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Non-equilibrium folding of individual DNA molecules recaptured up to 1000 times in a solid state nanopore

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Abstract
We investigate translocation of linear and circular double-stranded DNA molecules through solid state nanopores where each molecule is recaptured and re-translocated many times. Single molecules can be recaptured by switching voltage polarity for hundreds or even thousands of times. The large number of recapture events allows statistics on the translocation of individual molecules. Surprisingly, we observe that recaptured DNA molecules do not translocate in a linear head-to-tail fashion, but instead translocate as a folded blob where multiple parts of the DNA molecule simultaneously translocate through the pore in parallel. This folding is observed through the presence of up to 13 DNA double strands from the same molecule simultaneously inside the pore, as well as many smaller fold numbers occurring during the course of a translocation event. The strong folding is particularly prominent when the molecule is recaptured at short timescales, i.e. shorter than its characteristic time to relax to its equilibrium configuration. At longer recapture times, both the amount of folding and the mean duration of translocation approach the values observed in non-recapture experiments. The data shows that the translocation time of a molecule depends on the molecule's conformation at the start of the translocation process, with extended molecules having a longer translocation time. The observations can be attributed to a high-density non-equilibrium DNA configuration that arises in the close vicinity of the nanopore immediately after translocation, which dissipates on a timescale given by the Zimm relaxation time.

S Online supplementary data available from stacks.iop.org/Nano/24/475101/mmedia

(Some figures may appear in colour only in the online journal)

1. Introduction

Solid state nanopores are a versatile tool for biophysics with a broad range of applications [3, 19]. In this technique biomolecules are placed into one of two chambers separated by a membrane containing a nanometer-scale pore. Upon application of an electric field across this membrane, charged molecules experience an electrophoretic force pulling them through the pore, as shown in figure 1(a). Experimental research using nanopores has been able to provide a number of insights into the relevant polymer physics. These include, for example, the observations that long polymers translocating through large pores have a power-law length dependence of their translocation time [14], a diffusion-limited capture process [2], and a strong preference for end capture of molecules which has been attributed to configurational entropy [10].

Despite this body of work, a number of important open questions remain. For example, (1) how does the local translocation velocity vary as the molecule passes through the pore? Or (2) why are the distributions in the translocation times for one particular length so disperse? The former question is critical for many nanopore applications, for example DNA analysis, where one aims to map the measured temporal signals into the spatial domain, while understanding the latter question is key to determining if the differences observed between two translocation events are due

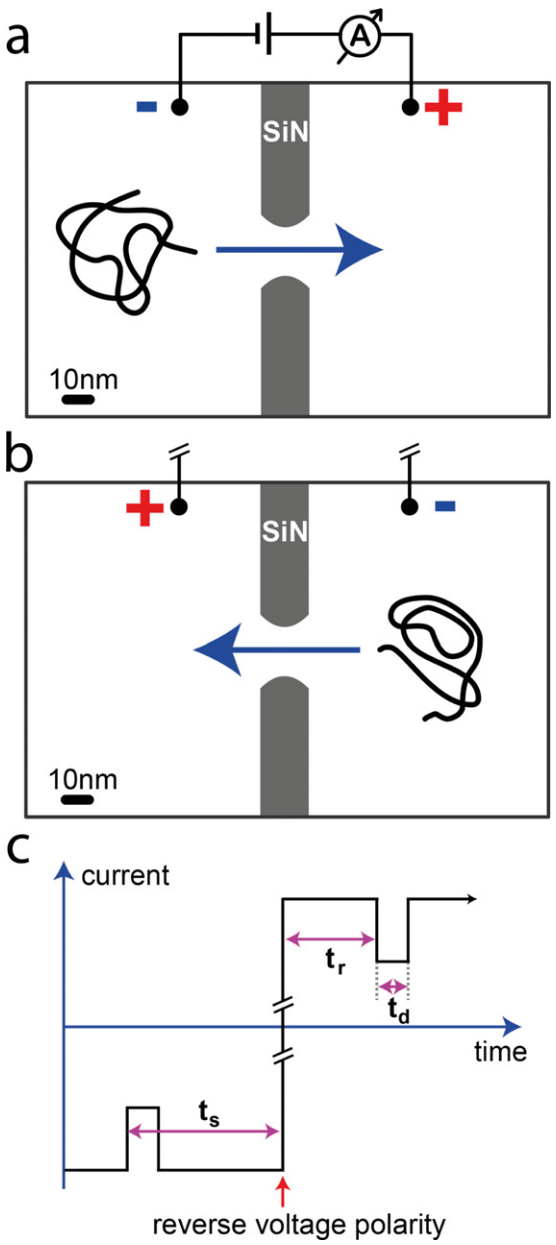

Figure 1. (a) Schematic representation of the nanopore setup, with two aqueous reservoirs separated by a SiN membrane containing a pore. (b) Once the passage of a DNA molecule has been detected, the electric field is switched and the same molecule is recaptured to translocate once more through the pore. (c) An idealized current trace showing a recapture event and the various timings.

to the physics of the translocation process or actual physical differences between two molecules. Both questions relate to the conformational state and behavior of the polymer coil outside the pore as the translocation occurs.

