Published in final edited form as:

Nano Lett. 2018 February 14; 18(2): 660–668. doi:10.1021/acs.nanolett.7b03987.

DNA Translocations Through Nanopores Under Nanoscale Pre-Confinement

Kyle Briggs[†], Gregory Madejski[‡], Martin Magill[§], Konstantinos Kastritis[§], Hendrick W. de Haan[§], James L. McGrath[‡], and Vincent Tabard-Cossa^{*,†}

[†]Department of Physics, University of Ottawa, Ottawa, ON, Canada

[‡]Department of Biomedical Engineering, University of Rochester, Rochester, NY, USA

[§]Faculty of Science, University of Ontario Institute of Technology, Oshawa, ON, Canada

Abstract

To reduce unwanted variation in the passage speed of DNA through solid-state nanopores, we demonstrate nanoscale pre-confinement of translocating molecules using an ultra-thin nanoporous silicon nitride membrane separated from a single sensing nanopore by a nanoscale cavity. We present comprehensive experimental and simulation results demonstrating that the presence of an integrated nanofilter within nanoscale distances of the sensing pore eliminates the dependence of molecular passage time distributions on pore size, revealing a global minimum in the coefficient of variation of the passage time. These results provide experimental verification that the inter- and intra-molecular passage time variation depends on the conformational entropy of each molecule prior to translocation. Furthermore, we show that the observed consistently narrower passage time distributions enables a more reliable DNA length separation independent of pore size and stability. We also demonstrate that the composite nanofilter/nanopore devices can be configured to suppress the frequency of folded translocations, ensuring single-file passage of captured DNA molecules. By greatly increasing the rate at which usable data can be collected, these unique attributes will offer significant practical advantages to many solid-state nanopore-based sensing schemes, including sequencing, genomic mapping, and barcoded target detection.

*Corresponding Author: tcossa@uOttawa.ca, 613-562-5800 x6964.

Author Contributions

VTC and KB designed the experimental study. KB performed nanopore experiments and wrote the first draft. KB and MM analyzed nanopore experiments. GM and JLM developed NPN material. GM and KB developed nanofiltered pore device assembly protocols. HWdH designed the simulation study. KK and MM performed and analyzed simulations. GM generated schematic renders. All authors contributed to revision of the manuscript

Competing Financial Interest

JLM declares a competing financial interest as a co-founder and equity holder of SiMPore Inc., a commercial manufacturer of NPN and silicon-based membrane materials. VTC and KB declare a competing financial interest in the form of a patent on the nanofiltered nanopore device. All other authors declare no competing financial interest.

Supporting Information

Supplementary Section S1: Physical Properties of Nanofilters.

Supplementary Section S2: Simulation Setup.

Supplementary Section S3: Analysis of Passage Time Distributions.

Supplementary Section S4: Size Separation of dsDNA by Mean Passage Time.

Supplementary Section S5: Slower Passage Times in Small Control Pores.

Supplementary Section S6: Long Passage Time Events.

Supplementary Section S7: Folding Distributions.

Video Legend: Brief descriptions of all supplementary videos.

Graphical Abstract

Keywords

nanopore; nanotechnology; nanofabrication; DNA; entropy; nanoconfinement

When a single biopolymer such as DNA translocates a nanopore, the dynamics of molecular transport are complex^{1–5}. The speed during passage is thought to be dependent on the fraction and conformation of the molecule outside the pore⁶, as well as being subject to thermal fluctuations and transient interactions with the pore walls and membrane materials^{7–9}. The net effect is for the molecular motion to be afflicted by a wide distribution of passage speeds, both due to inter- and intra-molecular velocity fluctuations¹. Such spread in passage times confounds simple translation of time to molecular position, complicating mapping applications, and greatly limits the ability of the nanopore to distinguish charged molecules by size compared to traditional gel-based electrophoresis techniques.

Most experimental efforts aimed at controlling the speed of molecular translocation through a solid-state nanopore have focused on slowing DNA by various means, including interfacing the pore with a gel^{10,11}; by judicious choice of electrolyte, both aqueous¹² and ionic-liquid¹³; by laser-modulating the surface charge density¹⁴; by adjustment of the viscosity¹⁵; or by using different membrane materials^{16,17}. While these methods are able to slow DNA translocations to varying degrees, they generally do so at the cost of wider passage time distributions.

Few studies have considered the factors that contribute to the wide distributions of passage times. Experimentally, the choice of salt solution has been shown to have a significant effect on the width of passage time distributions¹², while a pore with diameter matched to the size of DNA limited DNA self-interaction and reduced the variation in passage time¹⁸. Barcoded molecules have also been used to explore intra-molecular variation in passage times, revealing speed up toward the end of the translocation¹⁹, and it was found that Brownian motion alone is insufficient to explain the observed variations^{20,21}. Simulation work, on the other hand, has demonstrated that polymers are perturbed from equilibrium by the extended electric field gradient during the capture process²², changing the passage time distribution compared to equilibrium predictions. In addition, molecules which are extended prior to translocation have longer passage times due to increased drag forces⁶.

