{y} surface within the nanopore interior. Each adsorbed BSA protein produces a discrete drop in the current measured through a single nanopore. We found that the resulting current state followed one of two patterns. Either it was a stable constant value for long periods, or it fluctuated. We judge that the current fluctuations were due to a movable, unattached part of BSA that does not show significant interactions with the Si\textsubscript{x}N\textsubscript{y} surface. The fluctuations of the resulting current state of the nanopore were voltage dependent and obeyed a simple energetic landscape that is tilted along the applied electric force.\textsuperscript{71} When we used nanopores with a diameter of \~9 nm, a long-lived current drop was accompanied by an alteration of the frequency of short-lived current spikes. These short-lived spikes were attributed to BSA partitions into the nanopore interior without significant interactions of the protein with the Si\textsubscript{x}N\textsubscript{y} surface. On the contrary, the frequency of long-lived captures of BSA did not undergo a simple dependence on the protein concentration in aqueous phase. We interpret these events result from nonspecific, random and spontaneous adsorption of single BSA proteins to the Si\textsubscript{x}N\textsubscript{y} surface of the nanopore interior.\textsuperscript{58-60}
Results

Figure 3.2: Single-channel electrical recordings with a 12 nm-diameter Si₃N₄ nanopore, revealing long-lived BSA captures. (A) A uniform, stable and fluctuation-free single-channel current was observed in the absence of the BSA protein. (B) Short-lived and long-lived gating current blockades were detected when 180 nM BSA was added to the cis side of the chamber. (C) The dwell-time histogram of the long-lived current blockades. The transmembrane potential was +150 mV. A two-exponential fit was made, giving time constants of $\tau_1=110 \pm 11$ ms and $\tau_2=440 \pm 62$ ms with the associated probabilities of $P_1=0.58 \pm 0.05$ and $P_2=0.42 \pm 0.05$, respectively. The fit was based upon a log likelihood ratio (LLR) test, with a given confidence level of 0.95. The buffer solution contained 1 M KCl, 10 mM potassium phosphate, pH 7.4. For the sake of the clarity, the single-channel electrical traces were low-pass Bessel filtered at 400 Hz.
The excluded volume of free BSA proteins

When a positive voltage was applied across the Si₃N₄ membrane, a uniform, event-free single-nanopore current was recorded (Fig. 3.2A). With the addition of BSA to the cis chamber, which was grounded (Appendix A, Fig. A3), two types of interactions were observed: very short-lived current spikes and long-lived current blockades (Fig. 3.2B). As BSA has an effective negative charge of 12e at pH 7.4,² it is expected that, at a positive potential, the electric field within the nanopore interior will drive the negatively charged BSA through the nanopore. Short-lived events occurred at positive, but not negative voltages, confirming that the BSA protein has a net negative charge under the conditions used in this work. Dwell times for these events were near the resolution of our setup (~15 μs) and did not conform to a simple exponential. These findings are in accord with previous experiments performed with solid-state nanopores and BSA.⁶⁸,⁷³,⁷⁴ The amplitude of the short-lived current blockades varied significantly (Fig. 3.2B), suggesting that BSA traverses the nanopore under different structural conformations. The frequency of short-lived current blockades scaled linearly with the BSA concentration, confirming that single BSA proteins were the cause of the events (Appendix A, Fig. A4-A5).

The average excluded volume (Λ) of the BSA proteins may be estimated using the following equation:¹³
which depends on the amplitude of the current blockade made by the BSA proteins ($\Delta I_b$), the effective length of the nanopore ($H_{\text{eff}}$), the applied transmembrane potential ($V$), and the conductivity ($\sigma$) of the solution within the interior of the nanopore. It should be noted that this is an approximate equation in which the protein is assumed smaller than the diameter of the nanopore (see Chapter 1).

A typical maximum value of $\Delta I_b$ was 2500 pA. If we use this value, and $\sigma = 112 \text{ mS/cm}$, $V = +150 \text{ mV}$, and $H_{\text{eff}}=20$ nm, which is the thickness of the Si$_x$N$_y$ membrane, then the expected excluded volume is $\Lambda \approx 595 \text{ nm}^3$. The events with an amplitude greater than 2000 pA were rare (<1%), so that they might be attributed to a very low concentration of dimers and trimers in the BSA sample (Appendix A, Fig. A3). Using a value of 224 nm$^3$ for the excluded volume of BSA, we employ equation (1) to find that the expected amplitude of the current blockade $\Delta I_b \approx 941$ pA. It is worth mentioning that $H_{\text{eff}}$ could be greater than 20 nm, if the applied electric field extends beyond the wall of the nanopore. The access resistance of the nanopore is $\rho/d$, where $\rho$ is the resistivity of the KCl solution and $d$ is the nanopore diameter. Under the experimental conditions used in this work, the access resistance of a nanopore with a diameter of 12 nm is $7.44 \times 10^6 \Omega$. This value is of the same order of magnitude as the resistance of the nanopore ($1.58 \times 10^7 \Omega$), which was calculated using a cylindrical geometry. Therefore, we need to take into account the access resistance of the nanopore. This is equivalent to making the nanopore $\pi d/4$, or roughly $0.8d$, longer. For a typical nanopore with a diameter of 12 nm, then the effective length $H_{\text{eff}}$ is 29 nm,
which gives $\Delta I_b \approx 429 \text{ pA}$. This value is close to $470 \pm 40 \text{ pA}$, the median value of
the short-lived current blockades measured at a transmembrane potential of $+150 \text{ mV}$ (Appendix A, Table A1 and Fig. A5). It is also notable that the amplitude of
the current blockades ($\Delta I_b$) of the short-lived events is diminished, because the
events are near the time resolution of the instrument.\textsuperscript{138} Therefore, they are altered by the rise time of the filter.

The long-lived captures of BSA proteins

Long-lived current blockades occurred at every nanopore diameter greater
than 8 nm and showed several general attributes across the investigated range. Significantly, unlike the short-lived current blockades, the long-lived events did not show a simple linear relationship with the BSA concentration. Instead, long-
lived current blockades had a sudden onset that occurred between low (10 nM) and high (180 nM) concentrations of BSA. The concentration at which such onset occurred did not appear to be affected by the diameter of the nanopore
(Appendix A, Table A2). For example, measurements on nanopores from 9 to 12 nm in diameter had onsets varying from 10 nM BSA to 180 nM BSA. On the other hand, nanopores from 14 to 16 nm in diameter had onsets as low as 20 nM BSA and as high as 180 nM BSA. While the onset of events could occur between these ranges, it was much more probable at high BSA concentrations. For nanopores with diameters between 12 and 16 nm, only 2 of 27 nanopores tested had an onset below 20 nM BSA, whereas 80% displayed long-lived current blockades at 180 nM BSA (Appendix A, Table A2).
As expected, at very low BSA concentrations, the long-lived current blockades were rare. We tentatively interpret the “onset” of long-lived current blockades to be the adsorption of a single BSA molecule to the pore wall. The onset means that, at concentrations lower than the onset concentration, no BSA adsorbed to the pore surface within the timeframe of the experiment (10-minute single-channel electrical trace) and for the number of nanopores used in this work. Given the complexity of the nonspecific, random and spontaneous adsorption at the liquid-solid interface, involving a variety of electrostatic and hydrophobic forces, we think that a quantitative description, including model predictions of the far-from-the-equilibrium single-molecule events at very low BSA concentrations near the “onset” is quite difficult.

Thanks to the nonspecific nature of the BSA-nanopore binding interactions, the amplitude of the long-lived current blockades varied from nanopore to nanopore, indicating that different fragments of BSA produced such events in different nanopores (Appendix A, Fig. A6). Moreover, the long-lived current blockades were typically smaller in amplitude than the short-lived current spikes, between 100 and 400 pA, at a transmembrane potential of +150 mV. The sudden onset of the BSA-produced, long-lived current blockades was often followed by a sudden cessation of such events, demonstrating that long-lived events occurred in a reversible fashion (Appendix A, Fig. A7) and suggesting that these events were due to the adsorption of a single BSA molecule to the pore wall. The long-lived current blockades were either accompanied by additional current fluctuations between the resulting current state and a lower current state, with durations in the range of tens to hundreds of milliseconds (Fig. 3.2), or not
accompanied by additional current fluctuations. The nature of gating for each event appeared to be different both for different nanopores and for different adsorption events within the same nanopore. If $\tau_{on}$ is the average inter-event time interval and $\tau_{off}$ is the average duration of the current blockade, then the apparent rate constants of association and dissociation are $k_{on}=1/\tau_{on}$ and $k_{off}=1/\tau_{off}$ respectively. The observed “on” rate constants were in the range $0.3 - 769.1$ s$^{-1}$ ($n=9$ experiments). The observed “off” rates were in the range $4.1 - 4170$ s$^{-1}$ ($n=9$).

Moreover, we also observed multiple, subsequent and discrete current blockades at greater BSA concentrations, eventually producing the clogging of the nanopore (Appendix A, Fig. A8).

**Critical diameter of the nanopore for protein detection**

We did not observe BSA-induced current blockades with nanopores narrower than ~8 nm in diameter. The hydrodynamic diameter of BSA at pH=7.4 is ~ 9 nm,~72 close to the critical diameter ($d_c=8$ nm) that separated observable from non-observable BSA-produced current fluctuations. Our inability to probe BSA-induced current blockades with nanopores smaller than $d_c$ is interpreted as the exclusion of proteins from the interior of the narrow nanopores. Recent experiments performed in this laboratory have shown that globular proteins with dimensions greater than the diameter of the nanopore produce no significant alterations in the unitary conductance or single-channel current fluctuations.~75,76
Figure 3.3: Representative single-channel electrical recording with a 9 nm-diameter Si$_x$N$_y$ nanopore. The electrical trace was low-pass Bessel filtered at 2 kHz. 10 nM BSA was added to the cis side of the chamber. The other experimental conditions were similar to those presented in Fig. 3.2.

In contrast, for nanopores whose diameter is 9 nm, the low-amplitude long-lived current blockades had a detectable effect on the frequency of the short-lived current spikes (Fig. 3.3). Thus, a single BSA molecule adsorbed to the interior of the nanopore produces a prolonged current blockade, creating an experimentally detectable energetic penalty for further BSA molecules to traverse the nanopore. The BSA protein that is attached to the surface decreases the effective diameter, reducing the frequency of the protein partitions into the nanopore interior. The trace in Fig. 3.3 is partitioned into four sections: A, B, C and D, which delineate the states of the long-lived current blockades. In state A, no long-lived current drop is observed (Fig. 3.3 A). A first long-lived current drop is observed at the beginning of state B. A counting of events was performed for each section using
single-nanopore electrical data at a bandwidth of 10 kHz. At the beginning of state C, a second long-lived current drop occurs, accompanied by a drop in the frequency of short-lived current blockades from $32.2 \pm 0.4$ (Fig. 3.3, B) to $2.3 \pm 0.3$ s$^{-1}$ (Fig. 3.3, C). At the beginning of state D, the current rises and the frequency of short-lived current spikes increases to $21.7 \pm 0.4$ s$^{-1}$ (Fig. 3.3, D). For nanopores with a diameter much greater than 9 nm, very long-lived current blockades produced by single BSA proteins captured into the nanopore interior had no impact on the frequency of the large-amplitude, short-lived current blockades.

**Voltage-dependence of the long-lived captures of BSA proteins**

The frequency, amplitude and duration of long-lived current blockades, observed with a single 12 nm-diameter nanopore, were probed at voltages of $+100$ mV, $+200$ mV, $+300$ mV and $+400$ mV. Representative single-channel electrical traces are presented in Fig. 3.4. At progressively higher voltages, the probability of maintaining the lower state was increased, as judged by the longer-duration events recorded at this level. The probability of the open (upper) state was $0.71 \pm 0.01$ (n=1134 events), $0.38 \pm 0.01$ (n=3996), $0.26 \pm 0.01$ (n=614), and $0.12 \pm 0.01$ (n=24) at a transmembrane potential of $+100$, $+200$, $+300$, and $+400$ mV, respectively. An event-detection protocol was performed using ClampFit 10.2 (Axon) to count all current values above a threshold current. Each time the current passed above the threshold and then below, it was counted as an event. The sum of the duration of events above the threshold was taken and then this value was divided by the total sampling time. The free energy that is associated with the conformational fluctuation from the upper to the lower state could be
estimated using the formula $\Delta G = -RT \ln (k_{\text{off}} / k_{\text{on}})$. The values for $\Delta G$, at transmembrane potentials of +100, +200, +300 and +400 mV, were -0.24, -0.93, -1.66 and -4.30 kcal/mol, respectively. The total number of net negative charges of the BSA protein at pH 7.4 is 12. Assuming that only half of the charges are located on the protein domain that is attached to the Si$_3$N$_4$ surface, then the corresponding electrical force that alters these switching fluctuations is 13.2 pN at a transmembrane potential of +400 mV. In this calculation, the electrical force is $F = neV / H_{\text{eff}}$ and the access resistance of the nanopore was taken into consideration. Here, $n$ denotes the net number of negative charges that are not attached to the surface. It should be noted that this simple relationship between force and transmembrane potential is quite approximate, since it assumes a linear voltage drop across the nanopore length.
**Figure 3.4:** The voltage dependence of the long-lived current fluctuations. The single-channel electrical traces from the top panels are recorded at +100 mV (A) and +300 mV (B). These experiments were carried out with a 12 nm-diameter nanopore. The BSA concentration in the cis chamber was 20 nM. The middle panels represent a schematic model of the voltage-dependent partitioning of the negatively charged, unattached part of the BSA protein into the nanopore interior at a transmembrane potential $V=0$ mV (A) and $V >> 0$ mV (B). These panels show the attached BSA protein in the open (A) and closed (partitioned) (B) states, respectively. The bottom panels illustrate free energy landscapes of the BSA-nanopore complex at zero (A) and much greater than zero (B) voltages, respectively. The other experimental conditions were similar to those presented in Fig. 3.2.
A cartoon representing the qualitative alterations in the dynamics of a single BSA protein attached to the Si$_x$N$_y$ surface of the nanopore interior is shown in Fig. 3.4. The applied transmembrane potential alters the probability of the open state of the nanopore. In the absence of an electric field, there is a significant entropic barrier for the movable part of the BSA protein to partition into the interior of the nanopore (Fig. 3.4A), because more protein configurations are allowed in the aqueous phase than inside the nanopore. Therefore, the nanopore-BSA complex has a high probability to lie in the open state. However, the presence of a sufficiently intense electric field ($E \sim 7.5 \times 10^6$ V/m) tilts the energetic landscape along the force coordinate, lowering the activation free energy of the nanopore-BSA complex to undergo a transition from the open state to the closed state, and increasing the probability of the movable negatively charged BSA protein to partition into the nanopore interior (Fig. 3.4B).

