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Odijk excluded volume interactions during the unfolding of DNA confined in a nanochannel

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Abstract

We report experimental data on the unfolding of human and *E. coli* genomic DNA molecules shortly after injection into a 45 nm nanochannel. The unfolding dynamics are deterministic, consistent with previous experiments and modeling in larger channels, and do not depend on the biological origin of the DNA. The measured entropic unfolding force per friction per unit contour length agrees with that predicted by combining the Odijk excluded volume with numerical calculations of the Kirkwood diffusivity of confined DNA. The time scale emerging from our analysis has implications for genome mapping in nanochannels, especially as the technology moves towards longer DNA, by setting a lower bound for the delay time before making a measurement.

TOC image

1 Introduction

The interest in understanding the physics of DNA confinement in channels near the persistence length is driven by the importance of this system for genomics applications.^{1–9} To date, most work has focused on the equilibrium properties of DNA when the molecules are confined within the nanochannel.^{10–12} The companion non-equilibrium problem of loading the DNA from a microchannel to a nanochannel,^{13–16} or the related problem of piston-like compression of DNA within a nanochannel,^{17,18} has received considerably less

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Supporting Information

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Video data for Fig. 3.

attention. In the present contribution, we show that the deterministic model developed previously by Levy *et al.*¹⁶ for confinement in channels larger than the persistence length also holds for stronger confinement where the channel size is similar to the DNA persistence length. In addition to providing a useful tool for reducing physical artifacts during genome mapping, our experiments provide a test of Odijk's excluded volume theory¹⁹ in the context of a readily accessible experiment.

The physics of DNA confinement near the persistence length has been the subject of considerable controversy, which has only recently been resolved.²⁰ Explicitly, the seminal experiments on DNA confinement in nanochannels^{21,22} exhibited substantial deviation from the predictions of Odijk's theory for strong confinement^{23–25} and de Gennes' theory for weak confinement.²⁶ This conundrum led to a number of plausible theoretical explanations in the generic context of a wormlike chain confined in a channel.^{27–35} Confinement near the persistence length is now understood in part as the emergence of a backfolded Odijk regime,^{19,36–39} where the chain is modeled by the deflection segments of the classic Odijk theory²³ but in the presence of weak excluded volume between deflection segments.¹⁹ The theory for the backfolded Odijk regime,¹⁹ when corrected³⁶ for the global persistence length,⁴⁰ does an excellent job of modeling the thermodynamics of wormlike chains with high monomer anisotropy, i.e. where the persistence length is large compared to the effective backbone width.^{36,38,39} DNA is only modestly anisotropic at high ionic strengths (and, interestingly, more isotropic as the ionic strength decreases).^{33,41} As a result, confined DNA only marginally satisfies the criteria for the backfolded Odijk regime,³⁶ a likely reason for continued deviations between the predictions of Odijk's theory and experimental data obtained for DNA.^{30,42,43} A recently proposed model,²⁰ which treats the DNA configurations via a correlated telegraph process of hairpin formation and weak excluded volume, provides a complete collapse of all simulation data onto a universal curve as a function of a single scaling parameter that measures the typical number of collisions per hairpin. Like the backfolded Odijk theory,¹⁹ the telegraph theory also relies on the principle of weak excluded volume during confinement in channels near the persistence length, and the backfolded Odijk regime^{19,36} emerges naturally from this telegraph model as a limiting case.

The unfolding of DNA after injection into a nanochannel provides an attractive opportunity to test Odijk's backfolding theory within a readily accessible experimental system. DNA can be efficiently loaded into small nanochannels using an entropy gradient.¹³ In the first step, an array of pillars initially unravel the coiled DNA into a somewhat stretched conformation. The DNA are then electrophoretically driven into the 45 nm wide by 45 nm deep channel of the BioNano Genomics IrysChip®.¹ The physics of this process is the small-channel equivalent to previous work using nanochannels in excess of 100 nm that are connected to loading microchannels.^{14–16} When the DNA cross the interface leading to smaller confinement, the leading end of the molecule can be folded into a hairpin configuration, as seen in the representative micrograph of Fig. 1a, and the hairpins can be quite large (Fig. 1b). As noted by Levy *et al.*,¹⁶ the ensuing rapid unfolding within the nanochannel is caused by excluded volume interactions between the two ends of the hairpin. Thus, measuring the hairpin unfolding dynamics provides insights into the excluded volume interactions between overlapping segments of DNA, a key component of Odijk's theory¹⁹ and the telegraph

model.²⁰ Importantly, these insights are obtained using DNA as the model polymer, with the accompanying advantages of fluorescence microscopy. While confined DNA does not provide a sufficiently wide range of channel sizes and ionic strengths to permit an unambiguous test of the backfolded Odijk regime in equilibrium,³⁶ we test a key part of the equilibrium theory here by using instead an out-of-equilibrium system.