Unfortunately, while the dominant mechanism responsible for high variability in passage time is thought to be the large conformational entropy available to DNA molecules prior to translocation through the nanopore²³, experimental verification has remained difficult due to

the complexity of fabricating devices with sufficiently confining geometries in the vicinity of a nanopore^{24–27}. Recent work, employing the confining geometry of nanopipette-based conical nanopores, has provided evidence that polymer entropy prior to translocation is related to mean passage time, but did not consider the standard deviation²⁸. In this work, we present the first experimental verification that entropy reduction through pre-confinement of DNA reduces the passage time variation inherent in nanopore transport processes. Confinement is achieved by taking advantage of the extreme flexibility and permeability of ultrathin (50 nm) nanoporous silicon nitride (NPN) membranes^{29,30} to place them within nanoscale distances of a solid-state nanopore sensor while still allowing fluidic contact in an innovative single-molecule biosensor configuration.

The nanofiltered nanopore device architecture realizes a two-membrane system, comprised of an ultra-thin, nanoporous layer of 50-nm thick NPN membrane, separated by a 200-nm gap from a 20-nm thick silicon nitride (SiN) membrane. This essentially creates an architecture comprising two pores in series. NPN is a recently developed highly porous nanomembrane technology with tunable pore sizes (20 – 80 nm) and porosities (1–40%)²⁹. The gap between the nanofilter and SiN membranes is achieved through the lithographic patterning of a hexagonal grid of 1 μm diameter holes in a 200-nm thick SiO_2 layer deposited on top of SiN. A backside etch then creates a freestanding SiN membrane beneath cylindrical SiO_2 cavities spaced 5 μm apart. The nanofilter is passively held in place directly above these oxide wells, most likely by Van der Waal's forces. A schematic and electron microscopy images of the device assembly process are shown in Figure 1.

Unless otherwise noted, the nanofilter membranes used in this work had a porosity of 5%, with an average pore diameter of 49 ± 8 nm (mean $\pm$ standard deviation), corresponding to a number density of ~ 25 pores/ μm^2 . With this setup, each 1 μm diameter circular oxide microwell ($0.785 \mu\text{m}^2$) contains 20 ± 5 pores. Wetting of the space between the membranes is achievable due to the extraordinary high gas permeability of the ultrathin nanofilter membrane³¹. This device is mounted in a fluidic flow-through cell, exposed to an aqueous salt solution, and a single nanopore is fabricated in the SiN membrane using controlled breakdown (CBD), described elsewhere^{32,33}. The CBD process produces a single nanopore in one of the oxide wells at random³⁴. Due to the low electrical resistance of ultrathin highly porous NPN, the presence of the nanofilter has negligible effect on CBD and on the electrical characteristics of the resulting single nanopore (referred to hereafter as the sensing pore). Further details of the NPN material properties, pore fabrication process, and nanosensor electrical characterization are presented in Supplementary Section S1.

Simulations were also conducted to better understand the behavior of nanofiltered pores. A standard coarse-grained polymer model was used to model the DNA chains³⁵. The driving electric field was solved numerically between the nanofilter and the sensing pore, and approximated analytically elsewhere. Only a single pore in the nanofilter was explicitly represented in simulations, placed 150 nm off-axis from the sensing pore, which was located in the center of the microwell. Further details of the simulation setup are provided in Supplementary Section S2.

Double-stranded DNA (dsDNA) molecules of lengths varying from 100 bp to 4000 bp are introduced to the *cis* (nanofilter) side of the system and are driven through the system by a voltage bias of 200 mV in 3.6M LiCl pH 8 unless otherwise noted. Because the resistance of the sensing pore dominates that of the nanofilter, the current blockage that is sensed is due entirely to the interaction between dsDNA molecules and the sensing pore, while the nanofilter remains electrically invisible. Interestingly, the capture rate of nanofiltered devices is comparable to the capture rate of control devices in most cases. Detailed event counts and capture rates are available in Supplementary Table S1 and Supplementary Section S3.

Following Mihovilovic *et al.*³⁶, we define type 1 events to be single-file passage of dsDNA, type 21 events to be partially folded events in which the event begins in the folded state. More complex event shapes are indicated by longer strings of integers, which correspond to integer multiples of the single-file dsDNA blockage in the order in which they appear. Events containing levels which do not correspond to an integer multiple of the level 1 blockage are labelled anomalous and are excluded from subsequent analysis. These comprise less than 5% of events overall, and are typically attributed to interactions of DNA with the access regions of nanopores³⁷, or to fitting artefacts.

We first consider the kinetics of passage of molecules which pass the sensing pore in an unfolded type 1 configuration. Figures 2a–2c show schematic representations of possible conformations of dsDNA prior to translocation through the sensing pore. As can be seen in Figure 2d, when $N \lesssim 1300$ bp, there is a single population of passage times for single-file passage which is well characterized by a log-normal distribution. For $N \gtrsim 1300$ bp (Figures 2e and 2f), a one-sided tail appears in addition to the log-normal component, comprising events with very long passage times. We attribute the log-normal portion of the passage time distributions to unhindered passage through the two serial membranes. Details of the analysis, along with event counts, are shown in Supplementary Section S3.