Storm *et al* [15] showed that the observed non-linear dependence of translocation time on the molecule's length could be explained by the hydrodynamic drag of the polymer coil outside the pore balancing the applied electrophoretic force pulling the polymer through. Due to the large polymer coil outside the pore, we expect the velocity at the start of the translocation process to be lower, because the large coil needs to unravel and move towards the pore, compared to the velocity at the end of the translocation process where the coil has shrunk, providing less resistive force and increasing the translocation velocity significantly. Indeed, modeling simulations carried out by Lu *et al* predicted this type of behavior [9]. Their work suggests that such behavior should be present in all translocations. Additionally, they attributed the large distributions observed in translocation times (the second question) of equal length molecules to the large variety of molecular configurations possible at the start of the translocation process, with the translocation time increasing as the distance from the center-of-mass of the molecule to the pore entrance (at the start of the translocation process) becomes larger.

In this work we apply a technique which allows us to systematically alter the molecular configuration of a single molecule, switching between the normal relaxed, extended form and a much more condensed conformation. We carry out many repeated measurements on the same molecule in different configurations, in order to observe how this affects the translocation time and other parameters.

In 2007, Gershow and Golovchenko demonstrated that it was possible to recapture a translocating dsDNA molecule by quickly reversing the electric field after a molecule had passed through the nanopore [4], as illustrated in figure 1(b). By controlling the duration between a translocation event and the electric field switching time, they showed that the process of capturing the DNA into the nanopore fits a drift-diffusion transport model. This allowed them to probe the capture length, the point beyond which the electrophoretic force could no longer overcome diffusion due to Brownian motion. They were able to recapture ∼5 kbp DNA molecules up to 22 times.

Here we show that it is possible to obtain much higher numbers of recaptures (hundreds, or even thousands) of long double-strand DNA molecules. This enables us to obtain large-number statistics of the translocation characteristics of individual molecules. Our data shows that both the translocation time and the amount of folds observed are highly dependent on parameters of the recapture process which can be experimentally controlled. This behavior is found to differ if the molecule is in a non-equilibrium state versus in a relaxed state.

2. Recapture experiments

We use 20 nm diameter pores in 20 nm thick SiN membranes, fabricated as previously described [6]. After drilling the pores with a TEM, the membrane's surface was manually painted with a layer of PDMS in order to reduce the membrane capacitance, thus lowering the noise and increasing the signal-to-noise ratio [16]. Membranes were mounted into a PMMA flowcell where they separated two aqueous chambers each containing an Ag/AgCl electrode. Double-stranded DNA molecules were added to one reservoir and translocation events were detected as a drop in the nanopore ionic conductance. After detection of a translocation event, the electric field's polarity was reversed, leading to the recapture of the molecule that had just translocated through the nanopore. The nanopore's ionic current was recorded with an Axopatch 200B amplifier and digitized with a National

Instruments 6251 DAQ card controlled by a custom LabView script. Recorded current data were filtered with a 10 kHz Gaussian low pass filter and analysis was carried out in Matlab with level fitting done in OpenNanopore [11].

We automated the translocation event detection in order to perform multiple recaptures. Briefly, our script scans the previous 1 ms of 3 kHz-filtered current data for events where the current deviates more than 5σ away from the baseline, where σ is the standard deviation of the filtered baseline current. Upon detection of an event, an interval of time (t_s) was allowed to pass before the polarity of the electric field was switched (figure 1(c)). This switching interval was varied during the course of multiple recaptures, in order to probe the dependence of various parameters on the recapture time. The control script attempted to recapture the molecule indefinitely until the molecule escaped.

We investigated four different types of molecules: linear and circular dsDNA with a contour length of 48.5 kbp or 97 kbp. Lambda dsDNA (48.5 kbp) was purchased from New England Biolabs and diluted to a concentration of 3.7 pM in 1 M KCl, 10 mM Tris, 1 mM EDTA at pH 8. The lambda DNA solution was left at room temperature and not heated, resulting in a fraction of molecules in a circular configuration [18], as well as higher order structures such as dimers [5]. In order to ensure that only a single molecule was being studied during the recapture process, we measured the rate of translocation events at 50 mV applied voltage before attempting any recaptures. The rate of events was determined to be very low: below 0.0014 Hz, or approximately 1 event every 10 min.