2 Theory

Levy *et al.*¹⁶ previously proposed a deterministic model for the unfolding of a hairpin within a nanochannel, which we use in our analysis with a somewhat different notation. This model only makes modest assumptions about the details of the confinement, making it valid for any degree of confinement that leads to a deterministic unfolding process.

Following the notation in Fig. 2, we define the span of the unfolded portion of the chain as $X_U(t)$ and the span of the folded portion of the chain as $X_F(t)$. At any given point in time, the total extension of the chain is defined the sum of these two lengths,

$$X = X_U + X_F \quad (1)$$

The unfolded portion of the chain contains a contour length L_U and the folded portion of the chain contains a total contour length L_F that is assumed to be equally distributed between the two arms.¹⁶ The sum of these lengths is the total contour length of the chain,

$$L = L_U + L_F \quad (2)$$

We assume the chains are sufficiently long so that extension and contour length are proportional,¹⁹ whereupon

$$X_U = \alpha_U L_U \quad (3)$$

and

$$X_F = \alpha_F \left(\frac{L_F}{2} \right) \quad (4)$$

The parameters α_U and α_F account for the incomplete extension of the unfolded and folded regions of the chain, respectively, in a 45 nm channel,¹⁹ and are typically referred to as the fractional extension.

The first step of the analysis is to determine the fractional extensions α_U and α_F . The parameter α_U is readily obtained from measurements of the equilibrium extension of the unfolded chain,

$$X_E = \alpha_U (L_U + L_F) = \alpha_U L \quad (5)$$

since, as explained in the experimental methods, we know the total contour length L from mapping the Nt-BspQI nick sites on the DNA molecule to the appropriate reference genome.⁴² To determine α_F , we substitute Eqs. 3–5 into Eq. 2, which leads to¹⁶

$$\frac{X_F}{X_E} = \frac{\alpha_F}{2\alpha_U} \left(1 - \frac{X_U}{X_E} \right) \quad (6)$$

Since we can measure both $X_F(t)$ and $X_U(t)$ from the dynamics of the unfolding and know α_U from measurements of $X_E(t)$ and Eq. 5, we can extract α_F from Eq. 6.

The second step of the analysis is to determine the ratio of the unfolding force to the friction, which provides the desired test of Odijk's theory for the excluded volume interactions.¹⁹ To this extent, one can consider the dynamics of the short arm of the hairpin. The entropic unfolding force f is balanced by the drag exerted on the arm,¹⁶

$$f = - \frac{dX_F}{dt} \zeta \frac{L_F}{2} \quad (7)$$

where ζ is the friction per unit contour length. This model assumes no hydrodynamic interactions between the two arms of the hairpin, which has generally proved to be a good assumption in other related contexts.^{44–46} Inserting Eq. 4 into Eq. 7 furnishes

$$\frac{dX_F}{dt} = - \frac{\alpha_F f}{\zeta X_F} \quad (8)$$

The solution to this equation is

$$X_F^2 = X_{F,0}^2 - \frac{2\alpha_F f}{\zeta} t \quad (9)$$

where $X_{F,0}$ is the initial length of the folded region. Equation 9 provides a method to measure f/ζ once we have obtained α_F from the previous step.¹⁶

3 Experimental Method

The experimental approach used here is similar to our recent work on topological events in nanochannel-confined DNA.⁴⁷ Genomic DNA were extracted from either *E. coli* cells (MG1655) or human cells obtained from a blood sample. The DNA was nicked and labeled using standard methods developed at BioNano Genomics.^{8,48} Briefly, the DNA were nick-labeled with a cy3-like dye at the sequence GCTCTTC using Nt-BspQI (New England