Figure 3 shows properties of unhindered dsDNA passage time distributions as a function of the number of base pairs N , representing fits to data extracted from a total of 1.3 million individual DNA translocations through 8 nanofiltered pores and 7 control pores.

The mean passage time τ for nanofiltered pores is fitted well by a single power law,

$$\tau(N) = \tau_0 N^p \quad (1)$$

which yields an exponent of $p_{exp} = 1.19 \pm 0.06$, as shown in Figure 3a. This exponent is in reasonable agreement with previous studies on regular solid-state nanopores^{36,38,39}. Error bars define the 95% confidence interval for the fit parameters. Control devices without the nanofilter (Figure 3b) behave similarly, but with slightly larger spread in mean passage times. Simulations (Figure 3c) also show power law scaling, though the exponent, $p_{sim} = 1.61 \pm 0.02$, is larger than in the experimental case. This is consistent with previous work, since simulations typically find scaling exponents which are larger than those extracted from experiments^{40,41}.

The effect of the nanofilter becomes more apparent when considering the standard deviation σ of the passage time (Figures 3d–3f), where the nanofilter usually results in significantly lower standard deviations than the control case. Experimental data with the nanofilter is fitted by a two-power law:

$$\sigma(N) = \sigma_0 \left(\left(\frac{N}{N^*} \right)^q + \left(\frac{N}{N^*} \right)^r \right) \quad (2)$$

The experimental best-fit exponents for this form are $q_{exp} = 0.5 \pm 0.3$ and $r_{exp} = 2.0 \pm 0.6$, in reasonable agreement with $q_{sim} = 0.3 \pm 0.5$ and $r_{sim} = 2.1 \pm 0.2$ for the simulated data. The standard deviation thus exhibits a cross-over between different power laws for small and large N , as discussed below.

As a consequence of the mean and standard deviation passage times scaling behavior, the coefficient of variation σ/τ exhibits non-monotonic behavior, and has a global minimum which can be seen in Figures 3g and 3i. The origins of this minimum can be explained by examining the simulation data for the translocation time and standard deviation (Figures 3c and 3f). At short lengths, the standard deviation grows weakly with increasing DNA length. In this limit, the DNA is rod-like and thus increasing the length of the DNA causes minute variations in the available conformations at the start of translocation. On the other hand, for rod-like polymers the friction coefficient grows approximately with the length of the rod⁴² and thus the translocation time does increase significantly. Hence, a weakly growing standard deviation normalized by an increasing translocation time yields a decreasing coefficient of variation. This rod-like limit breaks down around the Kuhn length, where variation of initial conformations increasingly influences the distribution of translocation times. Indeed, the standard deviation is seen to increase strongly with increasing length for $N > 300$ bp (slightly above the Kuhn length in simulations). The onset of this additional source of variation causes the increase in standard deviation to outpace that in the mean passage time, thus increasing the coefficient of variation. The combination of these effects is a minimum between these two regimes.

In the controls (Figure 3e), we see that deviations from the fit to nanofiltered pore data are always biased toward larger standard deviations (note the log scale). For a given molecular size, sensing pores equipped with a nanofilter set a lower bound for the standard deviation that is achievable by the sensing pore. To understand this trend, we consider recent simulation work which showed that the gradient in the electric field outside of nanopores can stretch out polymers as they are captured prior to translocation since polymer segments that are closer to the pore experience a larger electrophoretic force than those further away^{22,43}. The distance from the pore at which elongation occurs can be estimated by considering the capture distance at which the electrophoretic force overcomes diffusion, which is proportional to the square of the pore diameter⁴⁴. The experimental data in Figure 4 are collected for several devices in which the sensing pore diameter was varied between 3 and 14 nm. This translates into a variation of the capture distance over more than an order of magnitude. Hence, in the absence of the nanofilter, the DNA will be consistently elongated for large pores but much less so for small pores. The effects of this are experimentally

observed in the control data in Figure 4b: large pores exhibit a smaller variation in the conformation of the dsDNA prior to translocation and thus a smaller standard deviation in the translocation times compared to the more variable conformations expected for smaller pores. In the presence of the nanofilter, sufficiently long dsDNA must uncoil in order to thread through the nanofilter and is thus biased to approach the sensing pore in an elongated conformation independent of the sensing pore size. Consequently, the nanofilter reduces the variability in the initial conformation and improves the standard deviation beyond what can be consistently achieved with large sensing pores alone, and more importantly removes the dependence of passage time standard deviation on sensing pore size and stability. We demonstrate that this effect can be used to improve the resolution and the reliability of nanopore polymer size spectroscopy, an application which is discussed in Supplementary Section S4.

It is also interesting to note that the normalized mean passage time has a pore size dependence, with smaller control pores actually leading to slower translocation than their nanofiltered counterparts. Since an elongated polymer is expected to have more drag and thus take more time to translocate⁶, this is counterintuitive. We attribute this observation to additional friction arising from interactions between the coiled polymers that approach small control pore both with itself (coil-coil interactions) and the surrounding membrane (coil-membrane interactions), as depicted in Figure 4d. Because these polymers are not elongated, their conformation renders them more prone to interact with the membrane surrounding the mouth of the pore, potentially leading to temporary weak sticking, whereas elongated polymers will not be subject to this slowing effect. The proposed mechanisms slowing translocation in the control pores are explored in more detail in Supplementary Section S5.