To test the reproducibility of the two-state gating, a set of experiments with nanopores ranging in diameter from 10 to 25 nm were used in the following conditions: 1M KCl, 10 mM potassium phosphate buffer at pH 7.4. BSA was added to the chamber to a concentration of 450 nM and a positive bias of +150 mV was applied. This high concentration of BSA was used to ensure the threshold for the onset of long-lived events was met. Long-lived events occurred in every nanopore at this concentration. Two-state gating occurred in 38% of the nanopores ($n=13$) tested within the 10 minute timeframe of the measurement (Appendix A, Fig. A6). In those nanopores that showed two-state gating, the
duration of the gating differed. The average duration of gating was $20 \pm 15$ sec, with values as short as 0.72 seconds and as long as 43 seconds.

**Discussion**

In the last decade, protein adsorption on silicon nitride surfaces has been examined by a variety of experimental techniques, including electron microscopy,\textsuperscript{77,78} ellipsometry,\textsuperscript{79} fluorescence labeling,\textsuperscript{61} and planar polarization interferometry (PPI).\textsuperscript{80} In general, these approaches reveal surface organization and nonspecific, random adsorption phenomena of proteins at the liquid-solid interface.\textsuperscript{58-60} In contrast, in this work we rely on the detectable single-channel current fluctuations produced by the interactions between single BSA proteins and the nanopore interior.

We interpret that the short-lived current blockades observed in the presence of BSA represent partition of individual proteins into the nanopore interior, but without a significant interaction with the Si$_x$N$_y$ surface. The duration of the short-lived current spikes was close to the time resolution of our instrument (~15 µs).\textsuperscript{81} This limitation precluded us from obtaining reliable voltage dependence data of the short-lived current blockades due to a large number of missed events at greater transmembrane potentials.\textsuperscript{82} Assuming a two-barrier, one-well free energy landscape for the BSA partitioning into the nanopore, the voltage dependence would enable a rough estimate of the frequency of protein translocations from one side of the chamber to the other as well as the frequency of protein collisions with the nanopore entrance.\textsuperscript{83} Remarkably, using an
optimized chemiluminescence assay, Fologea and colleagues showed that the BSA proteins traverse nanopores with wide diameters of about 16 nm. In this study, they also demonstrated the alteration of the BSA charge induced by pH modification near the pI of the protein.

The single-molecule measurements with BSA proteins carried out in this work also show that the solid-state nanopore might hold the potential for a rapid assay for determining the hydrodynamic radius of folded proteins in solution. We were not able to detect transient, short-lived current blockades with nanopores smaller than $d_c = 8$ nm. However, we were able to detect current blockades with much shorter polypeptides using narrower nanopores. For example, we observed transient current blockades produced by NCp7, a 55 residue-long nucleocapside polypeptide of the HIV-1 virus, with solid-state nanopores in the range of 3-4 nm (Appendix A, Fig. A9). Therefore, our inability to detect short-lived current blockades with nanopores smaller than $d_c = 8$ nm was not caused by an experimental artifact.

We interpret that the long-lived current blockades represent strong binding events between the BSA protein and the Si$_x$N$_y$ surface of the nanopore interior in the form of nonspecific, random and spontaneous protein adsorption. This interpretation relies on several lines of experimental evidence: (i) the dwell time of these biding events covers a very broad range, from tens of milliseconds to several minutes; (ii) in some experiments, very long-lived discrete shifts in the unitary current of the nanopore were still persistent after BSA was removed from the chamber bath by perfusion. Such electrical signatures comprising step-wise
changes of the single-channel current were not found within nanopores without BSA added to the chamber; (iii) binding events were strong enough that the application of a large reverse voltage (~750 mV) did not dislodge the protein from the nanopore. Very recently, Pedone and colleagues found similar long-lived captures of avidin proteins within the Si$_x$N$_y$-based synthetic nanopore, which differed from the short-lived ballistic flights of proteins through the nanopore. They interpreted that the long-lived events represent transient or semi-permanent adsorptions of avidin onto the interior surface of the nanopore. The dwell time for transient events was in the range of tens of milliseconds, whereas their amplitude was well defined.
Figure 3.5: Diagrams show the proposed mechanism for the long-lived protein captures. The upper panels indicate the position of adsorbed BSA (red) within the nanopore interior (grey), in cross-section. (A) BSA is attached within the interior of the nanopore causing a long-lived current blockade (middle panel) without additional long-lived current fluctuations of the resulting current state. The short-lived current spikes were in a sub-millisecond range. 20 nM BSA was added to the cis chamber; (B) BSA is attached to the nanopore interior, but in a different orientation than in (A). Additional current fluctuations occur (middle panel) in which a movable “unattached” part of the BSA protein wiggles between the nanopore interior and the aqueous phase, while the other end remains attached to the Si$_x$N$_y$ surface of the nanopore interior. This results in a gating of the current between the open and the partially occluded (closed) state (Fig. 3.4). The left-hand bottom panel presents an all-points amplitude histogram of the trace in (A). The right-hand bottom panel is a dwell time histogram of the trace in (B), with $\tau_{\text{off},1} = 240 \pm 6.9$ ms ($P_1 = 0.70 \pm 0.02$) and $\tau_{\text{off},2} = 3020 \pm 730$ ms ($P_2 = 0.31 \pm 0.04$). 60 nM BSA was added to the cis chamber. The fit was based upon a log likelihood ratio (LLR) test$^{57,58}$ with a given confidence level of 0.95. The diameter of the nanopore was 15 nm, as judged by the least square linear fit to an I-V curve (Appendix A, Fig. A1). The other experimental conditions were similar as those presented in Fig. 3.2.
In the case of long-lived current blockades with no further fluctuations, the BSA is in a stable conformation (Fig. 3.5A, the top panel). However when fluctuating, the BSA is likely in an unstable conformation, with only part of the BSA molecule adhering to the Si$_x$N$_y$ surface (Fig. 3.5B, the top panel). We tentatively interpret that the fluctuating BSA protein undergoes conformational transitions between two states (Fig. 3.5B) and that these transitions are modulated by the transmembrane potential (Fig. 3.4). The typical transmembrane potential in this work was +150 mV, corresponding to an electric field of ~7.5×10$^6$ V/m. This electric field induces an overall force of ~14.4 pN on the 12 net negative charges of the BSA at pH 7.4. Prior force spectroscopy measurements have shown that proteins rupture at elongation forces of several pN. Therefore, we think that a force of 14 pN would be able to at least partially unfold the BSA proteins during their transit across the nanopore interior so that the proteins traverse the nanopore under various partially unfolded conformations. Recently, Talaga and Li proposed that the electrical forces present under physiologically pertinent applied transmembrane potentials can unfold the translocating proteins.

We judge that the BSA molecules enter a flattened conformation upon nanopore wall adhesion, decreasing the excluded volume of the molecule. This accounts for the lower amplitude of the long-lived events as compared to the value that corresponds to the short-lived current blockades. Again, a linear dependence of the frequency of the short-lived current spikes on the BSA
concentration in aqueous phase indicates that these short BSA-induced events cannot be attributed to nonspecific protein adsorption.

The decrease in the excluded volume of the BSA protein upon its adsorption to the silicon nitride surface is presumably caused by the loss of water around the portion of the polypeptide backbone attached to the solid surface. During nonspecific adsorption, it is likely that the BSA protein undergoes a conformational transition from a large-volume hydrophilic structure to a small-volume hydrophobic molecular structure.\textsuperscript{58,59,83} The hydrophilic structure is globular and highly hydrated, whereas the hydrophobic structure is “adsorption competent” and exhibits a smaller volume due to dehydrated groups in the BSA protein.\textsuperscript{55,56,58,59,85} This process is entropically driven due to the loss of structure (e.g., content of \(\alpha\)-helix), which is triggered by the modification of the stabilizing hydrophobic contacts in the globular conformation in aqueous phase.\textsuperscript{63}

Although, we observed that the amplitude of the long-lived current blockades (e.g., non-fluctuating states) is between 100 and 400 pA, at a transmembrane potential of +150 mV (\textbf{Fig. 3.2B}, \textbf{Fig. 3.3}, \textbf{Fig. 3.5A}), the two-state gating events (e.g., fluctuating states) are often higher, in the range of 200 - 900 pA (\textbf{Fig. 3.5B}; \textbf{Appendix A}, \textbf{Fig. A6}). These values are consistent with our interpretation, since a partially adsorbed BSA protein is expected to have a larger accessible volume than a fully adsorbed BSA protein (\textbf{Fig. 3.5}).

BSA is a low-structural stability protein and generally tends to adsorb onto a broad variety of solid-state surfaces.\textsuperscript{85} The results obtained in this work confirm
prior scanning electron microscopy,\textsuperscript{77} ellipsometry\textsuperscript{79} and interferometry\textsuperscript{80} studies of BSA adsorption on silicon nitride surfaces. Micic and colleagues have found that BSA in solution spontaneously adsorbed onto the surface of silicon nitride cantilevers of the AFM tips.\textsuperscript{77} This process continued until a uniform layer of proteins was formed over the surface of the tip. In general, proteins adsorb onto Si$_x$N$_y$ surfaces more readily than to stoichiometric nitride films.\textsuperscript{79} Since the BSA-nanopore interaction is a non-equilibrium process, it would be instructive to assay macroscopic current measurements on an array of nanopores\textsuperscript{38} fabricated in a silicon nitride membrane. For example, individual long-lived bindings of BSA to the Si$_x$N$_y$ surface, measured at the single-molecule level, could be observed by continuous decay in the macroscopic current flowing through the nanopore array. The rate of change of the macroscopic current might provide information about the apparent “adsorption” reaction rate constant. We anticipate that these kind of measurements will not only provide an estimate for the strength of the protein-surface interaction, but will also illuminate the nature of the adsorption process by revealing the experimental conditions in which the adsorption rate is substantially altered.

In the past, locking a polymer into a single nanopore and observing its partitioning into the nanopore interior,\textsuperscript{86,87} thermal fluctuations,\textsuperscript{88} temperature-induced conformational alterations,\textsuperscript{89} and interactions with various ligands\textsuperscript{28,65,71,90,91} have been pursued. Very recently, Lin and colleagues were able to lock a single-stranded RNA (ssRNA) molecule within the interior of the $\alpha$-hemolysin protein pore to probe its helix-coil transitions at the single-molecule
Interestingly, they observed a much slower kinetic rate, nearly three orders of magnitude smaller than those rates measured in aqueous phase. This result is somewhat counterintuitive, since the confinement of biopolymers is known to catalyze their unfolding-folding transitions. Their finding might be determined by other experimental factors, such as the interaction of ssRNA with the hydrophilic side chains of the interior of the α-hemolysin protein pore. The paper of Lin and colleagues appears to share a similar approach with the design of the experiments presented in this work. For example, we are able to probe the nonspecific attachment of a single BSA protein within the interior of a solid-state nanopore and monitor conformational fluctuations of the tether in real time using time-resolved, single-channel electrical recordings.

The findings presented in this article suggest that caution must be practiced in the sensing of polypeptides with solid-state nanopores, in which there might occur various nonspecific interactions of different domains of the translocating proteins with the silicon nitride surface. One obvious way to overcome this challenge is the functionalization of the surface of the solid-state nanopore to prevent these long-lived captures of single proteins into the nanopore interior.

**Conclusion**

In summary, we show that the BSA proteins interact strongly with the Si₃N₄-based nanopores. Certainly, more experimentation is needed to decipher the different contributions to the adsorption of BSA proteins onto the interior surface of the nanopore. For example, the precise nature of the interaction between a BSA molecule and the Si₃N₄ surface might be determined by obtaining the
enthalpic and entropic contributions to the kinetic and thermodynamic 
constants, revealing information about which process in protein adsorption onto 
an inorganic surface is dominant. The long-lived captures differ in nature from 
the short-lived current spikes, which are attributed to protein excursions into the 
nanopore interior without a significant interaction with the nanopore surface. 
Moreover, the absence of transient BSA-induced current blockades with 
nanopores that feature a diameter smaller than 8 nm indicates that the 
hydrodynamic diameter of the BSA proteins, under the experimental conditions 
employed in this work, is ~8 nm. This finding is in excellent agreement with 
prior experimental studies using electrophoresis NMR and solid-state 
nanopores.
Chapter 4

Sampling a Biomarker of the Human Immunodeficiency Virus (HIV) across a Synthetic Nanopore
Introduction

Nanopore-based detection has proven to be a successful method for probing a variety of molecules of biological interest, such as DNA, RNA and proteins. Of particular appeal is this technique’s ability to probe these molecules without the need for chemical modification or labeling, to do so at physiological conditions, and to examine single molecules at a time; this allows the possibility for results to come to light that would otherwise be masked in bulk measurements. Recent work in the field has exploited these properties in order to probe dynamic bimolecular interactions in real time. In addition to these studies, an emerging interest in adapting DNA and RNA aptamers for use with nanopores has arisen.

Figure 4.1: TEM images of nanopores. (A) A bright-field TEM image of a thinned silicon nitride region with a small nanopore; (B) A high-angle annular dark field scanning TEM (HAADF-STEM) image of a thinned nitride region; (C) A TEM bright-field image of an ~3 nm-diameter nanopore in a silicon-nitride membrane; (D) A TEM bright-field image of an ~10 nm diameter nanopore in a silicon nitride membrane. All images were taken using a Technia F-20 S/TEM instrument.
Herein, we exploit the nanopore-probe technique to examine the single-molecule detection of the nucleocapsid protein (NCp7), a biomarker of the human immunodeficiency virus 1 (HIV-1). Single solid-state nanopores were created in a silicon nitride membrane using electron-beam ablation (Fig. 4.1). NCp7 is a 55-residue domain of the gag and gag-pol polyproteins in HIV-1 (Fig. 4.2A). It plays an important role in the selection of viral genomic RNA for packaging during the HIV-1 infection cycle. NCp7 contains two zinc knuckles that bind specifically to the exposed guanosines of RNA stem-loops in the packaging domain of genomic RNA at physiological salt concentrations. In this work, the detection scheme was based upon the specific interactions of NCp7 with three 20-nucleotide RNA aptamers of varying binding affinity, which were derived from the stem-loop 3 (SL3, also known as Ψ) from the packaging domain of the retroviral RNA (Fig. 4.2B-D). Thus, SL3 is a naturally occurring aptamer that binds NCp7 with high affinity (Fig. 4.2E). The results obtained from the nanopore measurements were compared to those derived previously from a well-established titration technique based on quenching the fluorescence of Trp37 in NCp7 by loop bases of bound SL3. In addition, N-ethylmaleimide (NEM), which is known to inhibit the binding interactions of NCp7 to DNA and SL3 RNA was added to the solution to test whether inhibition of the binding could be monitored in real time. Beyond the potential medical interest of this system, aptamers represent a highly versatile class of biosensing components, since they can be targeted to a wide variety of analytes. Aptamers are chemically stable and easily produced single-stranded nucleic acid molecules, representing a promising alternative to traditional antibody-based approaches.
used in molecular biomedical diagnosis and other biotechnological applications.\textsuperscript{129} By using modern screening techniques, they allow for the design of high specificity to numerous substrates, including peptides and proteins. Therefore, the methodology established in this paper is applicable to a wide range of systems.