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Biolabs) and the IrysPrep™ Reagent Kit (BioNano Genomics). Following the nick labeling, the nicks are repaired by *Taq* ligase.⁸ The DNA backbone was labeled with YOYO-1 at a dye:base pair ratio of 1:37 by staining the DNA overnight. An oxygen scavenging system containing protocatechuate 3,4 dioxygenase from *Pseudomonas sp.* (PCD, Sigma) and protocatechuic acid (PCA, Sigma) was added to the loading buffer (BioNano Genomics, ionic strength = 48 mM) to reduce photobleaching of the labeled nicks and photocleavage of the DNA molecules. The final concentration of PCD and PCA in the loading buffer were 0.3 μM and 324 mM, respectively. These additives allowed for the removal of oxygen from the buffer for roughly 1 hour in the nanochannel array.⁴⁹ The addition of the photobleaching additives reduced the concentration of the loading buffer to 88% of its standard strength. The ionic strength of the loading buffer, including the anti-photobleaching agents, is 89.2 mM. Some labeled DNA samples were stored at 4 °C for several days in loading buffer without PCD/PCA present.

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The dyed DNA were loaded into the BioNano Genomics Irys® chip, which contains 12,860 nanochannels with an approximately 45 nm cross-section and an entropy-gradient loading system,¹³ using a custom electrophoresis script provided with the Irys® System (BioNano Genomics, Inc.). The chip was mounted on the automated, inverted microscope described in our previous work,⁴⁷ and the experiments were conducted at room temperature. Shortly after loading the DNA into the array of nanochannels, images were collected on a custom built imaging system that uses an Olympus IX-71 with an Olympus 60× air objective (NA=0.90) and a Zyla 5.5 sCMOS camera (Andor). The labels at the nick sites were excited with a sapphire 532 nm, 200 mW laser from Cohorent while the YOYO-stained DNA is excited by a OBIS 488 nm, 150 mW laser from Cohorent. Movies were taken of the full field of view of the camera by alternating between the 532 nm and 488 nm laser. Roughly 50 frames of each color (green/blue laser) were collected at a frame rate of 0.35 to 0.75 frames/s with the exposure time at 532 nm being 80 ms and the exposure time at 488 nm being 50 ms. The slow frame rate was a compromise between collecting enough data to capture the unfolding event and preventing photocleavage of DNA from the 488 nm laser. We confirmed that the DNA are not stuck to the channel walls by electrokinetically ejecting them from the channels following the measurement.

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The locations of the nick labels on the unfolded DNA allow us to determine the sequence via genome mapping.¹ The *E. coli* DNA molecules were aligned to MG1655 while the human DNA molecules were aligned to the hg19 human reference using a custom alignment algorithm available from BioNano Genomics based on the work of Valouev.⁵⁰ For both *E. coli* and human, molecules with molecular weights of at least 100 kbp with at least 7 labels were aligned to the reference and accepted with a minimum *p*-value of 10⁻⁹. We convert the number of base pairs on a molecule from genome mapping into the DNA contour length *L* using a rise of 0.34 nm/bp. Although YOYO increases the backbone length due to intercalation, the very low dye loading level in our experiment minimizes this effect.

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The video data obtained from the YOYO channel were then analyzed using the custom image processing code DM-static (BioNano Genomics). In our prior work,⁴⁷ we developed a code that automatically identifies regions of anomalous brightness through a hierarchical clustering algorithm. This clustering analysis did not identify any knots in the DNA that

correspond to intensities that exceed the two-fold increase corresponding to a hairpin.⁴⁷ However, we do not have the sensitivity to detect very small knots that might be present at the ends of the chains,⁵¹ and simulations of the conditions relevant for genome mapping suggest that many small knots are present for the long chains and small channel sizes.⁵² We used this code on the first image of each movie to identify molecules that exhibit folds at the start of the video. Overall, we found that approximately 32% of the molecules exhibited folds. This is markedly higher than our previous experiments,⁴⁷ where only 7% of the molecules exhibited anomalously bright fluorescent regions anywhere within the molecule, which includes both folds at the ends of the molecules as well as knots and folds in the interior. The much higher rate of folds here is simply the result of observing the DNA shortly after injection.

We then selected 896 *E. coli* DNA molecules and 106 human DNA molecules that were initially folded molecules. Figure 3 shows a typical example of an unfolding molecule. Using the image analysis protocol, we segment the image of the molecule in Fig. 3a into two pieces, an unfolded region (with the normal level of fluorescence) and a folded region (with the anomalous level of fluorescence). We track these two lengths as a function of time. For all of the molecules we analyzed, the folded length decays to zero within the duration of the movie.