In order to further elucidate the origin of the minimum in the coefficient of variation and to confirm that molecules which pass through the nanofilter tend to be more elongated than those that do not, additional simulations were conducted wherein the dsDNA was initialized by equilibrating with one end fixed in the sensing pore (Figure 4c). In this configuration, DNA are much less elongated on average. However, in these simulations where the nanofilter plays little to no role, the crossover in the standard deviation of the passage time and the global minimum in the coefficient of variation are still observed, albeit at slightly shorter chain lengths. The fact that this cross-over behavior is also visible as the lower bound in the control experiments for both standard deviation (Figure 3d) and the coefficient of variation (Figure 3g) point to a molecular property of the polymers, namely the polymer stiffness, being responsible for the presence of the minimum in the coefficient of variation, rather than the presence of the nanofilter. The nanofilter is the mechanism by which the sensing pore can achieve the minimal standard deviation required to actually map the transition. Therefore, fundamental physics of polymer translocations are revealed by the presence of the nanofilter. This crossover behavior has always been present but has until now been indistinguishable from noise.

In principle, pre-stretching of the polymer is not the only way in which to achieve a reduction in the standard deviation of the passage time. As long as the conformational entropy is reduced, the passage time will be more consistent regardless of the subset of conformation space that is selected. Different confining geometries prior to translocation

through the pore can in theory produce similar effects²³. However, Figure 4a suggests that membrane interactions may play an important role in variability in the translocation process. Pre-stretching is capable of mitigating membrane interactions while simultaneously reducing conformational entropy, which need not be the case for any confining geometries.

While large control pores can sometimes approach the performance of nanofiltered pores, the results presented in Figures 3 and 4 show that the presence of the nanofilter idealizes the sensing pore, allowing the system to consistently achieve a minimal standard deviation and coefficient of variation. In addition, a remarkable feature of nanofiltered pores is the fact that this minimization happens independent of sensing pore size. In several cases the pore size was observed to grow over the course of a single experiment (Supplementary Table S1), without compromising the improvement to the passage time characteristics. This independence of the sensor on both pore size and pore stability, made possible by the presence of the nanofilter within nanoscale distances of the sensing pore, is a feature of clear importance to many solid-state nanopore-based technologies.

We attribute the long events which appear in Figures 2e and 2f to translocation which has one end of the dsDNA temporarily adsorbed to the nanofilter during capture of the other end by the sensing pore. Several other plausible explanations for the long events are discussed and ruled out in Supplementary Section S6. To support this picture, we define adsorbed events as events with passage times exceeding four standard deviations above the mean for the log-normal portion of the passage time distribution on a log-scale. Figure 5a shows the sublevel blockage depth as a function of duration for all of the events with long passage times for 3000 bp molecules translocating a nanofiltered pore. It can be compared to the full set of sublevels for a similar control pore (Figure 5b), which does not have the extended tail of long events. While most long events show a single blockage level (Figure 5c and 5h), blockage states corresponding to occupation of the pore by more than one dsDNA molecule are present in some events. These deeper blockage levels are always very short compared to the unfolded sublevel, and for the majority of events appear only at the very start of events (type 21, Figure 5f and 5i). These latter events correspond to a dsDNA adsorbed on the nanofilter which is captured in a folded configuration very near an end, and then proceeds to occupy the pore in an unfolded conformation for the remainder of its length. However, there is a significant population of complex events which contain levels corresponding to two and three times the single occupation level in the middle of an extended stretch of single-occupancy, which cannot generally be explained by interactions of a single molecule with the pore (Figure 5g and 5j). We attribute these superimposed events to simultaneous translocation of a second, unhindered dsDNA molecule while the pore is partially occluded by a single DNA molecule adsorbed on the nanofilter. To support this, we note that the passage time distributions for superimposed events are similar to those for regular unhindered events (Figure 5c). The inter-event time distributions for superimposed events are also consistent with the unhindered translocations (Figures 5d).

As dsDNA length increases even further, the passage time distribution eventually becomes dominated by long events as all of the molecules thread through multiple pores in the nanofilter and arrive with both ends in the sensing pore in a double-threaded configuration. The sensing pore then exhibits very predictable clogging modes closely matching the

expected blockage level for folded dsDNA, which we attribute to simultaneous capture of both ends of the DNA by the sensing pore while it is double-threaded through two pores in the nanofilter. Additional discussion of clogging by long molecules is presented in Supplementary Section S6.

In addition to augmenting the standard deviation of molecular passage time distributions through a nanopore, the large degree of conformational entropy available to semi-flexible polymers also leads to folded translocation, in which a polymer is captured from somewhere along the length of the molecule and translocates in a hairpin conformation. This is particularly problematic for genomic mapping and barcoding schemes in which a DNA molecule is tagged with a marker, either to determine the presence or location of a particular sequence^{45–47}, the presence of a target²⁰, or to map the velocity profile of translocating molecules¹⁹. In such applications, folded translocations are generally excluded from data analysis, which can result in having to ignore many translocation events entirely. As a result, ensuring single-file passage of dsDNA through pores of any size is of high technological importance for many diagnostic and genomics applications^{20,45,47,48} that rely on detecting and mapping bound probes. While it is possible to restrict folding by using a nanopore too small to allow it, this is often incompatible with bulky labeling schemes and can be sensitive to pore instability^{49,50}.