Using PDMS-coated nanopore membranes, we observed that each time the voltage polarity was reversed, the current baseline required around 2.5 ms to settle. Due to this effect, events recaptured less than 3 ms after switching were, although still detected by the recapture script, not included in the data set since their dwell times and amplitudes could not be properly determined. For each recapture event, the switching time (t_s), recapture time (t_r), and dwell (translocation) time (t_d) was recorded, as shown in figure 1(c). The switching time (t_s) is the time between an event and the subsequent voltage reversal. The recapture time (t_r) is the time between a voltage reversal and the next event. The dwell time (t_d) is defined as the time in between the leading and trailing edges of an event.

3. Results

Over the course of these recapture experiments, many (>30) molecules were recaptured, each producing current traces such as shown in figure 2(a). For the analysis, we selected eight molecules that were each recaptured at least 50 times. Four different types of dsDNA molecules were analyzed: two linear lambda molecules, four circular lambda molecules, one linear lambda dimer molecule, and one circular lambda dimer molecule. The average number of recaptures for each type of molecule was 64, 99, 1067, and 561 respectively. An example of a 97 kbp linear molecule translocation event is shown in figure 2(b), and an example for the 97 kbp circular molecule is given in figure 2(c). More event examples, for each type of molecule, are provided in supplementary section 4 (see SI available at stacks.iop.org/Nano/24/475101/mmedia). The presence of the 97 kbp lambda DNA dimers within the population was not surprising as these have been observed in the past [15] in translocation experiments where the lambda DNA solution, like in the current preparation, was not heat treated but kept at room temperature. Additionally, these longer molecules are selected out in experiments where we screen for high number of recaptures, since they diffuse slower and are thus less likely to escape.

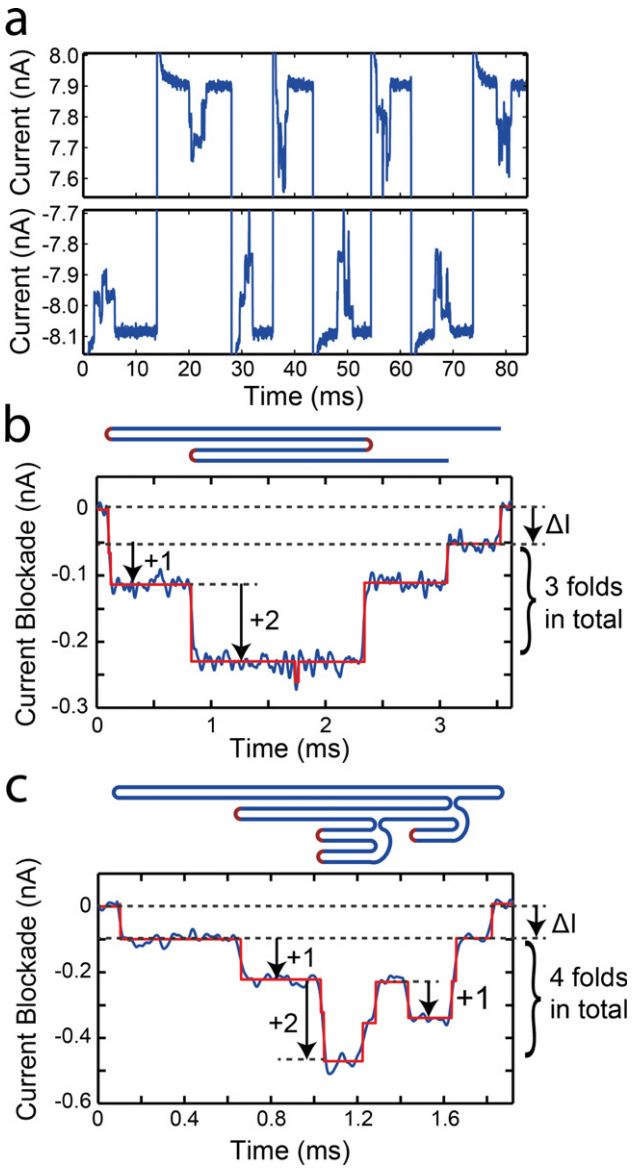

Figure 2. (a) A typical current trace showing a 97 kbp linear molecule being recaptured 7 times through a nanopore. (b) An example current trace of an event for the 97 kbp linear molecule. A possible molecule conformation is shown above in blue. (c) An example event for the 97 kbp circular molecule. A possible molecule conformation is shown above in blue.