In this work, the binding affinities of NCp7 with SL3 and two related RNA aptamers were extensively studied using two types of nanopores: (i) small nanopores, whose internal diameter was smaller than 6 nm, with a thickness of the silicon nitride membrane less than 15 nm, and (ii) large nanopores, whose internal diameter was in the range 7 through 15 nm, with a thickness greater than 20 nm. The translocation of the aptamers though small nanopores in ultrathin membranes was examined in detail. We present the titration experiments by adding increasing concentrations of NCp7 to the solution. The events observed when the aptamers were added to large nanopores in thicker membranes are also shown. Next, the events attributed to complexes of the SL3 RNA aptamers with the NCp7 protein are described. Finally, we employed a single small-diameter nanopore and \textit{N}-ethylmaleimide (NEM) to perform real-time sampling of the inhibition of specific interactions between NCp7 and the high-affinity SL3 RNA aptamer. The ability of our system to detect the efficacy of NEM without labeling suggests that nanopores may be used to study drug inhibitors of binding protein-aptamer interactions.
Figure 4.2: The primary sequence of the HIV-1 NCp7 protein and three variants of the SL3 RNA aptamers. (A) The diagram displays the amino acid residues of the Zn$_2$:NCp7 (1-55) protein. Positively charged amino acids are underlined; (B) The diagram indicates the nucleotide sequence of the high-affinity SL3 (GAG) aptamer; (C) The low-affinity SL3 (CUG) aptamer; (D) No-affinity SL3 (AUA) aptamer; (E) The panel shows the three-dimensional structure of the NCp7 protein bound to the SL3 stem-loop recognition element of the genomic Ψ RNA packaging signal, as determined by heteronuclear magnetic resonance spectroscopy (adapted from PDB 1A1T). The scale bar indicates the approximate dimension of the complex, with the largest cross-sectional dimension of approximately 5.5 nm.
Results and Discussion

_Small nanopores._ Nanopores with diameters less than 6 nm were formed in thin nitride membranes (~8-15 nm thick). Experiments were performed with 200 mM NaCl on the cis side of the chamber and 1 M NaCl on the trans side. The buffer on both sides was 5 mM NaH$_2$PO$_4$ at pH 7.0. The cis solution matched the salt and pH conditions used in previously published fluorescence-based titration experiments.$^{119}$ The higher molarity of NaCl salt on the trans side was advantageously employed for both the drastic improvement in the signal-to-noise ratio of the acquired data as well as the substantial increase in the capture rate of the RNA aptamer.$^{21}$ Conductance values for small nanopores under these conditions ranged from 4.5 through 13.3 nS (n=41). Small nanopores used in these experiments exhibited stable single-channel current signatures at voltages up to 400 mV. At positive applied voltages, NCp7 did not alter the single-channel current signature of small nanopores (Appendix B, B1), confirming its net positive charge. However, at negative voltages, it caused rapid fouling of the nanopore (Appendix B, B2) owing to protein adsorption on the inorganic surface of silicon nitride.$^2$ In contrast, SL3 RNA aptamers did not change the single-channel electrical signature of small nanopores at negative voltages (Appendix B, B3), confirming their net negative charge. However, when the applied voltage was positive, the SL3 RNA aptamers produced distinguishable current blockades. **Fig. 4.3A** shows a typical signature of the single-channel electrical trace after the addition of 500 nM SL3 (GAG) aptamer at a positive transmembrane potential of +200 mV.
Figure 4.3: Sampling SL3 RNA aptamer - NCp7 interactions using a small nanopore. (A) A representative single-channel electrical trace demonstrating SL3 RNA aptamer–produced current blockades. The chamber contained 500 nM high-affinity SL3 (GAG) aptamer. The internal diameter of the nanopore was smaller than 6 nm, whereas the thickness of the silicon nitride membrane was smaller than 10 nm; (B) Histogram showing the change in inter-event time when 500 nM NCp7 was added to 500 nM SL3 (GAG) aptamer. A fitting of the histogram for 500 nM SL3 (GAG) alone (green) gives an inter-event time of $\tau_{on} = 149 \pm 15$ ms, while the fitting of the same data after addition of 500 nM NCp7 protein (blue) shows an increased inter-event time of $\tau_{on} = 1030 \pm 60$ ms; (C) Trace showing a drastic reduction of event frequency corresponding to the above histograms. The applied voltage was +200 mV. The buffer solution was 200 mM NaCl, 5 mM Na$_2$HPO$_4$, pH 7.0 on the cis side, and 1 M NaCl, 5 mM Na$_2$HPO$_4$, pH 7.0 on the trans side.

The frequency of the current blockades produced by SL3 RNA aptamers can be determined by $\tau_{on}^{-1}$ histograms, which can be fit to a single exponential using a log likelihood ratio test protocol (Fig. 4.3B).$^{27}$ Thus, the inverse of $\tau_{on}$ gives the
event frequency. Addition of NCp7 did not alter the dwell time or amplitude of the current blockades (Appendix B, B4). Instead, it changed their event frequency, as measured by a $\tau_{\text{on}}$ histogram. Fig. 4.3C shows a representative single-channel electrical trace demonstrating the drastic reduction in the event frequency of current blockades when 500 nM NCp7 is added to 500 nM SL3 (GAG) aptamer. This reduction suggests that titration experiments can be performed using these small nanopores. Voltage dependence tests of the dwell time of the SL3 RNA aptamer-produced current blockades displayed a biphasic signature, featuring a peak of the dwell time of the SL3 (GAG) aptamer between 200 mV and 250 mV (Appendix B, B5). This sort of biphasic signature of the dwell time has also been observed in the past with other charged polymers, such as polypeptides interacting with protein nanopores. Experiments with SL3 (GAG) (number of distinct experiments was n=5), SL3 (CUG) (n=3) and SL3 (AUA) (n=3) were performed such that the SL3 RNA concentration was fixed at 500 nM and the NCp7 concentration progressively increased from 0 nM to 125 nM, 250 nM, 375 nM, 500 nM, 750 nM, 1000 nM, and finally 1250 nM.

**Titration curves using small nanopores.** The binding affinities of NCp7 to each of the three SL3 RNA aptamers may be calculated using a titration curve. In previous fluorescence experiments, this was achieved by assuming the fluorescence intensity to be directly proportional to the free NCp7 concentration. For the small nanopores used in this study, a titration may be analyzed by assuming that the frequency of events is directly proportional to the
concentration of free SL3 RNA aptamer. The curves may then be fit using the equation:\textsuperscript{119,120,124}

\[
\frac{f}{f_0} = \left\{ - \left( P_t - R_t + K_d \right) + \left[ \left( P_t - R_t + K_d \right)^2 + 4R_tK_d \right]^{1/2} \right\} / 2R_t \tag{1}
\]

where \( P_t \) and \( R_t \) are the total NCp7 protein and SL3 RNA concentrations, respectively, \( f \) is the frequency of low-amplitude current blockades, \( f_0 \) and \( f_\infty \) are the event frequency at the initial time and at saturation with NCp7, respectively, and \( K_d \) is the dissociation constant. This equation assumes that \( P_t = P_f + P_{\text{bound}} \), \( R_t = R_f + R_{\text{bound}} \), and \( K_d = (R_f P_f)/(RP) \). Here, \( R_t \) and \( P_t \) are the concentrations of free SL3 RNA aptamer and NCp7 protein in aqueous phase, respectively. \( R_{\text{bound}} \) and \( P_{\text{bound}} \) denote the concentrations of bound SL3 RNA aptamer and NCp7 protein in aqueous phase, respectively. \( RP \) is the concentration of the NCp7-SL3 aptamer complex in solution.

Here, we assume that all events observed with the small nanopores are due to the SL3 RNA passing into the nanopore and that the NCp7-SL3 complexes are completely excluded, despite their net negative charge, so that the events sample the concentration in the chamber well. Two considerations justify this assumption: (i) there is a lack of change observed in the current amplitude or dwell time of the blockade events after NCp7 is added to the \textit{cis} chamber, and (ii) in work with larger nanopores (see next section) there is a significant alteration in the amplitude of current blockades that is probably due to blockage by the complex.
The $K_d$ values determined by the small nanopores may differ somewhat from values derived using the fluorescence data, because the protein’s fluorescence is quenched most efficiently when the NCp7 protein binds to the loop of the SL3 RNA aptamer. In contrast, additional reduction of current blockades may occur in the nanopores due to non-specific interactions of NCp7 with the SL3 stem bases in solution; there may also be SL3 stems bound weakly to the substrate surface, and those bound near a nanopore could bind NCp7 and reduce current flow. Surface-bound RNA could also form complexes that reduce the solution concentrations of RNA and protein, and the effective event frequency. The effects of non-specific binding of RNA and RNA-protein complexes to the surface, and of RNA stem bases to NCp7 are likely to be small at the 200 mM NaCl used here, as it has been shown that non-specific SL3-NCp7 interactions are largely suppressed at monovalent cation concentrations above 150 mM.\textsuperscript{119}
Figure 4.4: Titration curves of the SL3 RNA variants interacting with NCp7. (A) Titration curves derived with small nanopores using a concentration of 500 nM for different SL3 RNA aptamer variants. The event frequency ratio is the frequency of the SL3 RNA aptamer-produced current blockades after addition of NCp7 to the chamber normalized to the initial event frequency; (B) Curve derived from large nanopores using a concentration of 1000 nM for SL3 RNA (GAG). Curves are fit as described in the text. $K_d$ values for the 1:1 complexes are derived from best fittings and are given in Table 4.1.

Fig. 4.4A shows the data obtained for all three SL3 RNA aptamers by using small nanopores. In this plot, the event frequency ratio is the frequency of the SL3 RNA aptamer-produced current blockades after addition of NCp7 to the cis side normalized to the initial event frequency (eq. 2). The $K_d$ values calculated by fitting to the titration curves given in Fig. 4.4A are provided in Table 4.1. These
Table 4.1: The dissociation constant, $K_d$, of the NCp7 protein-SL3 RNA aptamer complexes. Values for small and large nanopore data were arrived at using best-fit curves to titrations shown in Fig. 4.4.

<table>
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<tr>
<th>SL3 RNA Aptamer</th>
<th>Small nanopore</th>
<th>Large nanopore</th>
<th>Fluorescence$^b$</th>
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<tr>
<td>SL3 (GAG)</td>
<td>2 ± 4 nM</td>
<td>278 ± 166 nM</td>
<td>28 ± 2 nM</td>
</tr>
<tr>
<td>SL3 (CUG)</td>
<td>1960 ± 300 nM</td>
<td>ND$^a$</td>
<td>850 ± 250 nM</td>
</tr>
<tr>
<td>SL3 (AUA)</td>
<td>ND$^a$</td>
<td>ND$^a$</td>
<td>20000 nM</td>
</tr>
</tbody>
</table>

$^a$ND stands for not determined data. For large nanopores, we were unable to fit the low-affinity aptamer data to eqn. (3). The binding affinity of the SL3 (AUA) to NCp7, which was obtained from fluorescence measurements, is rounded up to the nearest significant digit.

$^b$Fluorescence values are from the literature.\(^{120}\)

We observed a somewhat greater binding affinity of NCp7 to SL3 RNA (GAG) when using the nanopore-probe technique as compared to the fluorescence approach (Table 4.1). While this may reflect non-specific interactions discussed in the previous paragraph, it is also difficult to accurately determine $K_d$ values by fluorescence when $K_d < 20$ nM or $K_d > 300$ nM, where small measurement errors exert dramatic effects on the derived $K_d$.\(^{119}\) The same error considerations apply to the nanopore data. Briefly, at $K_d < 20$ nM, there are few measured data points that differ from a binding isotherm that corresponds to $K_d \to 0$ (affinity $\to \infty$), and the results of the fitting algorithm are largely
dependent on the one or two points at the curved elbow of the titration curves (e.g., the curve for SL3(GAG) in Fig 4.4A; a $K_d \rightarrow 0$ titration would consist of two straight lines intersecting at a 1:1 ratio of [NCp7]:[SL3]). The experimental error for $K_d > 300$ nM is dominated by uncertainty in the extrapolation to a saturating concentration of NCp7 (e.g., the curve for SL3 (CUG) in Fig. 4.4A). For low-affinity complexes it is not practical to continue the titrations to very large [NCp7] to determine the saturation limit, as such high concentrations would favor stoichiometries where more than one protein is bound per RNA.  

Recently, we derived the affinities of NCp7 to 24-nucleotide long DNA aptamers of varying affinity using an engineered protein nanopore derived from ferric hydroxamate uptake component A (FhuA) of Escherichia coli. Since, this engineered protein nanopore is cation selective, it was more convenient to use a detection mechanism based upon the partitioning of the positively-charged NCp7 protein into the nanopore lumen, which contrasts to the biosensing approach presented in this work. Several other studies have used smaller-sized nanopores for force-spectroscopy. In that technique, the electric field produced in the nanopore was used to directly dissociate molecules from each other. The time to dissociation was measured and interpreted to derive affinity data. In these experiments, we were not able to produce current blockades with lifetimes long enough to perform such an analysis. However, the force of the electric field at or near the nanopore orifice could conceivably act upon an NCp7-SL3 aptamer complex, catalyzing the dissociation process. This may explain why the event frequencies of the high-affinity aptamers, shown in Fig. 4.4A, do not go
to zero at saturating concentrations of NCp7. While there are differences in the $K_d$ values determined using fluorescence, the silicon nitride nanopores, and the engineered FhuA nanopores, the most significant result is that the overall trends are similar.