We observed complete elimination of dsDNA folding during translocation through the sensing pore in two nanofiltered pore devices, despite the sensing pore being sufficiently large in both cases to allow for folded passage. The first of these pores is discussed in detail in Supplementary Section S7. Both of these devices had in common a low event rate compared to the rest of the nanofiltered devices. Due to the variability of folding behavior between nanofiltered devices, the origin of this effect must lie with the particular details of each individual nanofilter. We hypothesize that the mechanism behind folding suppression is the variation in the local distribution of nanofilter pore positions in the vicinity of the sensing pore. As discussed in Supplementary Section S7, only a small number of nanofilter pores which are very close to the sensing pore are active and have a sufficiently high electric field to capture dsDNA, so the local nanofilter pore distribution can be different between devices. In particular, if a device has two active nanofilter pores which are very close together compared to the extent of the dsDNA, the probability of a dsDNA molecule threading through both nanofilter pores will be high. Because of the electric field gradient between the two membranes, double-threaded polymers will tend to favor capture by one end, and translocation will proceed unfolded due to the resulting elongation.

To test this hypothesis, we performed experiments using a different nanofilter material, which had slightly smaller pores (38 ± 12 nm) but higher porosity (16 ± 3 %), yielding more closely-spaced nanofilter pores which would promote double-threading. Due to the smaller nanofilter pores, a higher voltage of 400 mV was required to obtain a sufficiently high event rate to gather statistics.

A dramatic demonstration of the hypothesized physical picture is shown in Figure 6. The device suppressed folding almost completely during the first part of the experiment (Figures 6a and 6b). Subsequently, the sensing pore clogged and exhibited increased noise centered

around a single-occupancy clog for a few minutes. When the clog cleared the sensing pore, with the open pore current baseline returning to its previous value, subsequent events presented folding (Figures 6a and 6c). The most likely explanation is that one of the two closely-spaced nanofilter pores was permanently clogged during the period in which the sensing pore was clogged, and since the local properties of the nanofilter no longer promoted double-threading, folding was no longer suppressed.

In light of this, we expect that folding suppression can be achieved reliably if the nanofilter parameters can be chosen so as to promote double threading of DNA molecules en route to the sensing pore. As long as the edge-to-edge distance between adjacent active nanofilter pores is smaller than the free solution radius of gyration of the polymer we expect this probability to be high, while small nanofilter pores will promote unwinding of the dsDNA molecule as it passes into the space between the membranes. Further discussion of folding kinetics can be found in Supplementary Section S7.

One of the reasons pre-confinement of molecules through the use of two-membrane systems has proven experimentally challenging is the difficulty inherent in producing two adequately-spaced, precisely-sized, serial nanopores while having fluidic access to the nanoscale gap between them for the purposes of wetting. The nanofiltered pore device presented herein creates a composite structure which achieves an optimal balance between these requirements while keeping the complexity minimal: the ultra-thin, porous nanofilter material allows easy wetting and simple fluidic and electrical access to the intermediate space for the purposes of nanopore fabrication by CBD, which guarantees the presence of appropriately aligned nanopores without requiring visual confirmation, and eliminates both the requirement for precise nanopore size and the deleterious effects of size instability during sensing.

The combined simulation and experimental results obtained on this nanofiltered pore device demonstrate the strong influence the capture process has on the dynamics of DNA translocation and reveal the presence, previously hidden in the noise, of a minimum in the normalized variance of translocation times that is inherent to semi-flexible polymers. The presence of the nanofilter upstream of the nanopore sensor pre-stretches the polymer, which offers a significant reduction in variation of passage times. This composite structure will enable a broad range of applications and provide enhanced sensing capabilities. It can be leveraged to provide more precise polymer size separation and to suppress folded translocations, thus forcing single-file passage, of critical importance for many life science and health applications, including DNA sizing, barcoded target detection and genome mapping.

Methods

Nanofilter Assembly

The NPN nanofilter membranes and SiN/oxide microwell substrate chips (SiMPore Inc., West Henrietta, NY) are cleaned with a nitrogen flow, followed by 38W air plasma for 40s to make all surfaces hydrophilic. Both the substrate and nanofilter chips are placed into gentle contact using a custom aluminum jig and placed in a -15°C freezer for 2 minutes to

cool below room temperature. The assembly is then exposed to fine mist produced by a vaporizer, which condenses on the cooled surface and fills the cavity between the two membranes. As the liquid evaporates through the nanofilter, surface tension pulls the two membranes into contact, which then remain sealed together once all the liquid has evaporated. The two chips are then separated mechanically, and the nanofilter is left behind in contact with the substrate chip. In order to completely seal the nanofilter and to reduce chip capacitance, PDMS is then painted over the entire chip surface, leaving only the free-standing membrane portion exposed. This assembly can be stored until use.