We investigated the amount of folding for each molecule using two different approaches:

(1) The large number of recaptures for each molecule meant that current blockade histograms could be made

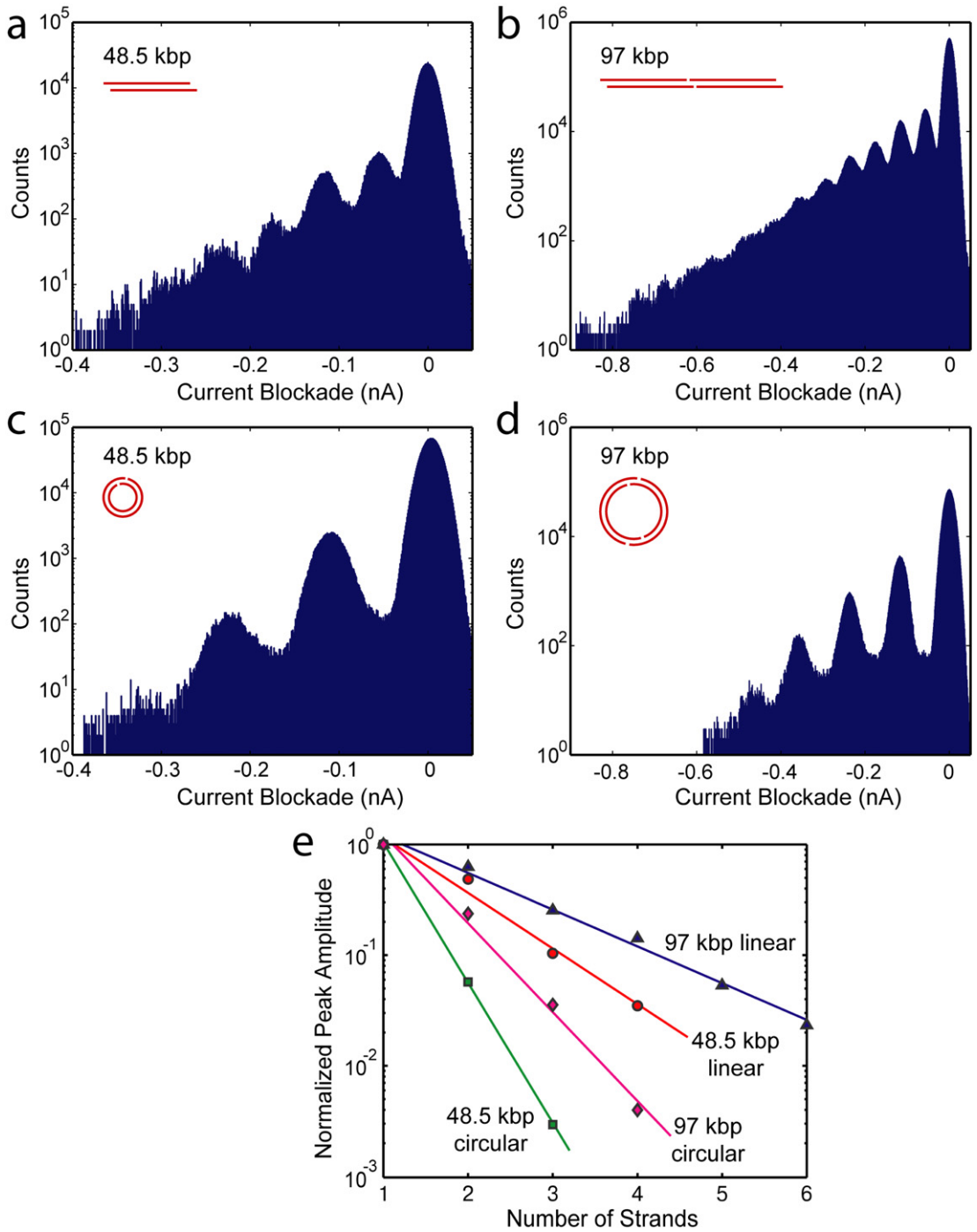

Figure 3. Current histograms for four types of molecules: (a) linear 48.5 kbp lambda dsDNA from a molecule recaptured 67 times. (b) Linear 97 kbp dsDNA from a molecule recaptured 1067 times, showing much higher folding levels. (c) Circular 48.5 kbp lambda dsDNA from a molecule recaptured 203 times. (d) Circular 97 kbp dsDNA from a molecule recaptured 561 times. (e) The frequency of occurrence for each peak on the current histograms. Peak amplitudes have been normalized using the amplitude of the largest (non-baseline) peak. The slope is a measure of the difficulty of inserting a new fold into the pore. The exponential decay constants of the fits are 2.91, 1.85, 1.16, and 0.76 for the 48.5 kbp circular, 97 kbp circular, 48.5 kbp linear, and 97 kbp linear molecules respectively.