**Figure 4.5:** Representative single-channel electrical trace showing the NCp7-SL3 RNA aptamer interactions using a large nanopore. (A) The addition of 1 µM NCp7 protein increases the frequency of large amplitude current blockades observed with a large-diameter (~7nm) nanopore in a thick (~30 nm) silicon nitride membrane when added to a solution containing 1 µM high-affinity SL3 (GAG) aptamer; (B) A scatter plot of current amplitude versus dwell time showing distinct event types. Low-amplitude current blockades are attributed to the SL3 RNA aptamer alone. Large-amplitude events are interpreted as current blockades produced by the NCp7-SL3 RNA aptamer complex. Square events represent measurements taken with SL3 (GAG) solution in the chamber, while circle events indicate data taken with both SL3 (GAG) and NCp7 added to the chamber. The applied transmembrane potential was +200 mV. The buffer solution contained 200 mM NaCl, 5mM NaH$_2$PO$_4$, pH 7.0 on the cis side, and 1 M NaCl, 5 mM NaH$_2$PO$_4$, pH 7.0 on the trans side.
**Large nanopores.** Nanopores with diameters greater than 10 nm enlarged significantly with time when drilled into ultrathin silicon nitride membranes, whose thickness was less than 10 nm. Therefore, thicker silicon nitride membranes, ranging from 20 through 30 nm, were used. A variety of nanopore diameters was tested with data collected on nanopores with diameters ranging from 7 nm to 15 nm, as measured by bright-field TEM. We were not able to isolate systematic trends based solely on the nanopore diameter due to our use of different thicknesses of nitride in these experiments. Experiments were performed with 200 mM NaCl on the cis side of the chamber and 1 M NaCl on the trans side. The buffer on both sides was 5 mM NaH$_2$PO$_4$ at pH 7.0. Under these experimental circumstances, large nanopores showed stable current versus voltage profiles when only buffer solution was in the chamber. The addition of NCp7 to the cis chamber did not produce any alteration in the current signature at a transmembrane potential of +200 mV. In contrast, the addition of the SL3 RNA aptamers created very short-lived current spikes of non-uniform, low amplitude, many of which exhibited dwell times near the rise-time of the filter (Fig. 4.5A). The addition of the NCp7 protein changed both the dwell time and current amplitude of the blockades (Fig. 4.5B). As expected, dwell time alterations were not reproducible in different-size nanopores. Importantly, the proportion of current blockades exhibiting a greater current amplitude increased with increasing concentrations of NCp7, suggesting that these events can be attributed to the NCp7 protein-SL3 RNA aptamer complex. Experiments with SL3 (GAG) (n=3) were performed such that the SL3 RNA aptamer concentration was fixed at 1000 nM and the NCp7 concentration progressively increased from 0 to 250 nM, 500 nM, 750 nM, 1000 nM, 1500 nM, 2000 nM, and finally 2500 nM.
We also performed similar single-channel electrical recordings using SL3 (CUG) (n=3) and SL3 (AUA) (n=3) aptamers (Appendix B, B6). One would expect a decrease in the frequency of low-amplitude, SL3 RNA aptamer-induced current blockades to be close to that observed with the small nanopores; however, this was not the case. The event frequency decreased, yet not in the same manner as that recorded with small-diameter nanopores and not reproducibly for each large nanopore (Appendix B, B7). Therefore, we speculate that some of the low-amplitude current blockades recorded with large nanopores are due to “bumping” events of the larger NCp7-SL3 RNA complex that cannot be separated by the current amplitude alone. Alternatively, there could be NCp7-SL3 RNA complexes that have small amplitude blockades due to short residence time and attenuation by the filter.

Titration curves using large nanopores. While smaller nanopores offer a straightforward method of binding affinity analysis, results with larger nanopores are more complicated to interpret. In large nanopores, events due to both the SL3 RNA and the NCp7-SL3 RNA aptamer complex are observed. In a previous study by Wanunu and coworkers, the binding of various aminoglycosides to an A-site RNA have been detected and discriminated by differences in current amplitude. In that study, an affinity curve was constructed, enabling the determination of reasonable binding affinity values matching bulk measurements. The major assumption was that the proportion of a drug molecule:A-site RNA complex and an A-site RNA entered the nanopore at the same rate, which is reasonable given their similar size. In contrast, using such an analysis in this work did not return reasonable values (Appendix B, B6), leading
us to reject such an assumption for further data analysis. A likely explanation is that the hydrodynamic radius of the NCp7-SL3 RNA complex is greater than that of the SL3 RNA aptamer alone, leading to different diffusion dynamics in solution and therefore a distinction in the capture rate between the two partitioning molecules. Major determinants of the difference in the capture rates between the SL3 RNA aptamer and the NCp7-SL3 RNA complex include different nature of the energetic barriers\(^6,133\) for the translocation of the aptamer and aptamer-target complex as well as a possible electro-osmotic effect.\(^{134,135}\)

An alternative method for calculating the \(K_d\)s with large nanopores is to measure the increase and saturation in large-amplitude current blockades with progressively higher concentrations of NCp7. Assuming 1:1 binding, the concentration of the NCp7-SL3 aptamer complex in solution can be expressed as:

\[
\frac{[\text{Complex}]}{[\text{Complex}]_{\text{max}}} = \frac{x + (1+C)\sqrt{x^2 + 2(C-1)x + (C^2 + 2C + 1)}}{x_{\text{max}} + (1+C)\sqrt{x_{\text{max}}^2 + 2(C-1)x_{\text{max}} + (C^2 + 2C + 1)}}
\]

where \([\text{Complex}]\) is the concentration of the NCp7-SL3 complex in solution and \([\text{Complex}]_{\text{max}}\) is the maximum concentration of the complex observed after saturation with NCp7. Here, \(x\) denotes the added concentration of NCp7 divided by the added concentration of the SL3 RNA aptamer. \(x_{\text{max}}\) indicates the highest added concentration of NCp7 divided by the added concentration of the SL3 RNA aptamer. \(C\) is the dissociation constant (\(K_d\)) divided by the added concentration of the SL3 RNA aptamer. Equation (2) was derived by the relation \(K_d = (R_f P_f)/(RP)\), as in equation (1), with the assumption that the concentration of the NCp7-SL3 aptamer complex is directly proportional to the frequency of the
large-amplitude current blockades. Fig. 4.4B shows a plot of the ratio of the frequency of the large-amplitude current blockades to the frequency of large-amplitude events at the maximum used NCp7-to-SL3 (GAG) ratio. Fitting using equation (2) for $C$ and multiplying by the concentration of SL3 (GAG) gives the value for $K_d$ of $278 \pm 166$ nM (Table 4.1). We were not able to obtain satisfactory fits to this equation for data acquired with the low-binding affinity SL3 RNA variants.

**N-ethylmaleimide addition to small nanopores.** Given that the binding affinities arrived at using small nanopores are in reasonable accord with those obtained by fluorescence data, the possibility of probing a drug candidate against NCp7 becomes feasible. NEM reacts covalently with the cysteine residues in the zinc-fingers of NCp7, unfolding the fingers and preventing the protein from making specific interactions with the SL3 RNA.\textsuperscript{136,137} We added a 6-fold molar excess of NEM to the test solution after suppressing events by the addition of the NCp7 protein to the SL3 (GAG) aptamer at an applied transmembrane potential of +400 mV. This experiment was repeated three times on the same nanopore of ~6 nm diameter. Addition of the NEM rapidly brought the event frequency to near its original value (Table 4.2), demonstrating that NEM suppresses the formation of the NCp7-SL3 complex (Fig. 4.6).
**Figure 4.6:** Effect of N-ethylmaleimide on formation of the NCp7-SL3 RNA complex. Event frequency modulation caused by the addition of 5 µM high-affinity SL3 (GAG) aptamer, with 10 µM NCp7, causing reduction in the event frequency. Introduction of 60 µM N-ethylmaleimide returns the event frequency to near its pre-NCp7 level. The applied transmembrane potential was +400 mV. The buffer solution contained 200 mM NaCl, 5 mM NaH$_2$PO$_4$, pH 7.0 on the cis side, and 1 M NaCl, 5 mM NaH$_2$PO$_4$, pH 7.0 on the trans side. The nanopore was ~6 nm in diameter in a 15-nm thick nitride.

<table>
<thead>
<tr>
<th>Concentration ratio SL3(GAG):NCp7:NEM</th>
<th>Normalized capture frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0:0</td>
<td>1</td>
</tr>
<tr>
<td>1:1:0</td>
<td>0.19 ± 0.10</td>
</tr>
<tr>
<td>1:2:0</td>
<td>0.03 ± 0.10</td>
</tr>
<tr>
<td>1:2:12</td>
<td>0.85 ± 0.20</td>
</tr>
</tbody>
</table>

**Table 4.2:** Normalized capture frequency of the SL3 (GAG) aptamer-produced current blockades in the presence of NCp7 and NEM. Event frequency of current blockades produced by the SL3 (GAG) aptamer was measured before and after addition of NCp7 and then NEM. The capture frequency was normalized to the value determined using solution that contained only the SL3 (GAG) aptamer. The initial concentration of the SL3 (GAG) aptamer was 5 µM. Error bars represent standard deviations for 3 separate single-channel experiments.
Concluding remarks

In summary, our studies have established that solid-state nanopores can be used to perform real-time measurements of the affinity of a small protein with short RNA aptamers, even when the overall charge of the protein analyte-aptamer complex is negative. The results obtained with small nanopores are a satisfactory match to the binding affinity values obtained in previously published fluorescence studies, suggesting that affinity can be monitored with nanopores, whose internal diameter is comparable or smaller than the largest cross-sectional diameter of the binding protein-RNA aptamer complex. This confirms that sensitive quantification of disease biomarkers, such as the HIV-1 nucleocapsid protein, can be accomplished using stable nanopores. The methodology can be expanded to other protein-RNA ligand complexes for a rapid, label-free determination of the dissociation constants with no further requirement for functionalization of the silicon nitride surface. Additionally, the demonstration of the inhibition of binding by NEM suggests that nanopores could be used for screening potential drug targets, especially as massively parallel nanopore devices become available.

Experimental Section

Fabrication of solid-state nanopores. First, 40-nm thick membranes of freestanding low-stress silicon nitride were created using standard photolithography techniques. Next, a 50 µm square section of nitride was thinned to either ~10 nm or ~20 nm using a FEI Strata 400 STEM focused ion beam (Fig. 4.1A and Fig. 4.1B). Nanopores were then created directly using a FEI Tecnai F20 S/TEM in a STEM mode, as described previously. Nanopore
diameter was measured in bright-field TEM mode (Fig. 4.1C and Fig. 4.1D). Nanopores were wetted using isopropanol and flushed with deionized water before being loaded into a custom-built Teflon chamber. The silicon-supporting chip was coated with a fast curing silicone elastomer (Kwik-Cast, World Precision Instruments, Sarasota, FL) to reduce the current noise. Large nanopores were treated for 10 minutes in piranha solution (a 3:1 ratio mixture of H₂SO₄:H₂O₂) before the isopropanol wash. Initial bath solution was 1 M NaCl, 5 mM NaH₂PO₄, pH 7.0 on both sides of the chamber. Current measurements were performed using Ag/AgCl electrodes connected to an Axon 200B amplifier (Axon Instruments, Foster City, CA) and filtered at 10 kHz with an 8-pole, low-pass Bessel filter, Model 900 (Frequency Devices, Ottawa, IL) before being digitized by an Axon 1400A digitizer (Axon). Only nanopores displaying low 1/f noise and stable current signatures were used in these experiments. In general, we found that the noise depends on two parameters: nanopore thickness and nanopore diameter. For thick nanopores, current noise was more pronounced. In thinner nanopores, less noise was detected. As was discussed by Smeets and coworkers (2008), it is thought that “wetting” of nanopores is closely related to the current noise, and large-diameter nanopores are generally easier to “wet” than small-diameter nanopores. A broad range of both nanopore diameter and thickness was used in these experiments. Thin nanopores, with a thickness smaller than 10 nm, had an excellent success rate and almost always displayed low current noise, while large-diameter, thicker nanopores have a success rate closer to 70% after piranha treatment.
Current versus voltage curves were constructed to confirm that nanopores matched the expected size according to the formula:\textsuperscript{142}

\[ I = V \left( \left[ \mu_{Na^+} + \mu_{Cl^-} \right] n_{NaCl} e \left( \frac{4h_{\text{eff}}}{\pi d^2} + \frac{1}{d} \right)^{-1} \right) \]  

where \( I \) is the open-state current of the nanopore and \( V \) is the applied transmembrane voltage. Here, \( \mu_{Na^+} \) and \( \mu_{Cl^-} \) are the electrophoretic mobilities of the Na\(^+\) ions and Cl\(^-\) ions, respectively. \( e, h_{\text{eff}} \) and \( d \) denote the elementary charge, the effective length of the nanopore and its diameter, respectively. \( n_{NaCl} \) is the number density of NaCl. Following confirmation of size, solution on the \textit{cis} (grounded) side of the chamber was changed to 200 mM NaCl, 5 mM NaH\(_2\)PO\(_4\), pH 7.0. All analytes were added to the \textit{cis} side of the chamber.

\textit{Preparation of NCp7 and SL3 RNA aptamers.} The NCp7 protein (Fig. 4.2A) was expressed, purified and had its concentration measured as described previously.\textsuperscript{124} The high-affinity SL3 (GAG) aptamer (Fig. 4.2B) used in this study is a mimic of the sequence that occurs in the packaging domain of genomic RNA, while the low-affinity SL3 (CUG) (Fig. 4.2C) and no-affinity SL3 (AUA) (Fig. 4.2D) aptamers represent mimics lacking one and both exposed guanosine(s), respectively, reducing the binding affinity of the NCp7-aptamer complex.\textsuperscript{120} The RNA stem-loops used in this study were purchased from IDT (Integrated DNA Technologies, Coralville, IA). SL3 RNA aptamer samples were dissolved in Milli-Q purified water (EMD Millipore Corporation, Billerica, MA). Prior to use in RNA experiments, samples were heated briefly to 90\(^\circ\)C, then cooled on ice for 15 minutes to form hairpins.
Given the highly specific interactions between NCp7 and SL3 genomic RNA region and that SL3 packing is highly conserved in different mutations of HIV, the zinc knuckles have become an attractive target for drugs seeking to treat HIV. NEM is an alkylating agent that covalently reacts with cysteine thiols; this causes irreversible ejection of zinc and the inability of the protein to adopt a biologically active conformation. In this work, its ability to inactivate the binding mechanism of NCp7 to SL3 allowed it to be used as a test molecule for assessing whether nanopores can perform real-time detection of drug candidates. At pH 7.0, NCp7 has a formal positive charge of +9e and the SL3 aptamers a charge of -19e each. The complex will therefore have a net charge of -10e. The solution structure of NCp7 bound to the SL3 RNA (GAG) aptamer was used to show that the volume is approximately 4 times that of the RNA aptamer alone, with the SL3 RNA aptamer approximated by a cylinder with a diameter of ~2 nm and a length of ~3 nm (Fig. 4.2E). It is difficult to obtain an accurate volume for the NCp7 protein alone. For example, in the NCp7-SL3 RNA complex, NCp7 is rather compact, while the unbound form is largely a random coil except for the two zinc fingers. The largest cross-sectional size of the NCp7-SL3 complex is ~5.5 nm.
Chapter 5

Inspecting FhuA ΔC/Δ4L using PEG
Introduction

A persisting challenge in nanobiotechnology is designing robust protein scaffolds that are tractable and versatile under a broad range of experimental circumstances. Recently, we extensively engineered ferric hydroxamate component A (FhuA) of *E. coli* by deleting a 160-residue cork domain (C) and four long extracellular loops (4L). We call this engineered pore FhuA ΔC/Δ4L (Fig. 5.1). FhuA is a 714-residue, monomeric β-barrel protein composed of 22 anti-parallel β strands, located in the outer membrane of *E. coli*. This protein is distinguished from other outer membrane family members by its numerous functional tasks, including the dual role of transporter and receptor. The major function of FhuA is to mediate the energy-driven, high-affinity Fe\(^{3+}\) uptake complexed by the siderophore ferrichrome. In addition, FhuA transports antibiotics, such as albomycin and rifamycin.