Just before use, the assembly is air plasma cleaned at 38W for 40s to make all surfaces hydrophilic. The cleaned assembly is then placed in a sealed container with ambient air (40% humidity) and this container is placed in the -15°C freezer for up to 5 minutes. The cooling condenses humidity in the microwell, wetting the gap between the two membranes.

Nanopore Fabrication by CBD

Nanopores are fabricated in nanofilter assemblies via CBD, which is described elsewhere³². Briefly, pores are formed in 1M KCl pH 10 using a slowly increasing voltage ramp from -10V to -18V applied to the nanofilter side of the assembly, with the trans side grounded. Typical fabrication times are between 5 and 10 minutes. Once fabricated, the salt solution is changed to 3.6M LiCl pH 8 and, depending on the initial pore size, diameter is adjusted using 3–4V 4s square voltage pulses until the desired pore in the range of 6 to 15 nm is achieved. Depending on the IV and noise characteristics, pores are sometimes aged before use⁷.

Data Acquisition and Analysis

NoLimits dsDNA molecules (Life Technologies Inc.) in the range of 100 bp to 4000 bp are pre-mixed to the desired concentration (between 3–76nM) and injected into the vicinity of the pore using a custom PEEK flow cell.

Data is acquired in MATLAB R2013a (32-bit) using the Chimera VC100 current amplifier with 200mV applied unless otherwise noted, sampled at 4.166 MHz, with a hardware 2-pole low-pass Bessel filter at 1MHz cutoff frequency. Data is post-filtered for analysis at 900 kHz using a digital low-pass Bessel filter, and analyzed to extract passage times and sub-level structure using both the adept2state module of MOSAIC^{51,52} (<https://pages.nist.gov/mosaic/>) for 100 bp molecules which do not fold, and a custom implementation of the CUSUM+ algorithm^{52,53} for the rest of the events (<https://github.com/shadowk29/CUSUM>). Both analysis programs are available freely online. Nonlinear fitting results are obtained using Origin 9.1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank James Roussie and SiMPore Inc. for generous donations of expertise and NPN membrane materials. Kyle Briggs acknowledges the financial support provided by the NSERC Vanier program for postgraduate fellowships.

Funding Sources

This work was supported in part by the Natural Sciences and Engineering Research Council of Canada (NSERC), and by the province of Ontario through their Early Researcher Award to VTC, by NSF PFI-BIC #1237699 to JLM, and by NIH R21EB024120 to both VTC and JLM.

References

1. Carson S, Wanunu M. *Nanotechnology*. 2015; 26:1–14.
2. Heerema SJ, Dekker C. *Nat. Nanotechnol.* 2016; 11:127–136. [PubMed: 26839258]
3. Dekker C. *Nat. Nanotechnol.* 2007; 2:209–215. [PubMed: 18654264]
4. van Dorp S, Keyser UF, Dekker NH, Dekker C, Lemay SG. *Nat. Phys.* 2009; 5:347–351.
5. Gershow M, Golovchenko JA. *Nat. Nanotechnol.* 2007; 2:775–779. [PubMed: 18654430]
6. Lu B, Albertorio F, Hoogerheide DP, Golovchenko JA. *Biophys. J.* 2011; 101:70–79. [PubMed: 21723816]
7. Briggs K, Kwok H, Tabard-Cossa V. *Small*. 2014; 10:2077–2086. [PubMed: 24585682]
8. He Y, Tsutsui M, Fan C, Taniguchi M, Kawai T. *ACS Nano*. 2011; 5:5509–5518. [PubMed: 21662982]
9. Aksimentiev A, Heng JB, Timp G, Schulten K. *Biophys. J.* 2004; 87:2086–2097. [PubMed: 15345583]
10. Waugh M, Carlsen A, Sean D, Slater GW, Briggs K, Kwok H, Tabard-Cossa V. *Electrophoresis*. 2015; 36:1759–1767. [PubMed: 25929480]
11. Squires AH, Hersey JS, Grinstaff MW, Meller A. *J. Am. Chem. Soc.* 2013; 135:16304–16307. [PubMed: 24143914]
12. Kowalczyk SW, Wells DB, Aksimentiev A, Dekker C. *Nano Lett.* 2012; 12:1038–1044. [PubMed: 22229707]
13. Feng J, Liu K, Bulushev RD, Khlybov S, Dumcenco D, Kis A, Radenovic A. *Nat. Nanotechnol.* 2015; 10:1070–1076. [PubMed: 26389660]
14. Di Fiori N, Squires A, Bar D, Gilboa T, Moustakas TD, Meller A. *Nat. Nanotechnol.* 2013
15. Fologea D, Uplinger J, Thomas B, McNabb DS, Li J. *Nano Lett.* 2005; 5:1734–1737. [PubMed: 16159215]
16. Larkin J, Henley R, Bell DC, Cohen-Karni T, Rosenstein JK, Wanunu M. *ACS Nano*. 2013; 7:10121–10128. [PubMed: 24083444]
17. Kwok H, Waugh M, Bustamante J, Briggs K, Tabard-Cossa V. *Adv. Funct. Mater.* 2014; 24:7745–7753.
18. Carson S, Wilson J, Aksimentiev A, Wanunu M. *Biophys. J.* 2014; 107:2381–2393. [PubMed: 25418307]
19. Plesa C, Loo N Van, Ketterer P, Dietz H, Dekker C. *Nano Lett.* 2015; 15:732–737. [PubMed: 25496458]
20. Bell NAW, Keyser UF. *Nat. Nanotechnol.* 2016; 11:1–28. [PubMed: 26740036]
21. Bell NAW, Keyser UF. *arXiv*. 2016:1–5.
22. Vollmer SC, de Haan HW. *J. Chem. Phys.* 2016; 145:154902. [PubMed: 27782455]
23. Sean D, de Haan HW, Slater GW. *Electrophoresis*. 2015; 36:682–691. [PubMed: 25461428]
24. Liu X, Skanata MM, Stein D. *Nat. Commun.* 2015; 6:6222. [PubMed: 25648853]
25. Langecker M, Pedone D, Simmel FC, Rant U. *Nano Lett.* 2011; 11:5002–5007. [PubMed: 21981323]