for each individual molecule, as shown in figure 3. These histograms display the blockade levels of the current relative to the local baseline level. Since recapture data contains events at both polarities, we take the absolute value of the current in order to use both positive and negative events. These histograms contain a large baseline peak at zero, corresponding to the portions of the current trace where there was no translocation occurring, as well as smaller peaks corresponding to the current blockade produced by an integer number of DNA strands present in the nanopore. Interestingly, the frequency of occurrence for each peak is found to decrease exponentially with the peak number (i.e. number of dsDNA

strands that is inserted), as is evident from the straight lines in figure 3(e). This plot was made by determining the height of each (non-baseline) peak in the current histograms and then normalizing the values using the height of the first non-baseline peak. The steepness of the slopes in this semilog plot are a measure for the difficulty of inserting a new fold into the pore, and we thus observe that it is harder to insert new DNA into the pore for circular dsDNA as compared to linear dsDNA, and that it is easier to obtain a higher number of folds for longer molecules compared to shorter molecules. The maximum number of dsDNA strands simultaneously present in the pore can be determined from the left-most current blockade peak in the current histograms of figures 3(a)–(d). This approach thus provides a direct measure of the maximum numbers of parallel dsDNA in the pore. It, however, does not indicate how often a certain molecule was folded along its length. For example, a molecule having many spatially separated short folds would produce a similar histogram as a molecule having one single long fold.

(2) A more proper representation of the latter can be produced by counting the number of folds present in each event. This is accomplished by counting the number of rising edges, above the initial dsDNA blockade ΔI present in each event, as shown in figures 2(b) and (c). Since an unfolded event caused by a simple head-to-tail translocation would only produce a single blockade level with value ΔI, any larger current values correspond to folds in the molecule. The magnitude of ΔI is determined using the single-dsDNA peak in the current histogram of that particular molecule. The magnitude of the rising edge divided by ΔI then provides the number of folds present at that point in the translocation process. Circular molecules have a baseline twice as large as that of linear molecules (i.e., $\Delta I_{\mathrm{circ}} = 2\Delta I_{\mathrm{lin}}$) since there are always 2 dsDNA segments present in the circular topology and each fold brings two more dsDNA segments into the pore. In our analysis, we determined the total number of folds n present in each event and calculated the average number of folds, henceforth called the 'fold count', using all events in a given population in order to quantify the amount of folding present. Although the number of folds in any single event is an integer value, the fold count is a non-integer real number. The standard error of the mean was used as a measure of the uncertainty in these values.

We analyze current blockades seen in the current histograms for each type of molecule. Linear and circular molecules could easily be distinguished from their current histograms, as shown by comparison of figures 3(a), (b), (c) and (d) respectively. The insertion of linear DNA molecules produced a current blockade of 0.056 nA, i.e. very comparable to the blockade of 0.061 nA seen in non-recapture control experiments performed on a pore of the same size (supplementary section 2 available at stacks.iop.org/Nano/24/475101/mmedia). Circular molecules, however, exhibited a blockade of 0.111 nA, i.e., as expected, twice as large, since there are always at least two dsDNA strands present inside the pore due to the circular topology. The current histograms reveal that the longer molecules tend to have a significantly larger number of parallel DNA strands simultaneously inside the pore. For the linear 48.5 kbp, the current histogram peaks indicate the presence of events where there are up to 5 DNA segments in the pore simultaneously, while this number increases to up to 13 for the dimer which is twice as long. In the case of the circular molecules, we see a similar trend, with the 48.5 kbp circular molecule having up to 6 dsDNA segments simultaneously and the 97 kbp molecule up to 8. Current histograms from non-recapture control experiments on 48.5 kbp linear dsDNA typically show the presence of up to 5 DNA strands in the pore at 50 mV.

What is causing these remarkably high numbers of parallel DNA strands in the pore that we observe in the recapture experiments? We suggest that this relates to the DNA configuration directly after translocation. Since the strength of the electric field quickly drops off with increasing distance away from the nanopore, a large amount of DNA is densely piled up in the close vicinity of the pore when a DNA molecule translocates through the nanopore. The molecule then relaxes to equilibrium on a timescale given by the Zimm relaxation time. The Zimm relaxation time [7] can be estimated as

$$\tau_{\mathrm{Zimm}} = \frac{\alpha M \eta_{\mathrm{s}} [\eta]_0}{RT}, \tag{1}$$

where M is the molecular weight, R is Boltzmann's constant, T is temperature, η_{s} is the solvent viscosity (0.89 cP for 1 M KCl), $[\eta]_0$ is the intrinsic viscosity, and α is a coefficient which depends on the type of molecule and experimental conditions. In a solution containing a polymer, the intrinsic viscosity is a measure of the polymer's contribution to the measured viscosity of the solution. The intrinsic viscosity of a particular polymer can be determined by measuring the viscosity of the solution at different concentrations of polymer and extrapolating the resulting trend to zero polymer concentration. For 48.5 kbp linear, 48.5 kbp circular, 97 kbp linear, and 97 kbp circular molecules, we find relaxation times of 67 ms, 15 ms, 205 ms, and 46 ms respectively. Details of this calculation have been provided in supplementary section 1 (available at stacks.iop.org/Nano/24/475101/mmedia). A different DNA configuration, and subsequently different translocation characteristics, can be expected when the recapture is smaller or larger than these Zimm times. In the following analysis these relaxation times mark the crossover points between equilibrium and non-equilibrium behavior.