4 Results

4.1 Fractional Extension of Unfolded Molecules

In our experiments, we do not know *a priori* the sequence of each DNA molecule, since they are obtained by randomly shearing the genomic DNA. Rather, we obtain the underlying sequence from the genome mapping of the nick labels. Since we know the intervening sequence between these labels and the optics of the microscope, the output of the genome mapping measurement is the number of kilobase pairs per μm of extension for a given molecule. For example, genome mapping for the molecule in Fig. 3 yields $L = 135.6$ kbp. Using a conversion of 0.34 nm/bp, we have $L = 46.1$ μm . In principle, we could then use the unfolded region of data such as those in Fig. 3 to obtain a value of α_U for a given molecule. However, the statistics from such an approach would not be very good. For example, in Fig. 3, we only obtained 27 measurements of α_U after the molecule has unfolded. In instances where the fold is large, we would have very few measurements of α_U .

Instead, we estimate α_U for a given experiment by measuring the span X_{ij} between two contiguous labels i and j on the backbone of a linear DNA molecule. To obtain these data, we used the first 10 frames in the movie for molecules that did not have folds and were successfully aligned to the reference. The reason for this choice was that, after 10 frames, significant numbers of labels on the nick sites were photobleached, which impairs alignment to the reference. When a given molecule is mapped to the reference genome we also know the contour length L_{ij} between those labels, and thus compute α_U for that segment from Eq. 5. The advantage of this strategy is that we can compile enormous statistics.

Figure 4 shows the distribution for one experiment where we obtained measurements of the fractional extension α_U from 306 252 nick pairs. In previous work,⁴² we have compared the

average extension and the variance about that extension in circa 45 nm wide channels to the various theories by Odijk.^{19,23} The results obtained here for the fractional extension are consistent with our previous studies, lying between the prediction for the classic Odijk regime²³ and the backfolded Odijk regime,^{19,36} and in very good agreement with the telegraph model.²⁰

Inasmuch as the distribution in Fig. 4 is very sharp, we chose to use the average of the distribution for α_U in the theory of Levy *et al.*¹⁶ Table 1 reports the values of α_U for each of our experimental data sets. The value of α_U varies somewhat between experiments, which can be due to small changes in the channel cross section between chips, differences in the samples (of both biological and physical origin), and differences in the temperature. In what follows, we use the value of α_U for a given data set to analyze the data for that data set, rather than further average across data sets.

4.2 Single Molecule Analysis

The approach we used here is inherently a single molecule analysis, where the dynamics of unfolding of a given molecule provide an estimate for the entropic force per friction per unit length, $f\zeta$, for that molecule. Figure 3 shows the results for one particular molecule extracted from *E. coli*. The span of the fluorescent region with double intensity provides the instantaneous measurement of the span of the unfolded regions, $X_U(t)$, while the span of the remaining fluorescent region provides the corresponding data for the folded region, $X_F(t)$. Eventually, the molecule unfolds and $X_F = 0$. For this particular molecule, the unfolding takes place between the frames at $t = 27.9$ s and $t = 29.3$ s. For time after the molecule has unfolded, we are simply measuring the equilibrium extension of the chain, $X_E(t)$, which fluctuates about its average. Naturally, $X_E = X_U$ following unfolding.

Given the value for α_U for this particular experiment from Table 1, we can then measure α_F from Eq. 6. Figure 5 provides the plot for X_F/X_E versus X_U/X_E for this molecule, which is well described by the linear equation predicted by Eq. 6. Indeed, the magnitude of the slope of the fit (0.542) is quite close to the intercept (0.543) in Fig. 5. In our analysis, we use the slope to compute α_F , with $\alpha_F = 0.93$ for this molecule.

The final step of the single-molecule analysis is to compute the entropic force per friction per unit length, $f\zeta$, using Eq. 9. Figure 6 shows the resulting analysis for the single-molecule data in Fig. 3. The intercept of the fit is slightly higher than the initial fold size, $X_{F,0} = 7.80$ μm , in Fig. 3. While we could constrain the fit using the intercept, this would overemphasize the importance of the initial condition. The slope of the fit, when combined with our previous measurement of α_F , provides the value of $f\zeta$ for this particular molecule, $f\zeta = 1.29$ $\mu\text{m}^2/\text{s}$.

4.3 Ensemble Data

Our analysis yields many measurements of the fractional extension of the folded region, α_F , and the entropic force per friction per unit length, $f\zeta$. Let us begin by examining how the folding of the chain affects the fractional extension, which is plotted in Fig. 7. The distribution is somewhat shifted to values $\alpha_F/\alpha_U > 1$, which would be expected due to the excluded volume between the two arms of the hairpin (the driving force for the unfolding

process). We also observe a number of instances where it appears that the fractional extension in the unfolded region is higher than that in the folded region. At first glance, this phenomenon is inconsistent with the effect of excluded volume interactions, as we would expect these effects to be stronger in the folded region. However, we note from Fig. 1b that many of the hairpin folds are relatively short. Such chains tend to unfold quickly and do not have time to sample the full configuration space for the confined chain. In this case, thermal fluctuations can produce fractional extensions that are larger for the folded region than the unfolded region of the chain.

In previous work, Levy *et al.*¹⁶ measured a value of $\alpha_F/\alpha_U = 1.36 \pm 0.14$ in a 183 nm nanochannel and 1.30 ± 0.14 nm in a 142 nm nanochannel, where the reported channel sizes correspond to the geometric mean of their rectangular channel dimensions. As we see in Fig. 7, the ratio α_F/α_U is much smaller for 45 nm nanochannels, with an average value $\alpha_F/\alpha_U = 1.12 \pm 0.26$ for *E. coli* and 1.1 ± 0.26 for human DNA. The reduction in the stretching enhancement in the folded region of the hairpin is consistent with the weakness of excluded volume in the backfolded Odijk regime when compared to blob regimes.^{19,20}

The key results of our analysis are the distributions for the entropic force per friction per unit length, which appear in Fig. 8. The distribution of f/ζ is relatively wide, reflecting both the effects of thermal fluctuations and the limited duration of some of the unfolding events. Moreover, the unfolding rate increases as the process proceeds, implying that the later stages of unfolding are highly unlikely to be locally equilibrated. Nevertheless, by sampling many molecules, we can arrive at a meaningful estimate for f/ζ . The data in Fig. 8 have an average value of $f/\zeta = 1.10 \pm 0.81 \mu\text{m}^2/\text{s}$ for *E. coli* DNA and $f/\zeta = 1.07 \pm 0.76 \mu\text{m}^2/\text{s}$ for human DNA.

5 Discussion

From a theoretical standpoint, the most important results obtained from our experiments are the values of the entropic unfolding force per friction per unit contour length, f/ζ , obtained from fits to individual molecules of the type seen in Fig. 6 and leading to the distribution in Fig. 8. The data exhibit a very wide spread in f/ζ , with a mean value near $1.1 \mu\text{m}^2/\text{s}$ for both DNA samples. This value is substantially lower than that obtained by Levy *et al.*¹⁶ in larger nanochannels, where they measured $f/\zeta = 1.8 \pm 1.0 \mu\text{m}^2/\text{s}$ in the 183 nm channel and $2.4 \pm 1.5 \mu\text{m}^2/\text{s}$. Our lower values of f/ζ are expected because the excluded volume decreases with increasing confinement,¹⁹ while the hydrodynamic friction increases (albeit slowly for DNA) as confinement increases.^{53–55}

One of the most attractive parts of studying the unfolding of DNA in nanochannels is that it allows us to test the Odijk excluded volume theory in a convenient experimental system. In order to produce quantitative predictions from the theory from Levy *et al.*,¹⁶ we need models for the entropic unfolding force f and the friction per unit contour length, ζ .

Let us consider first the friction term. In confined chain hydrodynamics, the long-range hydrodynamic interactions are screened by the channel walls.^{53,56} Interestingly, the monomer concentration within the hydrodynamic screening volume of the backfolded Odijk

regime is sufficiently low that the friction primarily arises from polymer-solvent interactions, rather than polymer-polymer interactions.⁵³ The most recent calculation of the friction coefficient for the backfolded Odijk regime, using the Kirkwood approximation,⁵⁷ is⁵⁵

$$\zeta = \frac{6\pi\eta}{1.75\ln(l_p/a)} \quad (10)$$

where η is the solvent viscosity, l_p is the DNA persistence length, and a is the hydrodynamic radius. While the Kirkwood approximation does not account for certain correlations in the hydrodynamics, simulations indicate that the Kirkwood approximation is increasingly more accurate as the confinement increases.⁵⁸ The hydrodynamic simulations begin to deviate from Eq. 10 near $\alpha = 0.8$, where α can refer to either the folded or unfolded region. Eventually, the friction transitions to the classic Odijk regime wherein^{10,55}

$$\zeta = \frac{2\pi\eta\alpha}{\ln\left(\frac{D}{2a}\right)} \quad (11)$$

with the fractional extension of the entire chain in a square channel given by²⁵

$$\alpha = 1 - 0.18274 \left(\frac{D_{\text{eff}}}{l_p} \right)^{2/3} \quad (12)$$

In the latter, we define D_{eff} as the effective cross-section available to the chain, as electrostatic interactions prevent the DNA from sampling the entire cross section.^{10,59} The actual friction per unit length lies somewhere between Eqs. 10 and 11. From a theoretical standpoint, it is worthwhile to consider the two limiting cases when we analyze our experimental data.

To compute the entropic unfolding force, we can adapt the model for overlapping blobs in the de Gennes regime^{16,60} to overlapping deflection segments in the backfolded Odijk regime. In the Odijk regime, the chain configuration is characterized by the deflection length (see Fig. 2)²³

$$\lambda = D^{2/3} l_p^{1/3} \quad (13)$$

To compute the entropic force, we recall the schematic of Fig. 2. The folded hairpin can be divided into pairs of overlapping deflection segments. The total number of pairs overlapping deflection segments is

$$N_{\lambda} \cong \frac{X_F}{\lambda} \quad (14)$$

Odijk's theory¹⁹ proposes that the excluded volume free energy for a pair of deflection segments is

$$\frac{F_{\lambda}}{k_B T} \cong \frac{v_{\text{ex}}(D/l_p)^{1/3}}{\lambda D^2} \quad (15)$$

where $v_{\text{ex}} = \lambda^2 w$ is the the Onsager excluded volume for a rod with a length equal to the deflection length λ and an effective width w . Typically, the excluded volume free energy is the ratio of the Onsager excluded volume to the available volume, which in this case is λD^2 . The additional factor of $(D/l_p)^{1/3}$ arises from the orientation of the deflection segments with the channel walls.¹⁹ The total free energy cost due to excluded volume is $F = F_{\lambda} N_{\lambda}$, which yields

$$\frac{F}{k_B T} = \frac{w X_F}{D^{5/3} l_p^{1/3}} \quad (16)$$

The entropic unfolding force corresponds to the change in free energy with respect to changes in the center of mass of the overlapped deflection segments,

$$f = \frac{\partial F}{\partial X_F} \cong \frac{k_B T w}{D^{5/3} l_p^{1/3}} \quad (17)$$

Note that the unfolding force due to overlapping deflection segments is qualitatively different than the case of overlapping blobs, where an analogous derivation leads to⁶⁰

$$f \cong \frac{k_B T}{D} \quad (18)$$

It is worth noting that the model used by Levy *et al.*¹⁶ combines the hydrodynamic friction in the Odijk regime, given by Eq. 11, with the unfolding force in the de Gennes regime, given by Eq. 18. These choices are not internally consistent but reflect the general understanding of confined DNA in the literature at the time of that publication.

To convert these arguments into a theoretical prediction for f/ζ , we take the effective width of the DNA backbone to be $w = 5.9$ nm, computed from Stigter's theory⁶¹ for an ionic strength of 89.2 mM, a viscosity of $\eta = 2.2$ cP from rheological measurements of the buffer,⁶² hydrodynamic radius of $a = 1.45$ nm,³³ and a persistence length of $l_p = 52.5$ nm from Dobrynin's theory.⁶³ For the backfolded Odijk regime, Eq. 10 gives a friction per unit length

of $\zeta = 0.0066$ Pa s. Invoking the common approximation $D_{\text{eff}} = D - w$ in Eq. 12, the classic Odijk regime prediction of Eq. 11 gives a friction per unit length of $\zeta = 0.0043$ Pa s. The small difference between Eqs. 10 and 11 is expected because this particular channel size lies at the point where the friction begins to transition between these two regimes.⁵⁵ The entropic force predicted by Eq. 17 is $f \approx 0.012$ pN. The theoretical value of $f\zeta$ is thus of the same order of magnitude as the experiments, albeit with a somewhat larger prefactor ($2.8 \mu\text{m}^2/\text{s}$ for classic Odijk and $1.8 \mu\text{m}^2/\text{s}$ for backfolded Odijk). Given the uncertainty in the physical parameters, the accuracy of the theory is remarkable.

While the unfolding appears to obey the deterministic model proposed by Levy *et al.*,¹⁶ we observe a very wide distribution in the values of $f\zeta$ in Fig. 8. The spread in the data reflect an underlying stochastic process that samples the excluded volume force and friction, even though the coarse-grained dynamics of the unfolding are effectively deterministic. Explicitly, in order for Eq. 17 to be a realistic model for the entropic force, the overlapping portions of the DNA need to be able to equilibrate at each instantaneous value of X_F . Given the rapid speed of the unfolding process and the slow dynamics of internal segments of DNA, this quasi-equilibrium assumption is violated. Indeed, it is impossible to simultaneously have sufficient residence time in a given configuration such that Eq. 17 is strictly valid and for the overall dynamics to be deterministic; if the overlapping arms are able to diffuse and explore the lateral configurational degrees of freedom, they will also diffuse longitudinally. A similar argument applies to the validity of Eqs. 10 and 11 for the hydrodynamic friction, as they emerge from models where the confined chain samples its entire configuration space. However, given the relatively good agreement between theory and experiment, it appears that such violations of local equilibrium are not a substantial source of error.

In our discourse, we focused entirely on hairpin unfolding within the nanochannels, while neglecting any consideration of hairpin formation. Indeed, we did not observe any hairpin formation within the nanochannels during the course of our experiments. This behavior is consistent with theoretical models for the free energy cost for forming a hairpin. Odijk proposed a mechanical model for the free energy required to fold a hairpin in square nanochannel, arriving at the result^{19,40}

$$\frac{F_{\text{mc}}}{k_{\text{B}}T} = E_{\text{m}} \left(\frac{l_{\text{p}}}{\bar{r}} \right) - 3 \ln \left(\frac{D_{\text{eff}} - \bar{r} \sqrt{2}}{D_{\text{eff}}} \right) - \ln \left(\frac{8}{3\pi} \right) \quad (19)$$

where

$$\bar{r} = \frac{l_{\text{p}}}{6} \left\{ \left[E_{\text{m}}^2 + 6 \sqrt{2} E_{\text{m}} \left(\frac{D_{\text{eff}}}{l_{\text{p}}} \right) \right]^{1/2} - E_{\text{m}} \right\} \quad (20)$$

is the average length of a hairpin chord and $E_{\text{m}} = 1.5071$ is a constant emerging from a numerical integration. Subsequent work suggested that Odijk's mechanical model is an overestimate of the energy penalty, proposing instead that the free energy cost is³⁶

$$F = F_{\text{mc}} - F_0 \quad (21)$$

where $F_0 = 4.91 k_B T$ is essentially independent of the ratio D_{eff}/l_p . Note that Chen has recently criticized this model by Odijk and proposed a revised model for a semiflexible chain in a tube⁶⁴ that is in excellent agreement with simulation data.³⁶ However, since the corrected free energy given by Eq. 21 captures simulation data as well³⁶ and is valid for a square geometry, we proceed with that model.

Using the physical parameters reported for our calculation of $f\zeta$ leads to $F_{\text{mc}} = 7.7 k_B T$. This mechanical energy barrier is substantial and would clearly prevent any hairpin formation. Indeed, it would imply a rather substantial electric field is required to drive the DNA into the nanochannel. The corrected value $F = 2.8 k_B T$ provided by Eq. 21 appears to be low in light of the experimental observation that no hairpins form. However, this conclusion is tempered by the limited resolution of the microscopy. It is possible that small hairpins indeed nucleate throughout the experiment, but the hairpins are quickly removed due to the ensuing excluded volume.

6 Conclusion

We have demonstrated here that the deterministic model proposed by Levy *et al.*¹⁶ for hairpin unfolding in channels much larger than the persistence length also holds for confinement in channels near the persistence length. At first glance, this may seem to be a surprising result because the model requires substantial excluded volume interactions to produce deterministic dynamics. The excluded volume in a blob regime seems obvious, since the polymer tends to fill the cross-sectional area, but perhaps less so in a deflection regime, as the deflection segments are thin threads of polymer. However, as demonstrated by Odijk,¹⁹ the absence of hairpins at equilibrium arises due to excluded volume. Indeed, comparison of Eq. 17 for deflection segments and Eq. 18 for blobs indicates that the excluded volume increases more rapidly with decreasing channel size for deflection segments than for blobs.

Our analysis shows that Odijk's excluded volume theory, combined with results for the friction of a confined semiflexible chain, leads to reasonable predictions for the entropic unfolding force per friction per unit contour length. We view these results as partial confirmation of the Odijk excluded volume theory. A complete confirmation of the theory would require disentangling the excluded volume effects from hydrodynamics, and performing the measurements at equilibrium. In the context of DNA, we are reasonably optimistic that the former limitation can be ameliorated by separately measuring the diffusion coefficient of confined DNA. While these are not simple experiments, owing to the very slow dynamics, they are feasible. The latter obstacle is challenging to surmount using DNA, and not possible using non-equilibrium measurements of the type we performed here.

In addition to its physical relevance, hairpin unfolding in nanochannels is important for the practice of genome mapping. In previous work,⁴⁷ we identified a number of topologies of DNA inside such nanochannels, which included folds at the leading end. If the folds are

sufficiently long, they can produce errors in the resulting genomic map. Explicitly, the operating principle behind genomic mapping in nanochannels requires that the sequence-specific labels inserted into the DNA be linearly ordered, such that the physical distance between labels can be converted to a genomic distance between them.^{1,65–67} In the presence of a hairpin fold, the sequence-specific labels on short end of the hairpin become interleaved with those on the longer end. In some cases, the molecule can be artificially “unfolded” or it can be converted into a “pseudomolecule” by removing the folded region from the image and mapping the remaining DNA.⁴⁷ A far more attractive option is to simply wait a sufficient period of time to remove all non-equilibrium hairpins. However, selecting an appropriate delay time requires balancing the increased genome mapping accuracy at long times with the concomitant lower throughput of the experiment. Based on the results obtained here, we estimate from Eq. 9 that about 200 s is sufficient to unfold a hairpin of initial length 20 μm and the unfolding time scales quadratically with that initial length. We anticipate that the knowledge gained here for hairpin unfolding times will prove useful for optimizing emerging experimental protocols.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Image of *E. coli* DNA shortly after injection into a 45 nanochannel. The direction of the DNA motion was from left-to-right. The regions of double intensity in the DNA indicate the presence of hairpin folds. (b) Distribution of initial fold sizes for all DNA molecules (*E. coli* and human) following injection into the nanochannel. Approximately 32% of the injected DNA molecules with molecular weights greater than 100 kilobase pairs contain a fold when imaged immediately after injection.

Figure 2.

Schematic illustration of the deterministic unfolding dynamics of a DNA molecule in a channel of size D based on the model proposed by Levy *et al.*¹⁶ The red portion of the chain is the unfolded portion with extension X_U and the blue part of the chain is the folded portion with extension X_F . For later reference, the effective width w and Odijk deflection length λ are indicated.²³

Figure 3.

Unfolding of a single DNA molecule by imaging the YOYO-stained backbone. (a) Snapshots of the unfolding DNA molecule. The folded region is indicated by the yellow squares. The images are spaced by a time delay of 3.75 s from top-to-bottom. The complete movie of the unfolding DNA is available as Supporting Information. (b) Dynamical evolution of the span of the folded segment, X_F , and span of the unfolded segment, X_U , in the nanochannel as a function of time. The initial fold length is 7.8 μm and the average final extended length of the molecule is 40.4 μm. From the mapping to the reference genome for *E. coli*, the contour length of this molecule is 135.6 kbp. The yellow shading indicates the times where the molecule is completely unfolded, from which we measure the equilibrium length X_E .

Figure 4. Probability density for the fractional extension of the unfolded region, α_U , obtained from the measurements of X_E for *E. coli* molecules after unfolding for Data Set 8. This figure was constructed from 306 252 measurements using a bin size of 0.005.

Figure 5. Approach to determine the fractional extension of the folded region, α_F , from the single-molecule data in Fig. 3. The solid line is the linear fit to the data, $X_F/X_E = -0.542X_U/X_E + 0.543$.

Figure 6. Approach to determine entropic force per friction per unit length, $f\zeta$, from the single-molecule data in Fig. 3. The solid line is the linear fit to the data, $X_F^2 = -2.25t + 63.27$.

Figure 7. Distribution for the ratio of the fractional extension in the folded region, α_F , to the unfolded region, α_U , for (a) *E. coli* DNA and (b) human DNA. The values of α_U for each data set are listed in Table 1.

Figure 8. Distribution for the ratio of the entropic force per friction per unit length, f_U/ζ , for *E. coli* DNA. Inset: Same data for human DNA.

Table 1

Measured value of the number of kilobase pairs of DNA per μm of extension in the channel and corresponding value of the fractional extension α_U for each experimental data set. Experiment 10 corresponds to the human DNA sample; the other experiments correspond to *E.coli* DNA.

Experiment	kbp/ μm	α_U
1	3.35	0.878
2	3.36	0.877
3	3.50	0.841
4	3.44	0.854
5	3.40	0.866
6	3.35	0.878
7	3.36	0.875
8	3.35	0.877
9	3.32	0.885
10	3.34	0.881