26. Harms ZD, Mogensen KB, Nunes PS, Zhou K, Hildenbrand BW, Mitra I, Tan Z, Zlotnick A, Kutter JP, Jacobson SC. *Anal. Chem.* 2011; 83:9573–9578. [PubMed: 22029283]
27. Pedone D, Langecker M, Abstreiter G, Rant U. *Nano Lett.* 2011; 11:1561–1567. [PubMed: 21388205]
28. Bell NAW, Chen K, Ghosal S, Ricci M, Keyser UF. *Nat. Commun.* 2017; 8:380. [PubMed: 28855527]
29. DesOrmeaux JPS, Winans JD, Wayson SE, Gaborski TR, Khire TS, Striemer CC, McGrath JL. *Nanoscale.* 2014; 6:10798. [PubMed: 25105590]
30. Gillmer SR, Fang DZ, Wayson SE, Winans JD, Abdolrahim N, DesOrmeaux J-PS, Getpreecharsawas J, Ellis JD, Fauchet PM, McGrath JL. *Thin Solid Films.* 2017; 631:152–160.
31. Fauchet MNK, S CC, F DZ, G TR, M JL, M P. *Nanotechnology.* 2012; 23:145706. [PubMed: 22433182]
32. Kwok H, Briggs K, Tabard-Cossa V. *PLoS One.* 2014; 9:e92880. [PubMed: 24658537]
33. Briggs K, Kwok H, Tabard-Cossa V. *Small.* 2014; 10:2077–2086. [PubMed: 24585682]
34. Briggs K, Charron M, Kwok H, Le T, Chahal S, Bustamante J, Waugh M, Tabard-Cossa V. *Nanotechnol.* 2014
35. Slater GW, Holm C, Chubynsky MV, de Haan HW, Dube A, Grass K, Hickey OA, Kingsburry C, Sean D, Shendruk TN, Zhan L. *Electrophoresis.* 2009; 30:792–818. [PubMed: 19260004]
36. Mihovilovic M, Hagerty N, Stein D. *Phys. Rev. Lett.* 2013; 110:1–5.
37. Carlsen AT, Zahid OK, Ruzicka J, Taylor EW, Hall AR. *ACS Nano.* 2014; 8:4754–4760. [PubMed: 24758739]
38. Storm AJ, Chen JH, Zandbergen HW, Dekker C. *Phys. Rev. E.* 2005; 71:51903.
39. Storm AJ, Storm C, Chen J, Zandbergen H. *Nano Lett.* 2005; 5:1–5. [PubMed: 15792402]
40. De Haan HW, Sean D, Slater GW. *Phys. Rev. E.* 2015; 91:1–10.
41. Ikonen T, Bhattacharya A, Ala-Nissila T, Sung W. *Phys. Rev. E.* 2012; 85:1–7.
42. Teraoka, I. *Polymer Solutions: An Introduction to Physical Properties.* John Wiley & Sons, Inc; 2002.
43. Farahpour F, Maleknejad A, Varnik F, Ejtehad MR. *Soft Matter.* 2013; 9:2750.
44. Rowghanian P, Grosberg AY. *Phys. Rev. E.* 2013; 87:1–8.
45. Morin TJ, Shropshire T, Liu X, Briggs K, Huynh C, Tabard-Cossa V, Wang H, Dunbar WB. *PLoS One.* 2016; 11:e0154426. [PubMed: 27149679]
46. Singer A, Wanunu M, Morrison W, Kuhn H, Frank-kamenetskii M, Meller A. *Nano Lett.* 2010; 10:738–742. [PubMed: 20088590]
47. Atas E, Singer A, Meller A. *Electrophoresis.* 2012; 33:3437–3447. [PubMed: 23109189]
48. Plesa C, Ruitenberg JW, Witteveen MJ, Dekker C. *Nano Lett.* 2015; 15:3153–3158. [PubMed: 25928590]
49. van den Hout M, Hall AR, Wu MY, Zandbergen HW, Dekker C, Dekker NH. *Nanotechnology.* 2010; 21:115304. [PubMed: 20173233]
50. Rollings R, Graef E, Walsh N, Nandivada S, Benamara M, Li J. *Nanotechnology.* 2015; 26:997–1003.
51. Balijepalli A, Ettegui J, Cornio AT, Robertson JWF, Cheung KP, Kasianowicz JJ, Vaz C. *ACS Nano.* 2014; 8:1547–1553. [PubMed: 24397836]
52. Forstater JH, Briggs K, Robertson JWF, Ettegui J, Marie-Rose O, Vaz C, Kasianowicz JJ, Tabard-Cossa V, Balijepalli A. *Anal. Chem.* 2016; 88:11900–11907. [PubMed: 27797501]
53. Raillon C, Granjon P, Graf M, Steinbock LJ, Radenovic A. *Nanoscale.* 2012; 4:4916–4924. [PubMed: 22786690]