We experimentally examined the amount of folding and the dwell time for each event as well as how these quantities vary as the molecule approaches equilibrium. Figures 4 and 5 show a strong dependence for both the fold counts and dwell times on the recapture time. For the 48.5 kbp circular molecule (figure 4(a)), we see the mean dwell time systematically increase from 1.4 ms at small recapture times to 1.85 ms at the largest recapture times probed. Correspondingly the fold count, shown in figure 4(b), reduces from about 1.3 at small recapture times to 0.4 at large recapture times. Interestingly, in the population distribution of folding, figure 4(c), we see the number of unfolded events reduced by a factor of 2 when

Table 1. The mean dwell time and the standard error for unfolded events in circular molecules, shown for events recaptured below and above the Zimm time.

Type	48.5 kbp circular		97 kbp circular	
Regime	$t_r < \tau_Z$	$t_r > \tau_Z$	$t_r < \tau_Z$	$t_r > \tau_Z$
t_d (ms)	1.68	1.78	1.67	2.41
S.E. (ms)	0.03	0.02	0.18	0.34

comparing events occurring above the Zimm time (60% of events unfolded) to those occurring below (31% unfolded). Detailed folding statistics for each molecule can be found in supplementary section 3 (available at stacks.iop.org/Nano/24/475101/mmedia). Analyzing the data from the 97 kbp molecules, we see the dwell time increase from 3 ms to 6 ms, and 2 ms to 3 ms for the linear and circular molecules, respectively, as shown in figure 5(a). Looking at the fold counts for these molecules, figure 5(b), we see a decrease from 9.5 to 5.5 folds on average for the linear molecule and from 2.5 down to below 1 fold for the circular molecule. The distribution of folds for the 97 kbp circular molecule, shown in figure 5(c) resembles that seen for the 48.5 kbp circular molecule (figure 4(c)), although with higher fold counts both above and below the Zimm time.

Control experiments for DNA translocation without recapturing show that the most probable dwell time for 48.5 kbp linear molecules at 50 mV was around 3.3 ms. Using the previously observed power-law length dependence [14] for the dwell time with an exponent of 1.26, we would expect a 97 kbp linear molecule to translocate with a dwell time of about 8 ms. Indeed, figure 5(a) shows the dwell time converging to a value of about 6 ms as the recapture time increases, though this value should be considered a lower limit in view of the limited statistics at the higher recapture times. The most probable dwell time for 48.5 kbp circular molecules was estimated to be around 1.7 ms at 50 mV, using the events from the control experiments which only contained the two-dsDNA current level. This estimate is in good agreement with the limiting value observed in figure 4(a).

We can also examine the effect of Zimm relaxation in the regime where folding plays no role. This allows us to analyze how the initial molecular configuration affects the translocation time of the molecule. For this, we selected only unfolded events and observe how their dwell time changes above and below the Zimm relaxation time. Due to the high fold counts in our recapture experiments, only the circular molecules had a sufficient number of unfolded events for this analysis. Unfolded events were separated into those occurring above and those below the Zimm relaxation time. We expect the molecules recaptured below the Zimm time to have, on average, a smaller coil radius then the molecules that were allowed sufficient time to relax. The mean dwell times and standard errors for these populations are shown in table 1. Indeed, in both cases we see the dwell time increase significantly when the molecule is recaptured only at times above its relaxation time. This observation agrees with the notion that a more spatially extended molecule takes a longer time to translocate.

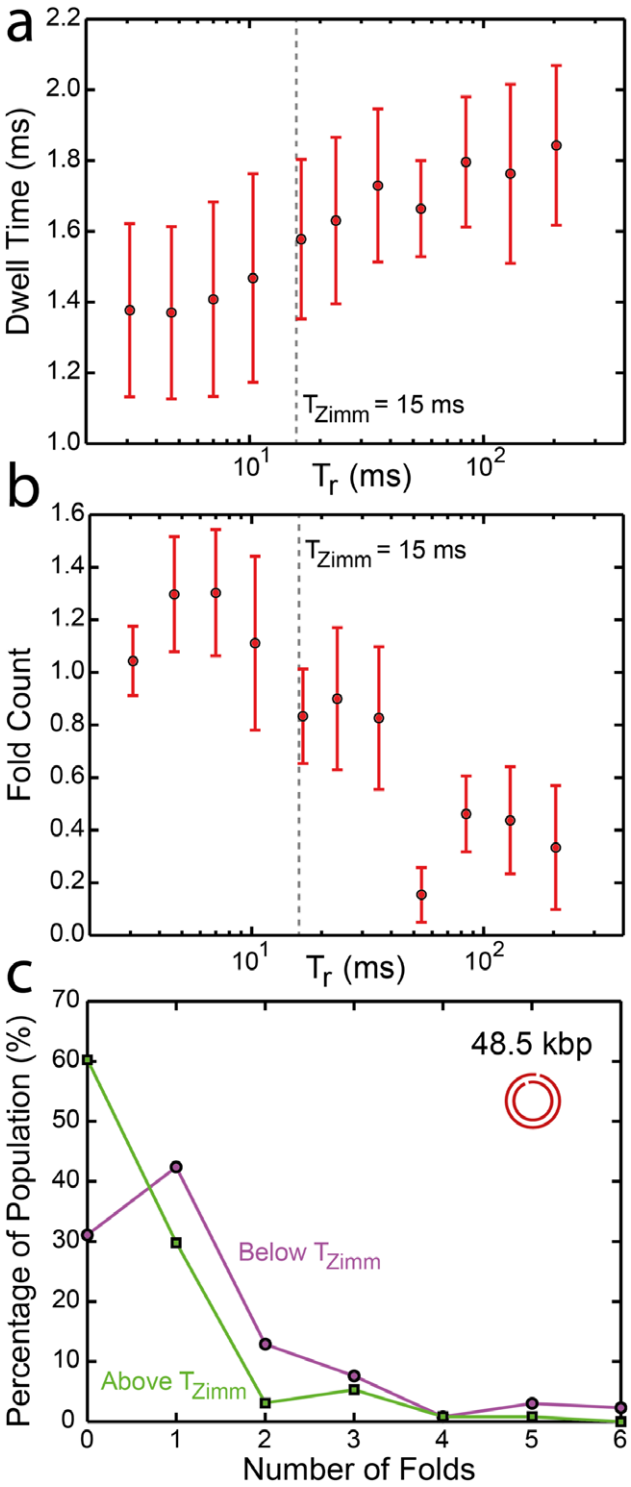

Figure 4. (a) The mean dwell time as a function of the recapture time for circular 45.8 kbp dsDNA molecules. At very large recapture times, we expect the dwell times to converge to about 1.7 ms, the most probable dwell time observed in non-recapture experiments. (b) The fold counts for events at a given recapture time. The error bars shown represent the standard error of the mean, determined using the dwell time (a) or fold count (b) of the events in each bin. (c) The percentage of molecules with a given number of folds, for recaptures occurring below the Zimm time (green squares) and above the Zimm time (magenta circles). Above the Zimm time 60% of events are unfolded while 30% have one fold. At times below the Zimm time, only 31% of events are unfolded and 42% of events have one fold.

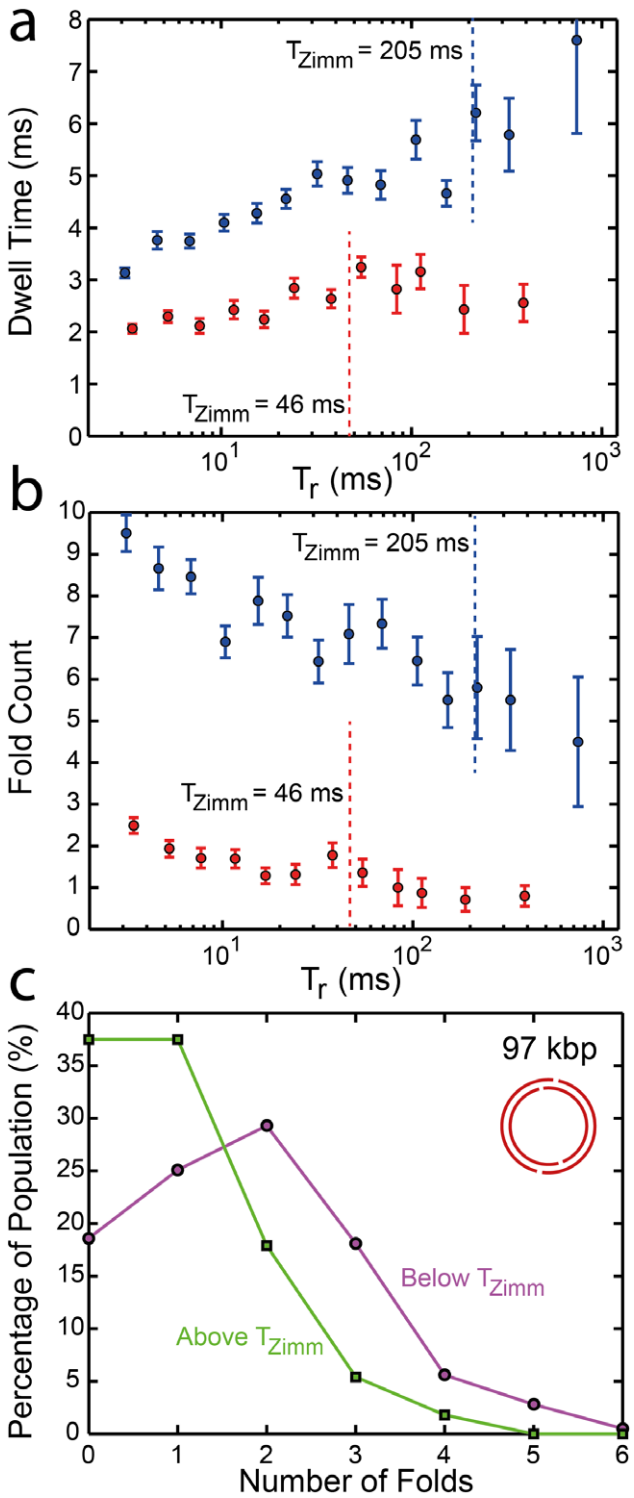

Figure 5. (a) The dwell time as a function of the recapture time for circular (bottom-red) and linear (top-blue) 97 kbp dsDNA molecules. (b) The fold counts for events at a given recapture time. For very short recapture times, the DNA density is very high in the close vicinity of the pore, which leads to very high fold counts. The error bars shown represent the standard error of the mean, determined using the dwell time (a) or fold count (b) of the events in each bin. (c) The percentage of molecules with a given number of folds for a 97 kbp circular molecule, for recaptures occurring below the Zimm time (green squares) and above the Zimm time (magenta circles).

4. Discussion and conclusion

These nanopore experiments provide a way to probe the non-equilibrium configurations of DNA and its approach to equilibrium. The exceptionally high fold counts that we observe in these experiments can be explained by looking at the recapture process. In the translocation process, the DNA molecule is transported through the nanopore, driven by the electric field which is very high right at the pore, but drops very quickly (with $1/r^2$) away from the pore. This causes a fast pile up of DNA material, leading to a large amount of DNA on the trans side of the pore. This effect is very prominent, since we are dealing with very long (16 or 32 μm) DNA molecules and applying very low voltages (50 mV). Note that translocation occurs fast, i.e., the DNA is piled up in a few ms, which is much faster than the Zimm equilibrium time for these long DNA polymers. As a result, the translocated DNA does not yet have time to equilibrate to its equilibrium configuration. This high-density pile up of DNA leads to multiple points along the molecule that can subsequently be recaptured into the pore, resulting in events with a large number of folds. Alternatively, at very long recapture times, the molecule is allowed to relax and the DNA blob will return to its characteristic large Flory radius on a timescale given by τ_{Zimm}, and the DNA densities and thus fold counts will approach those seen in non-recapture experiments where the molecules are in equilibrium when captured. Gathering sufficient statistics at very long recapture times is difficult, however, since longer switching times increase the probability of the molecule being lost. In practice, we were able to obtain data for recapture times between about 3 and 300 ms, with better statistics at the shorter t_r end.

Gershow and Golovchenko's original paper proposed the repeated interrogation of single molecules as a way to improve the accuracy of the measurement and as a way to probe time-dependent processes affecting the molecule. The data in this study shows that this approach may be viable in the future, but care must be taken to prevent unwanted effects such as folding that lead to complications. The use of short DNA molecules should reduce or eliminate the amount of folding observed, but will require the use of integrated nanopore amplifiers in order to achieve the fast switching speeds required [17, 12, 1]. Smaller-diameter pores introduce the complication of an entropic barrier present in the capture process [19], but may be useful to eliminate the folding observed, albeit with a higher risk of clogging and DNA–pore interactions. For probing protein–DNA structures [8], which require the use of large pores, analysis algorithms could take advantage of the fact that the location of folds as well as noise will change with each subsequent recapture event while the position of DNA-bound protein remains the same.

What do these results teach us about the influence of the molecular configuration on the translocation process? Our observations show that it is important to consider the timescales of the experimental process relative to the Zimm relaxation time, since the behavior of a polymer will be influenced by its state of (non)equilibrium. Additionally, the capture of a denser non-equilibrium DNA blob yields high

fold counts and short translocation times. The observation that the mean dwell time of unfolded events increases with recapture time supports the simulations of Lu *et al* [9]. It is clear that reducing the large translocation time variation seen in nanopore experiments will require an approach which can either control the initial molecular conformation or be able to apply a strong controlling force to the molecule. In the former approach, integration of a entry nanochannel with a nanopore [20, 21], or combining a nanopore with a thin film gel [13], could force a DNA molecule into an extended conformation before the start of the translocation process. Such a method should provide multiple advantages including longer translocation times, improved reproducibility from event to event, and better discrimination between molecules with different physical properties such as length.

We have shown in this work that it is possible to carry out large number of recaptures on a single DNA molecule and use this to gain insight into both the properties of the molecule as well as the physics governing the translocation process. While these results show that a number of issues such as high fold counts will need to be addressed, the high-number recapture technique promises to open a number of interesting avenues for future research and extend the versatility of nanopores as a biophysical technique.

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