We coupled genetic engineering with a rapid-dilution refolding to obtain a protein nanopore with unusual stability over a broad range of experimental conditions, from highly acidic to very basic pHs, as well as from low to high ionic concentrations in the chamber. This extensive engineering of the FhuA ΔC/Δ4L protein nanopore encompassed an overall deletion of ~33% of the wild-type protein. The engineered protein formed a pore in the lipid bilayer with a conductance of ~4 nS. One immediate question that we asked was how this newly redesigned cork-free β-barrel membrane protein differs from the native FhuA, (Fig. 5.1). It is possible that the deletion of the cork domain and several long extracellular loops may not only impact the orientation and the local
conformation of other extracellular loops, but also the inner dimensions of the β barrel along the central transversal axis.

**Figure 5.1:** Representation of the modifications to the FhuA protein nanopore. (A) Diagram showing the wild type crystal structure and modified (FhuA ΔC/Δ4L) protein structure with extracellular loops, L3, L4, L5 and L11 labeled. (B) Surface representation of the engineered FhuA ΔC/Δ4L protein nanopore and its expected orientation in a synthetic planar lipid bilayer. The cartoon was made using 1BY5.pdb of the native FhuA protein. On the right is graphed the expected internal radius of FhuA ΔC/Δ4L along the pore interior, as calculated using the HOLE program.
To further explore the size and geometry of the engineered FhuA ΔC/Δ4L protein nanopore, we systematically examined the interaction of water-soluble, flexible poly(ethylene glycol)s (PEGs) with its interior by polymer exclusion experiments. The crystal structure of the native FhuA shows an asymmetric outer membrane protein with an elliptical cross-sectional area that decreases from the *trans* to *cis* side \(^{144,145}\) (Fig. 5.1). Here, the *trans* side is the periplasmic side and the *cis* side is the extracellular side of the protein.

Interaction of PEGs with transmembrane protein pores and channels has been studied extensively in the past several decades. These investigations were primarily targeted to the following directions: (i) obtaining a mechanistic understanding of polymer partitioning into confined geometries, \(^7,11,27,87,88,150-156\) (ii) obtaining a quantitative approach for the impact of the osmotic effect of polymers on single-channel kinetics, \(^34,157\) (iii) determining the internal sizes of the transmembrane protein pores using polymer-induced change in their single-channel conductance \(^151,156,158-166\) or measuring polymer-induced alteration of the pore’s access resistance, \(^8,34\) (iv) probing the internal geometry of the transmembrane protein pores using chemical modification and cysteine scanning mutagenesis with functional polymers, \(^86\) (v) probing the dynamics of single polymers in confined spaces, \(^14,71,106\) and (vi) developing nanopore-based approaches for single-molecule mass spectrometry. \(^167,168\)

Here, the extent of the modification to the single-channel conductance in the presence of water soluble polymers was used to infer the diameter of the
engineered FhuA ΔC/Δ4L protein nanopore. PEGs of molecular weight lower than 2000 Da added symmetrically to both sides of the chamber reduced the nanopore single-channel conductance, confirming its large internal size. Asymmetric addition of PEG to the chamber suggests a conical internal geometry of FhuA ΔC/Δ4L with a minimum constriction of ~1 nm, which is located on the extracellular entrance, confirming the predictions derived from the crystal structure of the native FhuA protein. Estimates of the nanopore access-resistance using impermeable dextran polymers were employed to infer the average internal diameter of ~2.4 nm.

**Materials and Methods**

**Preparation of the engineered FhuA ΔC/Δ4L protein nanopores**

The construction of the plasmid for the expression of the engineered FhuA ΔC/Δ4L protein nanopores has been reported previously. The subsequent modifications of the protocol for obtaining the FhuA ΔC/Δ4L protein nanopore through rapid-dilution refolding have also been described. Briefly, the refolding of the FhuA ΔC/Δ4L protein was adopted from protocol developed by Arora and colleagues. 40 µl of His+ -tag purified denatured FhuA ΔC/Δ4L was diluted 50-fold into a 1.5% n-Dodecyl-β-D-maltopyranoside (DDM in 50 mM Tris-HCl, containing 200 mM NaCl, 1 mM EDTA, pH 8.0). The diluted protein samples were left overnight at 23°C to complete the refolding of the FhuA ΔC/Δ4L protein. Aggregated or misfolded proteins were removed by centrifugation. Samples were stored at -80°C in 50 µl aliquots.
**Single-channel electrical recordings on planar lipid bilayers**

Electrical recordings were performed on synthetic bilayers of 1,2-diphytanoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids, Alabaster, AL). Bilayers were formed across a single 100-µm diameter aperture in a 25-µm thick Teflon film (Goodfellow Corporation, Malvern, PA) separating the *cis* and *trans* compartments of an acetal chamber. The *cis* side of the chamber was grounded. The PEG-free initial solution was 1 M KCl, 10 mM potassium phosphate, pH 7.4. To achieve single-channel insertion, FhuA ΔC/Δ4L was added to the *cis* side of the chamber to a final concentration of ~0.19 ng/mL. A positive potential of +200 mV was applied using Ag/AgCl electrodes in 3 M KCl, 10% agarose bridges. Current recordings were performed using an Axon 200B patch clamp amplifier (Axon Instruments, Foster City, CA) in the voltage-clamp mode. Data was collected by an Intel Core Duo PC (Dell, Austin, TX) connected to a Digidata 1440A (Axon Instruments). Output was filtered using a Frequency Devices Model 900B 8-pole Bessel filter (Frequency Devices, Ottawa, IL) at 10 kHz. The acquisition rate was 50 kHz. Single-channel insertions were monitored by stepwise changes in the measured current. After insertion, channel conductance was measured at a membrane potential of +40 mV and channels with a single-channel conductance of 4.2 ± 0.2 nS were selected for this study.

After characterization of the conductance, either a solution containing poly(ethylene glycol)s (PEGs) or dextran replaced the original solution. The following reagents were used in this work: PEG 200, PEG 300, PEG 600, PEG 1000, PEG 1500, PEG 2000, PEG 3000, PEG 4000, PEG 6000, PEG 8000, PEG 10000,
PEG 12000 (Sigma-Aldrich, St. Louis, MO) (Appendix C). PEGs were added to 1M KCl, 10 mM potassium phosphate, pH 7.4 to a final concentration of 15% (w/w). 40000 Da dextran (Sigma-Aldrich, St. Louis, MO) was added to 1M KCl, 10 mM potassium phosphate, pH 7.4 to a concentration of 15% (w/w). Solution replacement was performed by perfusion using a Bio-Rad EP-1 Econo Pump (Bio-Rad Laboratories, Hercules, CA). Conductivity of the solutions was measured using an Orion 105A+ conductivity salinity meter (Thermo Electron Corporation, Marietta, OH). PEG solutions were perfused either symmetrically, with both sides of the chamber containing the same molecular weight PEG, or asymmetrically, in which the PEG on one side of the chamber was 12 kDa, while the examined polymeric species varied in the opposite chamber. Dextran solutions were perfused symmetrically to both sides of the chamber. All experiments were performed at room temperature 23 ± 1°C. The single-channel acquisition was performed using the Clampex 10.2 (Axon) software. Analysis of the single-channel electrical traces was carried out using the pClamp 10.2 (Axon), Origin 8.1 (Microcal Software, Northampton, MA) and Mathematica 7 (Wolfram Research, Inc., Champaign, IL) software.

Results

**Biophysical characteristics of the engineered FhuA ΔC/Δ4L protein nanopore**

Recently, we showed that the engineered FhuA ΔC/Δ4L protein nanopore inserts into a synthetic planar lipid bilayer as a monomer in a single orientation. Figure 5.1 shows the expected orientation and structure of the FhuA ΔC/Δ4L protein, with an open transmembrane β-barrel nanopore. It has a ~2.8 nm opening on the trans side, near to the bilayer surface, and a constriction of 1.2 nm.
near the cis end. These dimensions, which include the contribution of the residue side chains, suggest it is feasible to use polymers to assess the nanopore size. The partitioning properties of PEGs of varying molecular weight were explored by both their symmetric and asymmetric addition to the chamber.

At 1 M KCl, 10 mM potassium phosphate, pH 7.4, the FhuA ΔC/Δ4L protein nanopore was stable for extended periods at applied potentials between +100 mV and -100 mV. The FhuA ΔC/Δ4L nanopore exhibited a relatively quiet electrical signature (no major closures), which was decorated by brief (~100 µs) and low-amplitude (~ 20 pA at +80 mV) current deflections at both positive and negative voltages (Fig. 5.2A). The FhuA ΔC/Δ4L nanopore inserted into the bilayer with a range of conductances. To ease in the single-channel data analysis, nanopores within a narrow range of conductance were selected (4.2 ± 0.2 nS at +40 mV). Nanopores with this conductance are slightly non-ohmic, with current response at +100 mV and -100 mV differing by ~7%.

The introduction of PEG into the bath solution changed the single-channel conductance of the nanopore in a manner related to the PEG molecular weight, with lower-weight PEG solution creating a large decrease in the single-channel conductance and higher-weight PEG solution having less effect. Figure 5.2 provides representative single-channel electrical traces of the effect of symmetric addition of 15% (w/w) solutions of PEG on the single-channel conductance of the nanopore. In the case of 300 Da PEG, the single-channel conductance dropped to 60% of its PEG-free level (Fig. 5.2B), a decrease consonant with the
drop in the conductivity of the PEG-containing bulk aqueous phase to the PEG-free solution. The addition of PEG 4000 Da did not appreciably change the single-channel conductance of the nanopore (Fig. 5.2C), and the addition of PEG 12,000 Da did not change the conductance (Fig. 5.2D), indicating that greater-molecular size PEGs were not able to penetrate into the nanopore interior.

![Graph showing current (pA) vs. PEG molecular mass](image)

**Figure 5.2:** Representative dependence of single-channel electrical current on the PEG molecular mass for the engineered FhuA ΔC/Δ4L protein nanopore. With the addition of small-molecular mass PEG, the unitary conductance of the nanopore decreases. With addition of larger-molecular mass PEGs, the conductance nears its PEG-free conductance. All traces show the open-state current through a single FhuA ΔC/Δ4L nanopore. The applied transmembrane voltage was +80 mV. The unitary conductance of these FhuA ΔC/Δ4L nanopores was 4.2 nS in PEG-free solution. All experiments were performed with 15% (w/w) PEG in 1 M KCl, 10 mM potassium phosphate buffer, pH 7.4. Single-channel electrical traces were low-pass Bessel filtered at 1 kHz filter.

**Voltage dependence of the conductance ratio**

While the conductance ratio of the nanopore with PEG solution to PEG-free solution is nearly constant for voltages between +100 mV and -100 mV when measured in symmetric conditions, it is highly attenuated for lower-weight PEG
in asymmetric conditions. Figure 5.3 gives an example of such attenuation in a 300 Da PEG solution.

**Figure 5.3:** Conductance attenuation induced by osmotic stress in the FhuA ΔC/Δ4L nanopore. Asymmetric PEG conditions induce a measurable attenuation of the FhuA ΔC/Δ4L nanopore current response. Indicated are the currents measured at different voltages for the FhuA ΔC/Δ4L nanopore in: 300 Da PEG *cis*, 12000 Da PEG *trans*; 12000 Da *cis*, 300 Da PEG *trans*; and 300 Da PEG symmetric. Curves indicate that osmotic stress induces significant attenuation of the nanopore. Solutions were 15% (w/w) PEG in 1M KCl, 10 mM potassium phosphate, pH 7.4.
Figure 5.4: Conductance of the FhuA ΔC/Δ4L nanopore in the presence of PEGs of varying molecular mass. Conductance ratios represent the nanopore conductance with PEG solution normalized to the PEG-free conductance. Curves are constructed at +100 mV (A), 0 mV, where conductance values were interpolated (B), and at -100 mV (C). Experiments labeled cis were performed with the listed PEG mass on the cis side of the chamber and impermeable 12000 Da PEG on the trans side of the chamber. Alternatively, experiments labeled trans were performed with the listed PEG mass on the trans side of the chamber and impermeable 12000 Da PEG on the cis side. The bottom horizontal dashed line represents the ratio of the conductivity of the bulk solutions containing PEG to the PEG-free solution. Symmetric and trans curves were fit using equation (1) and (2) using Origin 8.5. Cis curves were fit using equations (5), (6) and (7) in Mathematica 7. PEG was added at 15% (w/w) with 1M KCl, 10 mM potassium phosphate, pH 7.4.
Molecular weight dependence of the conductance ratio

To mitigate the voltage-dependent attenuation, conductance values for nanopores in this work were taken at two different voltages, +100 mV, -100 mV and also interpolated to a potential of 0 mV. Figure 5.4 shows a summary of conductance changes due to the PEG presence in aqueous phase. The results indicate that FhuA ΔC/Δ4L is asymmetric, with the trans side larger than the cis side. In the case of both cis and trans experiments, PEG solutions with PEG molecular weight greater than 4000 Da do not appreciably change the conductance of the nanopore, suggesting PEG of weight greater than that value do not permeate into the nanopore interior.

For impermeable PEG, we suggest the following two factors contribute to the conductance change of the nanopore. Previous work has shown that PEG solutions increase the salt activity inside the nanopore interior leading to conductance ratios higher than expected for impermeable PEG.34 This increase in conductance is compensated by a decrease in conductance due to an increase in the nanopore’s access-resistance. To measure the effect of the change in access resistance to the nanopore due to the addition of PEG, impermeable dextran was used. 40000 Da dextran was added to 1 M KCl, 10 mM potassium phosphate, pH 7.4 to a final concentration of 15% (w/w). The decrease in bulk conductivity of the dextran-containing solution was comparable to that of the PEG solution (Appendix C, Table C1). While PEG increases the activity of potassium ions in the channel, dextran does not.34 Therefore the effect of impermeable polymers of PEG and dextran is different. The addition of dextran solution decreased the
measured conductivity of the engineered FhuA ΔC/Δ4L protein nanopore by (8.8 ± 2.4)%.
This decrease is attributed solely to the change in the access resistance associated with the bulk conductivity of the solution surrounding the nanopore. The change in the access resistance with dextran also provides a means of estimating the nanopore diameter (see Discussion).³⁴
The power spectral density of the current noise

Figure 5.5: Power-spectrum analysis of noise fluctuations in the engineered FhuA ΔC/Δ4L protein nanopore. (A) Representative power spectra at +100 mV. (1) Power spectra taken at 0 mV. (2) Power spectrum of a single FhuA ΔC/Δ4L nanopore, in PEG-free solution, and at +100 mV. (3) Power spectrum of a single FhuA ΔC/Δ4L nanopore in solution containing 1000 Da PEG at +100 mV. Note that the PEG-containing solution has a greater noise level. The sharp cutoff at 10000 Hz is due to the Bessel filter; (B) Trace indicates the excess S(0) noise in the power spectra of FhuA ΔC/Δ4L with PEG solutions. S(0) values were taken at +100 mV by averaging of the spectral values in the range of 100 Hz to 1000 Hz. Displayed values of each channel are calculated by subtracting the S(0) value at 100 mV from that at 0 mV. The horizontal dashed line represents baseline S(0) noise for PEG-free FhuA ΔC/Δ4L. Results indicate PEG-induced noise is highest in the impermeable regime.
Prior examinations of polymer partitioning into large nanopores have employed fluctuations in the single-channel current noise induced by PEGs using power spectral analysis.\textsuperscript{11,33,151} Such studies yielded kinetic information on polymer partitioning into and out of the nanopore. We analyzed the single-channel current of the engineered FhuA ΔC/Δ4L protein nanopore at +100 mV, selecting portions of the single-channel recordings that lacked the rapid downward deflections seen in figure 5.2. The power spectral density of the current noise of the FhuA ΔC/Δ4L protein nanopore was taken both with and without symmetric PEG solutions. Figure 5.5A presents the spectral densities of current fluctuations of a single FhuA ΔC/Δ4L protein nanopore at zero transmembrane potential (trace 1), at +100 mV in the absence of PEG (trace 2), and at +100 mV in the presence of 1,000 Da PEG (trace 3). Each analyzed sample was taken from a concatenation of the current recording to a total of 5 seconds in duration, with the 24 Hz-resolution bandwidth used for spectral analysis.

The results for all PEGs used here are presented in figure 5.5B in the form of the low-frequency spectral density $S(0)$. It was obtained from the spectra by averaging over the range $100 \text{ Hz} < f < 1000 \text{ Hz}$ and subtracting the background noise at 0 mV applied potential.\textsuperscript{169} These error bars reflect reproducibility of the PEG-induced noise from 3 separate traces.
Discussion

Determining the diameter of the nanopore using equilibrium partial filling approach

When introduced into solution at an identical concentration of 15% (w/w), differently-sized PEGs decreased the conductivity of that solution by the same amount (Appendix C, Table C1). It has also been observed that, for many transmembrane protein nanopores, the addition of small, easily penetrating PEG to solution results in a reduction in their single-channel conductance that is similar to the reduction in the conductivity of the bulk aqueous phase.\textsuperscript{11} Motivated by these observations, several studies have employed the reduction of single-channel conductance for modeling the partitioning of PEG into the channel interior.\textsuperscript{11,151,153,156,159,166} In these investigations, the decrease in the single-channel conductance is assumed to be linearly proportional to the monomeric concentration of PEG inside the channel:

\[
\frac{g(w)}{g(\infty)} = 1 - \chi p(w)
\]

(1)

where \(g(w)\) is the single-channel conductance of the nanopore at a given molecular weight \(w\), \(g(\infty)\) is the single-channel conductance of the nanopore for a completely excluded polymer, \(\chi\) is the proportional reduction in the single-channel conductance when PEG lies in its interior and \(p(w)\) is the partitioning function. Here, we follow the modified scaling model\textsuperscript{11,151}:

\[
p(w) = \exp(-w / w_0)^\alpha)
\]

(2)
where \( w \) is the PEG molecular weight and \( w_0 \) is a characteristic weight of the channel that separates the regime of partitioning from that of exclusion. \( \alpha \) is a scaling parameter used to sharpen the transition from one regime to the other.

Combining equations (1) and (2) we may fit the symmetric curves shown in Figure 5.4. Leaving \( \alpha, \chi \), and \( w_0 \) as free parameters, fitting results in the values shown in Table 5.1.

The effective diameter of PEG in solution scales as a \( 3/5 \) power to its weight.\(^{166} \) Thus the value of \( w_0 \) for FhuA \( \Delta C/\Delta 4L \) can be compared to that of previously studied nanopores with known diameters to calculate its size. Using the outer membrane protein F (OmpF) of \( E. \ coli \), which has a known crystal structure,\(^{170} \) as a reference, we can estimate the diameter of the FhuA \( \Delta C/\Delta 4L \) nanopore with the formula\(^{156,166} \):

\[
d = d_{\text{OmpF}} \left( \frac{w_0}{w_{\text{OmpF}}} \right)^{3/5}
\]

where \( d_{\text{OmpF}} \) is 1.4 nm and \( w_{\text{OmpF}} \) is 1360 Da.\(^{11} \) The estimated diameter is given in Table 5.1.

Figure 5.1 suggests that FhuA \( \Delta C/\Delta 4L \) is asymmetric in structure and therefore asymmetric addition of PEG was performed to probe each opening of the nanopore independently. As is apparent in figure 5.4, the reduction in single-channel conductance for small polymers, 200 and 300 Da, in both \( \text{cis} \) and \( \text{trans} \) conditions, does not drop the single-channel conductance of the nanopore by the same proportion \( \chi \) seen in the symmetric case. The dropped proportion is well above that of the drop for conductivity of bulk aqueous phase. This finding
indicates only partial filling of the nanopore with PEG. To estimate the size of the \textit{cis} and \textit{trans} openings, the fitting procedure must be modified to take into account the non-complete partitioning. A simple way to do this is to express:

\[ \chi = \frac{g(w)_{\text{max}} - g(w)_{\text{min}}}{g_{\text{noPEG}}} \]  

again allowing \( \chi \) to be a free parameter when using equations (1) and (2) to fit. \(^7,1^5^6\) \( g(w)_{\text{min}} \) and \( g(w)_{\text{max}} \) are the single-channel conductance of FhuA \( \Delta C/\Delta 4L \) in the presence of PEG in the regime of full penetration and complete exclusion, respectively. \( g_{\text{noPEG}} \) denotes the single-channel conductance of FhuA \( \Delta C/\Delta 4L \) in the absence of PEG. Values for \( \alpha, \chi \), and \( w_0 \) for the \textit{trans} curve are given in Table 5.1.

**Table 5.1. Size estimates of the FhuA \( \Delta C/\Delta 4L \) nanopore using PEG.**

<table>
<thead>
<tr>
<th>Voltage</th>
<th>PEG addition</th>
<th>( \chi )</th>
<th>( w_0 )</th>
<th>( \alpha )</th>
<th>Diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+100 mV</td>
<td>Symmetric</td>
<td>0.54 + 0.06</td>
<td>1330 + 240</td>
<td>0.8</td>
<td>1.4 + 0.1</td>
</tr>
<tr>
<td></td>
<td>Trans</td>
<td>0.38 + 0.06</td>
<td>1890 + 400</td>
<td>1.2</td>
<td>1.7 + 0.2</td>
</tr>
<tr>
<td>0 mV</td>
<td>Symmetric</td>
<td>0.54 + 0.06</td>
<td>1250 + 280</td>
<td>0.8</td>
<td>1.3 + 0.1</td>
</tr>
<tr>
<td></td>
<td>Trans</td>
<td>0.28 + 0.02</td>
<td>1730 + 190</td>
<td>1.5</td>
<td>1.6 + 0.1</td>
</tr>
<tr>
<td>-100 mV</td>
<td>Symmetric</td>
<td>0.48 + 0.03</td>
<td>1610 + 160</td>
<td>1.0</td>
<td>1.5 + 0.1</td>
</tr>
<tr>
<td></td>
<td>Trans</td>
<td>0.2 + 0.03</td>
<td>1800 + 430</td>
<td>1.2</td>
<td>1.6 + 0.2</td>
</tr>
</tbody>
</table>

**Determining the size of the \textit{cis} opening of the nanopore using non-equilibrium partial filling assumption**

In the above model, equilibrium partitioning of PEG into FhuA \( \Delta C/\Delta 4L \) is assumed. A more complex interpretation of partial filling can be found by taking into account that the partitioning is not an equilibrium process for asymmetric partitioning. Also, at asymmetric conditions PEG will not partition all the way through the nanopore, but instead only partially into the interior. Here, we
model the probability of PEG polymer partitioning at a given distance, \( x \), into the nanopore interior. In this case, the single-channel conductance of the nanopore may be expressed as a function of the integral along its squared cross-sectional radius \( R(x) \) and multiplied by the factor \( \chi(w,x) \): \[ g(w) = \left( \frac{\int_0^L dx}{\chi(w,x) \pi R^2(x)} \right)^{-1} \] (5)

such that \( \chi(w,x) \) represents the decrease in conductivity in the presence of a polymer multiplied by the probability of the polymer being there:

\[ \chi(w,x) = \chi_0 - p(w,x)(\chi_0 - \chi) \] (6)

where \( p(w,x) \) is the partitioning function for a polymer of a given weight, \( w \), to partition into the nanopore a distance, \( x \). \( \chi_0 \) and \( \chi \) denotes the conductivity of the solution without PEG and with PEG, respectively. We use the following partitioning function: \[ p(w, x) = 10 \exp \left( -\left( \frac{w}{w_{\text{trans}}} \right)^\alpha \right) \int_0^L \frac{R^2(x)dx}{\int_0^L R^2(x)dx} \] (7)

where \( \alpha \) is again the scaling factor and \( w_{\text{trans}} \) is the characteristic weight of the protein at the \textit{trans} opening. Equation (6) takes into account that the nanopore is not in equilibrium and makes use of the Fick-Jacobs approximation.\(^{164}\) If we assume that the nanopore is conical with a constant slope and that the larger \textit{trans} opening is accurately modeled by using eqns. (1-4), we may fit the values of \( \frac{g(w)}{g(0)} \) for the \textit{cis} data by using eqns. (5-8). By assuming a \textit{trans} opening of 1.8 nm
and allowing $\alpha$ and $\chi_0 / \chi$ to be free parameters, best fits give estimates of the cis diameter between 0.85 and 1.1 nm for the voltages used in figure 5.4.

**Determination of the nanopore diameter using impermeable dextran polymer**

To independently assay the nanopore diameter, the contribution of FhuA $\Delta C/\Delta 4L$’s access resistance to the overall nanopore resistance was measured using impermeable dextran. For a non-selective, cylindrical and ohmic nanopore, the access-resistance can be expressed as $1 / 4 \sigma r$, where $\sigma$ is the solution’s conductivity and $r$ is the nanopore radius. The introduction of impermeable dextran to the bulk, decreases $\sigma$ of the solution surrounding the nanopore by the factor $\chi_{\text{dextran}}$ yet the solution in the interior of the nanopore maintains a conductivity of $\sigma$. The formula:

$$d = \frac{g_{\text{dextran}}}{\chi_{\text{dextran}} \sigma (1 - g_{\text{dextran}} / g)}$$

may then be used to estimate the nanopore diameter $d$, where $g_{\text{dextran}}$ is the single-channel conductance of the nanopore when dextran is added to chamber, and $g$ is the nanopore conductance in PEG-free solution.$^{34}$ This calculation gives an estimated nanopore diameter of 2.4 ± 0.6 nm for FhuA $\Delta C/\Delta 4L$. The diameter of the FhuA $\Delta C/\Delta 4L$ nanopore estimated from dextran experiments is significantly larger than that obtained from PEG experiments (Table 5.2). This is most likely a result of the simplifying assumptions used in modeling the access resistance contribution of dextran. The PEG experiments suggest an asymmetrical and non-
conical nanopore, whereas a conical nanopore was assumed in the dextran calculation.

**Table 5.2. Size estimates of transmembrane protein pores using water-soluble polymers**

<table>
<thead>
<tr>
<th>Protein pore</th>
<th>PEG symmetric (nm)</th>
<th>PEG cis (nm)</th>
<th>PEG trans (nm)</th>
<th>Access resistance dextran (nm)</th>
<th>Crystal structure (nm)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FhuA ΔC/Δ4L</td>
<td>1.2 - 1.6</td>
<td>0.85 - 1.1</td>
<td>1.4 - 1.9</td>
<td>2.4 ± 0.6</td>
<td>NA</td>
<td>This work</td>
</tr>
<tr>
<td>α–hemolysin</td>
<td>&lt; 2.0</td>
<td>--</td>
<td>--</td>
<td>2.2 ± 0.4</td>
<td>1.4</td>
<td>9</td>
</tr>
<tr>
<td>Anthrax protective antigen (PA₁₆₃)</td>
<td>&gt; 1.2</td>
<td>--</td>
<td>--</td>
<td>2.3 ± 1.6</td>
<td>NA</td>
<td>32</td>
</tr>
<tr>
<td>Alamethicin</td>
<td>~ 1.4</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.7 x 1.1</td>
<td>13</td>
</tr>
<tr>
<td>OmpU</td>
<td>1.10</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>NA</td>
<td>33</td>
</tr>
<tr>
<td>OmpT</td>
<td>0.86</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>NA</td>
<td>33</td>
</tr>
<tr>
<td>Epsilon Toxin</td>
<td>2.0</td>
<td>0.8</td>
<td>2.0</td>
<td>--</td>
<td>NA</td>
<td>20</td>
</tr>
<tr>
<td>Syringomycin E channel</td>
<td>--</td>
<td>0.25-0.35</td>
<td>0.5-1.0</td>
<td>--</td>
<td>NA</td>
<td>31</td>
</tr>
</tbody>
</table>

NA stands for not available.

Previous studies have looked at the contribution of polymer interaction with membrane pores to the current noise of the system. In the case of α–hemolysin and alamethicin, polymer interaction was found to increase the noise when the weight of the polymer was near $w_0$.$^{151}$ However, in the case of OmpF, the interactions of the polymers with the protein interior were negligible.$^{11}$ We found that no significant noise fluctuations occur near the transition weight, $w_0$, suggesting that the nanopore behaves more like OmpF (Fig. 5.5B). In contrast, there is excess noise present at higher PEG molecular weight. While, all visible closures were excluded when analyzing this data, it is still possible that this excess noise occurred as a result of rapid nanopore closures that were not visible as spikes. Increased closing activity of the nanopore was observable when higher
molecular weight polymers were added (Appendix C, C3). Given that the polymer-pore interactions lead to skewing of the transition weight, \( w_0 \), to higher values, we believe that the limited interaction measured near the \( w_0 \) regime justifies the use of equation (3), as the comparison with OmpF is apt. It may also explain why estimates with the PEG experiments are smaller than those obtained from the dextran experiments, while \( \alpha \)-hemolysin, which exhibits a large noise response, shows the reverse.

**Concluding remarks**

We systematically examined the interior of the engineered FhuA \( \Delta C/\Delta 4L \) protein nanopore using polymer exclusion. Permeable and impermeable water-soluble polymers were added to the chambers symmetrically or asymmetrically. Results from asymmetric addition of PEG suggest the nanopore’s extracellular opening is smaller than the periplasmic opening; however, the values obtained in these estimates are smaller than those expected from the crystal structure. A possible explanation for this is that the deletion of the cork domain and extracellular loops may modify the inner dimensions of the \( \beta \) barrel along the central transversal axis. The elliptical nature of the FhuA \( \Delta C/\Delta 4L \) nanopore may also contribute to this underestimate, with the minor axes precluding the partitioning of larger PEG. This might explain the discrepancy between the PEG estimates and the dextran result, as the access-resistance calculation assumes a circular opening. Evaluation of the low-frequency spectral noise density, \( S(0) \), provided modest values for PEGs whose molecular weight is comparable or lower than the transition weight, \( w_0 \). This finding suggests that PEGs do not
significantly interact with the interior of FhuA ΔC/Δ4L, which is in accord with the hydrophilic nature of the nanopore walls with numerous positive and negative charges.
Chapter 6
Concluding Remarks
**Introduction**

While the last decade has seen a great deal of study using solid-state nanopores, there are still several problems to overcome before these devices can be integrated into commercial sensing systems. First, the ability to sculpt solid-state pores with atomic level precision is lacking. This means that each individual nanopore has slightly different characteristics, which affects the residence-time, capture-rate, and amplitude blockades of an analyte interacting with the pore. This requires that each pore be calibrated separately. There are some helpful developments in this problem. For example, Wei et al. developed a technique for placing a single binding site within a large solid state pore\(^{53}\) however, even here, the exact placement of the site within the pore is not reproducible. Second, there still exists the problem of nanopore fouling caused by non-specific adsorption of analytes, which makes further use of the nanopore impossible.\(^2\) Third, there is the problem of resolving different analytes. This can be seen when comparing the abilities of protein nanopores, which can sense individual nucleotides of DNA to their solid-state counterparts. This difficulty arrives mostly due to the rapid speed that DNA transverses the nanopore. Molecular dynamics simulations suggest that it may be possible to slow down DNA translocation through a nanopore by imbedding electrodes within the sides of the pore and ratcheting the DNA through,\(^{171}\) yet this has yet to be demonstrated experimentally. Improvements to the noise of the solid-state nanopore system have also been made recently by integrating the current measurement device on the nanopore chip.\(^{172}\)
There is no sign that development in the nanopore field is slowing. As these issues are resolved in the future, a greater and greater range of experimental possibilities will come within the ambit of nanopore sensing. Some of the more detailed possibilities will be discussed below.

**Future possibilities**

Among the most interesting possibilities is the use of nanopore to detect epigenetic information along DNA. Wanunu et al. described the sensing of DNA methylation with solid-state nanopores.\textsuperscript{173} As the development of nanopore DNA sequencing devices improve, the ability to see epigenetic markers along the DNA are likewise enhanced. Soni and Dekker recently published a work inspecting the structural properties of nucleosomes using solid-state nanopores.\textsuperscript{174}

Recently, the detection of circulating microRNAs in lung cancer patients using a nanopore has demonstrated the feasibility of nanopore technology for use in early detection of cancer-associated disease markers.\textsuperscript{175} The possible development of an inexpensive DNA sequencing device\textsuperscript{1,176} has great implications for personalized medicine. For example, a physician could take samples of cancer cells and compare any mutations these have with normal cells, which could show the way for treatment options. Should the development of nanopores lead to sensitivity to epigenetic changes, this technique might be even more powerful.

Given the possibilities, the best nanopore work is yet to come.
Immediate prospects

The previous chapters have explored the use of solid-state nanopores in studying BSA adsorption to silicon nitride and the inspection of binding of RNA aptamers to NCp7. Additionally chapter 5 detailed the characterization of the FhuA ΔC/Δ4L nanopore using PEG. In this section I would like to explore how to combine these two systems to create a hybrid nanopore, with FhuA ΔC/Δ4L embedded in a nitride membrane.

![Figure 6.1](image) Hybrid nanopore system. (A) dsDNA connected to the FhuA cysteine mutant is pulled into the nanopore. (B) dsDNA is removed forming a hybrid pore. (C) FhuA cysteine mutant activity in a lipid bilayer at +120 mV. Solution was 1 M KCl 10mM potassium phosphate, pH 7.4 (courtesy of Mohammad Mohammad).

To date there are two types of hybrid nanopores. First there was the demonstration by Hall et al. that α-hemolysin could be dragged into a nanopore by tethering to dsDNA\(^{51}\). Second, there have been two papers detailing the formation of DNA origami structures that could be placed inside a nanopore interior, again by dragging with DNA\(^{177,178}\). Can this also be done with FhuA ΔC/Δ4L? What advantages might it bring?
Recent experiments at Syracuse University by Mohammad Mohammad (data unpublished) have demonstrated the creation of a new FhuA ΔC/Δ4L variant with a cysteine containing loop near the cis end of the pore. This nanopore has been demonstrated to actively insert into synthetic bilayers in 1M KCl, pH 7.4, Figure 6.1C shows this nanopore at an applied voltage of 120 mV. The cysteine allows for the attachment of thiolated DNA to the nanopore.

What might we expect from an FhuA ΔC/Δ4L solid-state hybrid? Analysis with PEG suggest a smaller diameter of FhuA ΔC/Δ4L that would be expected from the simple removal of the cork form the FhuA wild type crystal structure. One possible reason for this difference is the excursion of loops from the exterior to the interior of the pore. It is possible that by placing the FhuA ΔC/Δ4L into the interior of the nanopore, these loops might adsorb to the silicon nitride sides, allowing for a larger FhuA ΔC/Δ4L interior. This might allow for the inspection of dsDNA. Additionally, the application of voltage across FhuA ΔC/Δ4L was limited by the breakdown of the bilayer at voltages greater than 250 mV, a hybrid nanopore has no such limitation. Therefore, voltages as high as 1000 mV might be possible across the hybrid pore.

Such a hybrid system could also be more easily integrated into devices and analyte solution could be changed without the worry of breaking the bilayer.
Figure 6.2: Change of residence time for dsDNA with nanopore functionalization. (A) dwell-time histogram and typical event for 1500 bp dsDNA in a 15 nm silicon nitride nanopore. (B) dwell-time histogram and typical event for 1500 bp dsDNA in 15 nm pore that has been coated with amine groups. A significant difference in residence-time was observed. Solution conditions were 1M KCl, 10 mM potassium phosphate, pH 7.4. Applied voltage was +100 mV. dsDNA was added to the cis side of the chamber. (Results courtesy of Nan Qin).

Another line of research we have pursued is the functionalization of nitride nanopores, using an adapted method from Wanunu and Meller. Working in collaboration with Nan Qin, we have preliminary data suggesting that the silanization of a nitride nanopore using 3-aminopropyltrimethoxysilane significantly slows the translocation of dsDNA (Figure 6.2). This may be
explored further, enlightening how charges affect both the entrance of dsDNA into the nanopore and the translocation dynamics of dsDNA.
Appendix A:
Characterization of the nanopores

Figure A1. Voltage dependence of the single-channel currents for nanopores of different diameters. Current response measurements were taken at 1 M KCl, 10 mM potassium phosphate buffer, pH 7.4.

Figure A2. Comparison traces of nanopores. A) A trace collected with a 10 nm-diameter nanopore with a “noisy” current signature, B) A trace collected with a 10 nm-diameter nanopore with a stable single-channel current. Measurements were taken at a transmembrane potential of +150 mV, with a buffer solution containing 1M KCl, 10 mM potassium phosphate solution, pH 7.4. The single-channel electrical traces were low-pass Bessel filtered at 10 kHz.

A nanopore was considered to be “wet” if the following three properties were observed. First, the nanopore had to show a stable conductance at a constant applied transmembrane potential. Transmembrane potentials of +150 mV were typically used for this test (Fig. A2). Second, the current response had to be linear (Ohmic) with the applied transmembrane potential (Fig. A1). Third, the conductance, as measured by a
straight-line, least-squares fitting of the I/V curve, had to correspond with the expected value of the conductance for a nanopore of the diameter measured via TEM. Expected conductance values were obtained in the following way. The nanopore diameter was measured with TEM. Conductance of nanopores satisfying the first two criteria was characterized at 1M KCl using the I/V curve protocol described above. The results were comparable to those found in the literature. For our purposes, if the nanopore conductance was within 20% of the expected value, the nanopore was considered acceptable.

**BSA Purity**

Lyophilized bovine serum albumin (BSA) was purchased from Bio-Rad (Hercules, CA). BSA was hydrated with double-distilled water to a concentration of 22 µM and stored at 4°C. Purity was confirmed by 10% SDS-PAGE gel electrophoresis (Fig. A3). Bands consistent with BSA monomers, dimers and trimers were observed.

**Figure A3.** SDS-PAGE gel electrophoresis of the BSA sample. (A) BSA trimers, (B) BSA dimers (C) BSA monomers. BSA-SDS was heated to 95°C for 5 minutes prior to running the gel.
The amplitude of the short-lived current blockades

Figure A4. Typical single-channel current trace (A) and typical event amplitude histogram (B) recorded with a 12 nm-wide solid-state nanopore when 60 nM BSA was added to the cis chamber. The buffer solution contained 1 M KCl, 10 mM potassium phosphate, pH 7.4. The applied transmembrane potential was +150 mV. The single-channel electrical traces were low-pass Bessel filtered at 10 kHz.

Figure A5. Dependence of the short-lived event frequency on the BSA concentration added to the cis chamber. The single-channel electrical data were recorded with a 12 nm-diameter nanopore. Least squares fitting gave a slope of 26 s⁻¹nM⁻¹ BSA. Other experimental conditions were similar to those presented in Fig. A4.

Our estimate for the frequency of the short-lived current blockades did not include the missed events due to the rise time of the Bessel filter. The frequency of the short-lived current blockades was calculated by dividing the number of current blockades by the duration of the trace. Assuming that the current blockades occurred stochastically, uncertainty values for frequency were calculated from the uncertainty in the number of current blockades given by \(N^{1/2}\), where \(N\) is the number of current blockades. This value for uncertainty assumes that the short-lived current blockades occurred independently and continuously following a Poisson process. Nanopores varied in sensitivity to BSA. Different nanopores of the same
diameter showed up to a 60-fold difference in the frequency of events under the same conditions. For example, we observed that the frequency of current blockades with two 12 nm-diameter nanopores (at +150 mV; 1M KCl, 10 mM potassium phosphate, pH 7.4; 180 nM BSA) was $0.5 \pm 0.03 \text{s}^{-1}$ and $37.5 \pm 0.25 \text{s}^{-1}$, respectively. The median amplitude of the short-lived current blockades was independent of the diameter of the nanopore ($470 \pm 40 \text{pA}$, for 19 nanopore diameters ranging 9-20 nm) (Table A1). This is to be expected, if these current blockades feature amplitude that is proportional to the excluded volume of the BSA proteins.

<table>
<thead>
<tr>
<th>Diameter (nm)</th>
<th>Median amplitude of blockades (pA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>524</td>
</tr>
<tr>
<td>10</td>
<td>488</td>
</tr>
<tr>
<td>10</td>
<td>554</td>
</tr>
<tr>
<td>12</td>
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<td>20</td>
<td>496</td>
</tr>
<tr>
<td>20</td>
<td>516</td>
</tr>
</tbody>
</table>

Table A1. Median amplitude values for nanopores of given diameter were found by performing a single-channel search with Axon ClampFit 10.2 analysis package. All measurements were performed at 1M KCl, 10 mM potassium phosphate, pH 7.4, with an applied voltage of +150 mV.

The characteristics of the long-lived current blockades

Figure A6. Examples of two-state gating in Si,N, nanopores. For all traces, the experimental conditions were 1 M KCl, 450 nM BSA, 10 mM potassium phosphate, pH 7.4. The applied potential was +150 mV. The single-channel traces were low-pass Bessel filtered at 2 kHz. Nanopores A, B, C, D and E had diameters of 22, 16, 11, 11 and 10 nm, respectively.
The long-lived current blockades did not occur in every nanopore, or at every concentration. When “fluctuating” events occurred, they often began suddenly and occurred at a high frequency for short intervals, only to cease again. We call these events “gating” and they do not appear to have a simple concentration dependence (Fig. A7). Two protocols were followed in concentration dependent measurements. In both cases, BSA was added to the cis side of the chamber. In the first data set, concentration experiments were performed in 1M KCl, 10mM potassium phosphate, pH 6 at a voltage of +120 mV. Initial concentration of BSA was 10 nM. A 10 minute current trace was performed at each interval.

<table>
<thead>
<tr>
<th>Diameter (nm)</th>
<th>Voltage (mV)</th>
<th>Concentration at onset (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>150</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>150</td>
<td>-NA-</td>
</tr>
<tr>
<td>10</td>
<td>120</td>
<td>59</td>
</tr>
<tr>
<td>12</td>
<td>150</td>
<td>180</td>
</tr>
<tr>
<td>12</td>
<td>150</td>
<td>-NA-</td>
</tr>
<tr>
<td>14</td>
<td>150</td>
<td>60</td>
</tr>
<tr>
<td>14</td>
<td>120</td>
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<td>14</td>
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<td>150</td>
<td>20</td>
</tr>
<tr>
<td>22</td>
<td>120</td>
<td>59</td>
</tr>
</tbody>
</table>

Table A2. Summary of the concentrations at which the onset of the long-lived current blockades occurred. In some experiments, nanopores experienced clogging before the long-lived current blockades occurred; these cases were excluded from analysis unless a BSA concentration of 180 nM was reached. “–NA–” signifies the pore clogged (Fig. S9).

Concentrations were raised in staggered intervals until clogging occurred. In the later data set, experiments were performed at 1M KCl, 10 mM potassium phosphate, pH 7.4. Initial BSA concentrations were 20 nM. They were raised in 20 nM increments until reaching 180 nM or clogging occurred. The majority of experiments ended when the nanopore “clogged” (Fig. A8), making it impossible to continue measurements. We interpret this state as an irreversible absorption of BSA molecules to the nanopore’s inner surface. BSA-induced short-lived current...
blockades ceased entirely in the clogged state. Experiments were not performed in the reverse order, meaning high concentration to low, due to clogging at high concentrations.

Figure A7. Representative states of pore. (A) BSA-induced current blockades prior to gating state, (B) Long-lived current blockades occurring during the “gating” state, (C) “Clustering” of gating events. Data was taken with a 10 nm pore in 1 M KCl, 10 mM potassium phosphate, pH 7.4. 20 nM BSA was added to the cis chamber. The transmembrane potential was +150 mV. The electrical traces were low-pass Bessel filtered at 10 kHz.

Figure A8. Trace showing the adsorptions of BSA molecules to interior of the pore wall, resulting in a final “clogging” of the pore in which short lived events end. This single-channel electrical trace was recorded with a 10 nm-wide nanopore. 450 nM BSA was added to the cis chamber. The buffer solution contained 1M KCl, 10 mM potassium phosphate, pH 7.4. The applied transmembrane potential was +150 mV. The trace was filtered at 2 kHz.

Observation of small polypeptides with narrower solid-state nanopores

BSA-induced current blockades were not observed with nanopores smaller than 8 nm in diameter. Our interpretation is that the bulk of the BSA is too large to enter nanopores smaller than this size. To confirm that this observation is not an artifact of our nanopores, we show events in a 4 nm-wide nanopore due to the 55 residue-long viral nucleocapside polypeptide NCp7 of the HIV-1 virus.120,124,179,180
**Figure A9.** The viral nucleocapside polypeptide NCp7 of the HIV-1 virus produces short-lived current blockades with a small nanopore. (A) Control trace without NCp7, (B) 100 nM NCp7 added to the cis chamber. The diameter of the nanopore was 4 nm. The buffer solution was 1M KCl, 10 mM potassium phosphate, pH 7.4. The applied transmembrane potential was -450 mV. When measuring with a 100 kHz filter, the median $\tau_{\text{off}}$ was 15μs. This places an upper bound on the $\tau_{\text{off}}$. 

![A graph showing current (pA) over time with labels indicating the control trace and NCp7 addition.](image-url)
Appendix B:

Sampling a Biomarker of the Human Immunodeficiency Virus (HIV) across a Synthetic Nanopore

B1. Control experiments with NCp7 and SL3 RNA aptamers using small nanopores

**Figure B1:** The NCp7 protein did not produce any alteration in the single-channel electrical signature of a small nanopore. Single-channel electrical trace demonstrates lack of current blockades at a positive bias of +100 mV for a nanopore after 1000 nM was added to the *cis* side. Solution in the chamber was 0.2 M NaCl, 5 mM NaH$_2$PO$_4$ on the *cis* side, and 1 M NaCl, 5 mM NaH$_2$PO$_4$ on the *trans* side. The diameter of the nanopore was 3.8 ± 0.3 nm.

**Figure B2:** NCp7 fouling of a nanopore. The single-channel electrical trace demonstrates NCp7 events at a negative bias of -100 mV for a nanopore after 1 µM NCp7 was added to the *cis* side. Solution in the chamber was 0.2 M NaCl, 5 mM NaH$_2$PO$_4$, pH 7.0 on the *cis* side, and 1 M NaCl, 5 mM NaH$_2$PO$_4$, pH 7.0 on the *trans* side. The diameter of the nanopore diameter was 3.8 ± 0.3 nm. Nanopores eventually clogged under these conditions.
B2. Comparison of the values of the dwell time and amplitude of the current blockades recorded with a small-diameter nanopore before and after addition of NCp7 to solution

Figure B3: The SL3 (GAG) aptamer does not produce current blockades at a negative voltage using a small nanopore. Single-channel electrical trace demonstrates lack of events at a negative bias of -100 mV for a nanopore after 500 nM RNA SL3 (GAG) was added to the cis side. Solution in the chamber was 0.2 M NaCl, 5 mM NaH2PO4 on the cis side, and 1 M NaCl, 5 mM NaH2PO4 on the trans side. The diameter of this nanopore was 3.7 ± 0.3 nm.

Figure B4: Comparison of the values of the dwell time and amplitude of the current blockades recorded with a small-diameter nanopore. Dwell time histograms for events in solution with 1000 nM SL3(GAG) RNA (blue) and after addition of 400 nM NCp7 (red). Fitting to a single term exponential gives dwell times of 210 ± 3 µs and 220 ± 3 µs, respectively. Event amplitude versus dwell time scatter plot is given in the inset. Solution was 0.2 M NaCl, 5 mM NaH2PO4, pH 7.0 on the cis side, and 1 M NaCl, 5 mM NaH2PO4, pH 7.0 on the trans side. 500 nM SL3 (GAG) aptamer was added to the cis side. The diameter of the nanopore was 4 nm.
B3. Voltage dependence of the dwell time of the current blockades produced by SL3 RNA aptamers

Figure B5: Voltage dependence of the dwell time of the SL3 RNA aptamer-produced current blockades. (A) Dwell-time histograms are shown; (B) The voltage dependence plots presenting that the dwell time displays a maximum value at applied voltages between 200 and 250 mV. Solution was 0.2 M NaCl, 5 mM NaH₂PO₄, pH 7.0 on the cis side, and 1 M NaCl, 5 mM NaH₂PO₄, pH 7.0 on the trans side. 500 nM SL3 (GAG) aptamer was added to the cis side. The diameter of the nanopore was 4.5 nm. Note that single-channel recordings with different nanopores displayed different dwell time durations.

B4. Large event ratio with smaller events for all three SL3 RNA aptamers
Assuming that the capture of NCp7 and its complex in large nanopores is representative of the concentration of the complex and free SL3 RNA aptamer in solution and also assuming 1:1 complexes, we could use equation 2 (the main text) to find $K_d$, making the substitution of $f_{\text{small events}} + f_{\text{large events}}$ for $f_0$. Fig. B6 shows a plot of the titration curves following this method. Using this approach, there is a substantial difference between the binding affinities of the high-affinity SL3 RNA aptamer to NCp7, as calculated with large nanopores and fluorescence (Table S1).

Table B1. The $K_d$ values calculated with large nanopores and using the event ratios.¹

<table>
<thead>
<tr>
<th>The SL3 RNA aptamer</th>
<th>Large nanopore</th>
<th>Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAG</td>
<td>1357±337 nM</td>
<td>28 ± 2 nM</td>
</tr>
<tr>
<td>CUG</td>
<td>607±67 nM</td>
<td>850 ± 250 nM</td>
</tr>
<tr>
<td>AUA</td>
<td>11400±2450 nM</td>
<td>20000 nM</td>
</tr>
</tbody>
</table>
Note that it is the NCp7 protein - SL3 (GAG) aptamer complex that gives the great distinction values from fluorescence data. The SL3 RNA (CUG) and (AUA) fittings give fairly reasonable values, despite their slopes being shallower than expected. 

Fluorescence-based values are from the previously reported data.²

The most probable culprit is the assumption that the nanopore samples the ratio of events correctly. There are several reasons for thinking this might not be the case. Most theories of analytes entering a nanopore break the process into 3 regimes: the diffusion regime, the attraction regime and the entering regime. All three will affect the capture rate of the analyte and for all three we may expect that the complex will behave differently than RNA alone. In the first regime the bulkier complex will have a slower diffusion rate. In the second it will feel less force because of its lesser charge density. In the third, while larger pores appear to admit the complex, there still may be a substantial energetic penalty for the complex to enter the pore when compared with the RNA alone.

---

**B5. Frequency of low-amplitude, SL3 RNA aptamer-induced current blockades observed with large nanopores**

The success in determining $K_d$ values by monitoring the reduction in event frequency with small nanopores suggests that a similar mode of analysis may be fruitful with large nanopores. However, monitoring the reduction in small-current amplitude events in larger nanopores does not lead to reasonable $K_d$ values for NCp7-SL3 RNA (GAG) interaction. Analysis was performed in which the original frequency of small amplitude current events was measured and titrations with NCp7 were performed. The frequency of small amplitude events was measured at each NCp7 concentration and a ratio was made to the original SL3 RNA frequency. The main difficulty with this approach is the reproducibility of event reduction when NCp7 concentration exceeds that of SL3 RNA (Fig. B7). It may be the case that NCp7-SL3 complexes are causing short-lived bumping events that increase the apparent frequency of SL3 RNA (GAG) events.

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**Figure B6: Titration curves for all three SL3 RNA aptamer variants using ratio of the number of small events to number of large events acquired with large nanopores.**

Squares represent the high-affinity SL3 (GAG) aptamer, triangles indicate the low-affinity SL3 (CUG) aptamer, and circles display the no-affinity SL3 (AUA) aptamer. The applied voltage was +200 mV. The solution in the chamber contained 200 mM NaCl, 5 mM NaH$_2$PO$_4$, pH 7.0 on the cis side, and 1 M NaCl, 5 mM NaH$_2$PO$_4$, pH 7.0 on the trans side. Curves are fits, as described in the main text.
Figure B7: Frequency reduction of short amplitude events in large nanopores. The three scatter plots represent different measurements of ratio of measured small amplitude events ascribed to SL3 RNA (GAG) in a single nanopore 10 nm diameter nanopore. The reduction in short event frequency varied significantly even on the same pore. Solution was 0.2 M NaCl, 5 mM NaH$_2$PO$_4$, pH 7.0 on the cis side, and 1 M NaCl, 5 mM NaH$_2$PO$_4$, pH 7.0 on the trans side. The original SL3
Appendix C:

FhuA purification and refolding:

![Coomassie blue-stained SDS-PAGE gel indicating the purified FhuA ΔC/Δ4L protein pore and its refolding.](image)

**Figure C1.** Coomassie blue-stained SDS-PAGE gel indicating the purified FhuA ΔC/Δ4L protein pore and its refolding. Protein samples are loaded on the gel without or after boiling for 5 minutes. Folded and unfolded FhuA ΔC/Δ4L proteins are indicated by arrows. MW stands for molecular weight standard.

**Selection Criteria**

The FhuA ΔC/Δ4L pore inserts with a range of different conductances at +40 mV. Figure S1 displays a histogram of the conductance of the pore at +40 mV. Only pores greater than 4 nS in conductance were selected. The reasoning behind this choice is that FhuA ΔC/Δ4L pores with a closely similar conductance are thought to be likely to have a similar structure in our protein preparation.
Figure C2. Histogram of different conductances of the FhuA ΔC/Δ4L engineered nanopore. The conductances are taken at +40 mV in 1 M KCl, 10 mM potassium phosphate buffer, pH 7.4. Histogram was constructed from a subset of data representing step-wise insertions of 98 different pores.

Purity of Reagents

PEG molecular weight ranges were following: PEG200 (M, 190-210), PEG300 (M, 285-315), PEG600 (M, 570-630), PEG1000 (M, 950-1050), PEG1500 (M, 1400-1600), PEG2000 (M, 1900-2200), PEG3000 (M, 2700-3300), PEG4000 (M, 3500-4500), PEG6000 (M, 5000-7000), PEG8000 (M, 7000-9000), PEG10000 (M, 8500-11500), PEG12000 (M, 11000-15000).
Table C1. Measured Conductivity of the Solutions

<table>
<thead>
<tr>
<th>Solution</th>
<th>Conductivity (mS/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG300</td>
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</tr>
<tr>
<td>PEG600</td>
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<td>56.8</td>
</tr>
<tr>
<td>Dextran</td>
<td>62.8</td>
</tr>
</tbody>
</table>

Table C1. Table of different measured bulk conductivities of the solutions used in this work. Normal 1 M KCl solution was buffered with 10 mM potassium phosphate, pH 7.4 and had a conductance of 98.6 mS/cm. Both PEG and Dextran solutions were 15% (w/w) in 1 M KCl, 10 mM potassium phosphate, pH 7.4.

Dextran Experiments

![Graph showing open channel current of a single FhuA nanopore at +80 mV potential.](image)

Figure C3. Open channel current of a single FhuA nanopore at +80 mV potential. The trace on the right shows the channel in 1M KCl, 10mM potassium phosphate, pH7.4. The trace on the left shows the same channel after solution has...
been changed to 15% (w/w) 40 kDa dextran in 1 M KCl, 10 mM potassium phosphate, pH 7.4. A reduction in open channel conductance of 9% was observed. Note the increased noise.

**Calculations of expected conductance**

A rough calculation of the expected conductance of the FhuA ΔC/Δ4L can be made assuming a symmetrical conical pore, or elliptical pore, that approximates the crystal structure.

1. **Calculation for pore conductance assuming a conical pore**

Here, $Q$ is conductance, $r_{eff}$ is the effective radius of the pore, $\sigma$ is the bulk conductivity, and $l$ is the pore length. The conductance of the pore may be approximated by

$$Q = \pi r_{eff} \frac{\sigma}{l},$$

where $r_{eff}$ is the “effective radius” of the nanopore.

Let $r_{eff} = \left( \frac{\pi}{l} \int_0^l \frac{dz}{S(z)} \right)^{-1/2}$ were $S(z)$ is the area of the cross-section of the pore at a point $z$ along the axis, then

$$Q = \frac{\pi \sigma}{l \left( \int_0^l \frac{dz}{S(z)} \right)^{1/2}},$$

assume the pore can be expressed as a simple symmetrical cone, where $a(z)$ is the perpendicular distance of the cone from the $z$-axis at point $z$.

Then,

$$Q = \frac{\sigma}{l \int_0^l \frac{dz}{\pi(a(z))^2}}.$$
Where \( \chi \) is a constant representing the slope of the cylinder given by,

\[
\chi = \frac{a(0) - a(l)}{l}.
\]

\[
Q = \frac{\pi \sigma}{4} \left( \frac{dz}{\chi(a(0) - \chi l)} \right)
\]

Relying on the crystal structure and bulk conductivity measurements, we may substitute in \( \sigma = 112 \times 10^{-11} \text{ S/m} \), \( a(0) = 16 \times 10^{-10} \text{ m} \), \( a(l) = 10 \times 10^{-10} \text{ m} \) and \( l = 50 \times 10^{-10} \text{ m} \), we find \( Q = 2.81 \text{ nS} \).

2. **Calculation for pore conductance using smooth-walled ellipsoid**

Following the same equation as above, we may ask what the expected conductance of a smoothly changing ellipsoid.

\[
Q = \frac{\pi \sigma}{4} \left( \frac{dz}{l \int_0^l \frac{dz}{S(z)}} \right)
\]
We will assume the cross-sectional area may be expressed as an ellipse with a major and minor axes given by \(a(z)\) and \(b(z)\) respectively. The integral will now become,

\[
\int_0^l \frac{dz}{a(z)b(z)}
\]

If we assume that \(a(z)\) and \(b(z)\) change with a constant slope we have,

\[
\int_0^l \frac{dz}{(a(0) - \gamma z)(b(0) - \xi z)}
\]

From the crystal structure we may estimate values of \(a(0) = 3.1 \times 10^{-9} \text{ m}, b(0) = 4.4 \times 10^{-9} \text{ m}, l = 4.5 \times 10^{-9} \text{ m}, \gamma = 1/15, \xi = 3/5.\)

\(Q = 16.2 \text{ nS}\)
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Vita

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