Figure 1.

a) 50 nm NPN membrane is aligned to a 20 nm SiN membrane, patterned with a 200 nm SiO₂ spacer. b) Condensation of water vapor penetrates the NPN membrane, inundates the nanocavity, and draws the NPN membrane to the SiO₂ spacer. c) The NPN membrane is torn away from the carrier chip by surface tension, and remains attached to the SiO₂ spacer and SiN surface. d) Nanopore fabrication by controlled breakdown with the nanofilter already in place. e) SEM image of a device after step c. A section of NPN membrane is torn away, revealing the SiO₂ spacer underneath. The pore may be formed in any oxide microwell in the darker central region, which corresponds to the free-standing SiN membrane. The device shown in this SEM image has a 2 μm microwell, whereas the devices used in the rest of the paper use 1 μm microwells.

Figure 2.

a, b, c) Schematic representations of 1000, 2000, and 3000 bp dsDNA traversing the nanofiltered pore device, respectively. Vertical distance and DNA length is to scale. d, e, f) Passage time histograms of unfolded type 1 events for the corresponding lengths of dsDNA. All three histograms are obtained using the same pore, while the pore grew during the course of the experiment (4.3h), from top to bottom, from 6.7 nm, to 7.3 nm, and finally to 8.0 nm, respectively. Insets: time series of dsDNA translocations (including folded events) for the corresponding histogram. Data recorded at 4.166 MHz sampling rate, digitally filtered with a low-pass Bessel filter at 900 kHz, and down-sampled to 2.5 MHz for plotting.

Figure 3.

Mean passage times for a) nanofiltered, b) control, and c) simulated nanofiltered pores as a function of DNA length. The solid blue lines are a fit of eq 2 to the data in a), while the dashed line in c) is a fit to the simulated data. Standard deviation of passage times for d) nanofiltered, e) control, and f) simulated nanofiltered pores. The solid red lines are a fit of eq 3 to the data in d). The dotted lines in d) show the two power laws separately, while the dotted line in f) shows the two-power fit to the simulated results. Coefficient of variation for g) nanofiltered, h) control, and i) simulated nanofiltered pores. The solid green lines are the quotient of the fits in a) and d), while the dotted line in i) is the quotient of the fits to simulated data.

Figure 4.

a) Experimental mean passage times normalized by the fit of eq 1 as a function of sensing pore size for both nanofiltered (black squares) and control pores (blue circles). Inset schematics show stretching of the polymer as it enters the capture radius of control pores for two different pore sizes. b) Experimental standard deviation normalized by the fit of eq 3 as a function of sensing pore size c) Simulated projection of the radius of gyration on the vector connecting the sensing pore and the center of mass of the DNA at the moment of capture, for molecules initialized with one end in the nanofilter (black squares) versus one end in the sensing pore (blue circles). d) Schematics illustrating the expected conformations

of polymers at the onset of translocation for small and large pores with and without the nanofilter. Red gradients depict the electric profile outside the pore, while blue dots represent the potential interaction sites between the polymer and the membrane outside the pore.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 5.

a) Heat map of sublevels within events, showing blockage depth as a function of sub-level duration for events which fall within the tail of long events for 3000 bp dsDNA in an 8.0 nm nanofiltered pore. b) For comparison, the sublevel breakdown for a control pore (no nanofilter), showing that the long tail of events is absent c, d) Distribution of passage times and inter-event times respectively for superimposed events within long single-level events (red) compared to the passage time distribution for unhindered events from the same pore (black). e–g) Examples of a single-level, unfolded type 1 event, a briefly initially folded type 21 event, and a more complex event, respectively, from among the 3000 bp double-threaded

events. h–j) schematic representations of the molecular conformations giving rise to each of the corresponding event signatures above. Red DNA represents a polymer which is adsorbed to the filter while other colors translocate freely.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 6.

a) Maximum blockage as a function of experiment time, showing near-complete suppression of folding during the first half of the experiment, followed by allowing folding after a period of prolonged clogging. The single-occupancy blockage level is around 2 nA for this 5.4 nm sensing pore. b) Heat map of the sub-levels for events from the folding-suppressed half of the experiment. c) Heat map of the sub-levels for events from the folding-allowed half of the experiment, following clogging of the sensing pore. d) Schematic of the hypothesized mechanism of folding suppression consisting of two closely-spaced nanofilter pores. e)

Schematic depicting how the folding suppression can be lost when one or more of the active nanofilter pores is clogged.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript