Superoiling DNA optically

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Cellular DNA is regularly subject to torsional stress during genomic processes, such as transcription and replication, resulting in a range of supercoiled DNA structures. For this reason, methods to prepare and study supercoiled DNA at the single-molecule level are widely used, including magnetic, angular-optical, micropipette, and magneto-optical tweezers. However, it is currently challenging to combine DNA supercoiling control with spatial manipulation and fluorescence microscopy. This limits the ability to study complex and dynamic interactions of supercoiled DNA. Here we present a single-molecule assay that can rapidly and controllably generate negatively supercoiled DNA using a standard dual-trap optical tweezers instrument. This method, termed Optical DNA Supercoiling (ODS), uniquely combines the ability to study supercoiled DNA using force spectroscopy, fluorescence imaging of the whole DNA, and rapid buffer exchange. The technique can be used to generate a wide range of supercoiled states, with between <5 and 70% lower helical twist than nonsupercoiled DNA. Highlighting the versatility of ODS, we reveal previously unobserved effects of ionic strength and sequence on the structural state of underwound DNA. Next, we demonstrate that ODS can be used to directly visualize and quantify protein dynamics on supercoiled DNA. We show that the diffusion of the mitochondrial transcription factor TFAM can be significantly hindered by local regions of underwound DNA. This finding suggests a mechanism by which supercoiling could regulate mitochondrial transcription in vivo. Taken together, we propose that ODS represents a powerful single-molecule method, termed Optical DNA Supercoiling (ODS), that advances our ability to study negatively supercoiled DNA. Since ODS is based on dual-trap optical tweezers, it is compatible with a wide range of functionalities that are difficult to combine with traditional methods of DNA twist control. This includes the ability to image supercoiled DNA with fluorescence microscopy and move the supercoiled substrate rapidly between different buffer/protein solutions. We demonstrate that ODS yields unique and important insights into both the biomechanical properties of negatively supercoiled DNA and the dynamics of DNA–protein interactions on underwound DNA.

Significance

Torsional stress plays a vital role in many genomic transactions, including replication and transcription, and often results in underwound (negatively supercoiled) DNA. Here, we present a single-molecule method, termed Optical DNA Supercoiling (ODS), that advances our ability to study negatively supercoiled DNA. Since ODS is based on dual-trap optical tweezers, it is compatible with a wide range of functionalities that are difficult to combine with traditional methods of DNA twist control. This includes the ability to image supercoiled DNA with fluorescence microscopy and move the supercoiled substrate rapidly between different buffer/protein solutions. We demonstrate that ODS yields unique and important insights into both the biomechanical properties of negatively supercoiled DNA and the dynamics of DNA–protein interactions on underwound DNA.

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buffer and/or protein solutions. This can hinder the study of complex biological processes involving sequential protein-binding reactions.

Here, we present a multifunctional nanomechanical assay, termed Optical DNA Supercoiling (ODS), that can rapidly and controllably generate negatively supercoiled DNA using a standard dual-trap optical tweezers instrument. Supercoiled DNA formed in this way is amenable to rapid buffer exchange, and can be interrogated with both force spectroscopy and fluorescence imaging of the whole DNA. We demonstrate that this approach can provide detailed insight into the local topology of underwound DNA as well as track the spatial dynamics of proteins such as transcription factors on underwound DNA. We therefore propose that this method represents a powerful assay to study the role of DNA topology in complex and dynamic genomic processes.

Results

Generation of Negatively Supercoiled DNA Using Dual-Trap Optical Tweezers. Our method exploits the intrinsic mechanical properties of DNA to induce a fixed reduction in \( L_k \) (i.e., \( L_k \) is fixed) through the binding of at least 2 biotin moieties (on each end of the DNA) to streptavidin-coated, optically trapped beads (24). Application of high tension (~115 pN) induces overstretching, resulting in ~70% elongation of the DNA length without any global change in \( L_k \) (24, 25). In the absence of torsional constraint, however, overstretching occurs at much lower forces (~65 pN) (26, 27), and is associated with cooperative unwinding of the double helix; in this case, the average molecular twist is reduced from ~10.5 bp per turn to ~37.5 bp per turn (25). Consequently, when torsionally constrained DNA is overstretched, the molecule experiences torsional stress, relative to the unconstrained state. This torsional stress can be, at least partly, released through unwinding of the DNA around a single biotin–streptavidin tether, which can happen for example when one of the biotin–streptavidin connections is disrupted (Fig. 1B). If the disrupted tether subsequently reforms, torsional constraint will be reinstated, but now with the DNA in a lower \( L_k \) than that of the B-form double helix (Fig. 1A). Crucially, the reduced \( L_k \) is preserved upon decreasing the tension (i.e., the DNA remains negatively supercoiled).

This process is demonstrated experimentally in Fig. 2A. Here, we present a sample force–distance curve of highly negatively supercoiled DNA obtained using ODS (i.e., following overstretching of end-closed torsionally constrained DNA). For comparison, the force–distance curves of both unconstrained DNA and nonsupercoiled torsionally constrained DNA are shown in black and blue, respectively. The magnitude of \( \sigma \) can be calculated with reference to the known force–extension curves of negatively supercoiled DNA (Fig. 2B and Methods) (25). Note that the maximum error in the absolute value of \( \sigma \) determined using this calibration procedure is typically between 0.03 and 0.045 (SI Appendix, Fig. S1 and SI Note 1). The maximum value of \( \sigma \) that can be generated using ODS is ~0.7; the same as that associated with fully overstretched unconstrained DNA (25). This value of \( \sigma \) will occur if the torsional stress is fully released by transient disruption of a biotin–streptavidin tether at high force, resulting in the red force–distance curve in Fig. 2A.

Importantly, the probability and timescale for tether disruption can be tuned by holding the torsionally constrained DNA molecule at high tension for different periods of time. For example, Fig. 2C compares sequential force–distance curves of the same DNA molecule, obtained using ODS, where after each extension the DNA molecule is held at ~150 pN for 10 s prior to retraction. The most likely value of \( \sigma \) generated upon retraction is typically between 0 and ~0.15 (Fig. 2D). However, as demonstrated in Fig. 2C, by repeating such stretch–release cycles with the same DNA molecule, the value of \( \sigma \) can be tuned over a much wider range (up to \( \sigma \sim 0.7 \)). The above behavior accounts for ~25% of cases, where supercoiling is only generated at forces >115 pN (i.e., beyond the end of the overstretching transition). In another 25–35% of molecules, one of the biotin–streptavidin tethers is even less stable, and will already transiently disrupt during overstretching—this allows the magnitude of \( \sigma \) to be even more precisely controlled, simply by tuning the extent to which the molecule is initially overstretched (Fig. 2E and F). A summary of the various force–distance behaviors of end-capped torsionally constrained DNA is provided in SI Appendix, Figs. S2 and S3 and SI Note 2.

ODS Yields a Stable Change in DNA Linking Number. The ability of ODS to produce and maintain a reduced DNA linking number relies on 2 assumptions. The first is that the supercoiling generated is not easily dissipated through free rotation of the optically trapped beads. It has previously been reported that for supercoiled DNA in the range of \(-0.1 > \sigma > -1.5 \), the torque is roughly constant at approximately ~10 pN nm (11, 13). The rotational velocity of the beads in response to such an applied torque is inversely proportional to the cube of the bead radius and can be calculated using the equations of rotational motion (SI Appendix, SI Note 3). From these equations, the timescale for a single bead

Fig. 1. Generation of negatively supercoiled DNA using dual-trap optical tweezers. (A, i) End-closed DNA is torsionally constrained between 2 optically trapped beads via at least 2 biotin–streptavidin bonds (brown ellipses) on each end of the molecule. (A, ii) The torsionally constrained DNA molecule (tcDNA) is overstretched via displacement of 1 of the beads, resulting in both overwound and underwound DNA structures (25). (A, iii) At high force (at least ~80 pN), a sufficient number of biotin–streptavidin bonds break (orange ellipse) such that only a single tether is present on at least 1 end of the DNA molecule. This results in the loss of torsional constraint. (A, iv) The DNA molecule unwinds by swiveling around the single tether (arrow). (A, v) Once the linking number has decreased, the broken biotin–streptavidin bond(s) can reform. The molecule is once again torsionally constrained, but now in a lower linking number than that of B-DNA. (A, vi) The tension is released by reducing the DNA extension, stabilizing the negatively supercoiled (sc) state. (B) Schematic illustration of the tethering geometry that results in the formation of constrained underwound DNA. One biotin moiety (attached to the endcap of DNA) transiently unbinds from streptavidin at high force, during which time a second biotin remains bound to another streptavidin unit. The dashed gray arrow represents DNA unwinding during the time that the biotin–streptavidin bond is disrupted.
rotation is calculated to be ~180 s when using 4.5-μm-diameter beads. Moreover, the timescale for converting the entire molecule from a supercoiled state to a nonsupercoiled state depends on the total number of base pairs in the molecule. Assuming a torque of ~10 pN nm and 4.5-μm-diameter beads, it should take ~170 h to fully convert a λ-DNA molecule (48,502 bp) from a highly supercoiled state (σ ~ -0.7) to a nonsupercoiled state through rotational motion of the beads (Fig. 3A, dark blue). The fractional change in $L_k$ over time also depends on the initial value of $\sigma$, as highlighted in Fig. 3A (shaded blue). Nevertheless, Fig. 3A indicates that, assuming long DNA and large beads are used, the total change in $L_k$ due to bead rotation is negligible on the timescale of most single-molecule experiments. Additionally, any small asymmetries in the shape of the beads will likely result in a preferred orientation in the optical traps, and thus further hinder rotational motion.

The second key assumption underpinning ODS is that the biotin–streptavidin bonds are stable at low forces: any transient loss of torsional constraint due to cleavage of a tether could lead to rewinding of the double helix and loss of supercoiling. Indeed, it is the transient cleavage of a biotin–streptavidin tether that is the basis of ODS. However, such cleavage is promoted by tension; it has been predicted previously that the lifetime of a biotin–streptavidin bond is several orders of magnitude lower at forces >100 pN than at forces <10 pN (28).

To test the above assertions experimentally, we measured the change in $L_k$ (and thus $\sigma$) over time by monitoring the DNA extension at a fixed force (5, 20, and 40 pN, respectively). At these tensions, negatively supercoiled DNA (for $\sigma$ in the range of ~0.1 to ~0.7) is longer than B-form DNA, and thus any change in $\sigma$ should be detected through a change in extension (SI Appendix, Fig. S4). In a small subset of cases (10%, $n = 3/30$) a clear change in $\sigma$ was observed within a 10-min time span. However, for the vast majority of molecules considered (90%, $n = 27/30$), negligible change in extension (and thus $\sigma$) was detected over a 30-min period. We note that the few molecules that are unstable typically lose their supercoiled state within a short period of time and can therefore be easily identified and screened for.

Unraveling the Structure of Negatively Supercoiled DNA Using ODS.

To demonstrate the power of ODS, we first apply this method to study the structural and mechanical properties of underwound DNA as a function of ionic strength and tension. The structural transitions of negatively supercoiled DNA are of particular interest as a result of their role in both transcription and replication (1–3, 9, 10). It has been reported previously that upon changing $\sigma$ from ~0.1 to ~1.5, torsionally constrained DNA held at ~5 pN undergoes a cooperative transition from B-DNA to an increasingly underwound state (11–13). The underwound regions have been reported to exist as several different structures, including left-handed conformations (L-DNA and Z-DNA) as well as less defined base-pair melted conformations (bubble-melted DNA) (11). However, the exact structures of underwound DNA and their
dependence on local (e.g., cellular) environment are unclear. To gain a better understanding of this, we recorded force–extension curves of negatively supercoiled DNA as a function of ionic strength (Fig. 4 A and B). These experiments reveal 2 notable features. The first is that, for NaCl concentrations below ~50 mM, the overstretching transition appears less cooperative (i.e., the change in force as a function of extension is more gradual and less smooth). Second, at very low ionic strength (<25 mM NaCl) hysteresis is observed when comparing force–extension and force–retraction curves (Fig. 4B). A similar hysteresis is also induced at elevated temperature (SI Appendix, Fig. S5). These observations indicate that underwound DNA exhibits a heterogeneous structure, and is at least partially base-pair melted at low ionic strength. By fitting the extensible worm-like chain model (up to 30 pN) to the force–extension curves of supercoiled DNA, quantitative information can be extracted (Fig. 4C and SI Appendix, Fig. S6). These fits reveal a significant decrease in the apparent DNA persistence length, from ~57 to ~30 nm as σ is varied from ~0.08 to ~0.65. Concomitant with this, the apparent DNA contour length increases by ~8% over the same range of σ. This is consistent with the presence of both L-DNA and bubble-melted DNA, which have each been reported to exhibit a lower persistence length and a greater contour length than B-DNA (13).

To gain more insight into the structure of underwound DNA, we imaged the binding of fluorescently labeled replication protein A (eGFP-RPA) to negatively supercoiled DNA. This protein has previously been used to identify bubble-melted domains of DNA under mechanical strain (11, 24, 26). We first confirm that at low concentrations (0.8 nM), RPA does not significantly perturb the structure of underwound DNA (SI Appendix, Figs. S7 and SI Note 4). Next, we compare the binding of eGFP-RPA to negatively supercoiled DNA at 5 and 55 pN, respectively (for σ between ~0.6 and ~0.7). Note that the binding affinity of RPA for base-pair melted DNA is tension independent (26). These experiments were performed by incubating the DNA molecule in RPA (0.8 nM) for 2 min and then recording a snapshot image (e.g., Fig. 4D). These images reveal 3 important observations. The first is that eGFP-RPA binding is observed at low forces (~5 pN). This suggests that, at low tensions, localized regions of the DNA are base-pair-melted and sufficiently accessible for RPA (which has a footprint of ~30 nucleotides (26)). This observation is consistent with recent studies of RPA binding by Vlijm et al. (SI Appendix, SI Note 5) (11). The second key finding is that a significant (~3–4-fold) increase in RPA binding to negatively supercoiled DNA is observed at 55 pN, compared with 5 pN (Fig. 4 D and E and SI Appendix, Fig. S8), indicating that force assists the further melting of the DNA. Third, eGFP-RPA binding (at both low and high forces) is observed primarily in the most AT-rich domains of the DNA molecule (Fig. 4F). Such sequences have been shown previously to promote base-pair melting under mechanical stress (24, 26). Together, the above findings support a growing body of evidence that negatively supercoiled DNA exhibits a heterogeneous structure at low forces (e.g., 5 pN) and low salt concentrations (e.g., 25 mM NaCl), with a coexistence of at least L-DNA, B-DNA, and bubble-melted structures (11–14).

**ODS Reveals Topology-Dependent Transcription Factor Diffusion.** Given the ability of ODS to combine supercoiled DNA manipulation with both wide-field fluorescence imaging and rapid buffer exchange, the method is well-suited to probe protein dynamics on supercoiled DNA. To demonstrate this, we investigate how negative supercoiling can influence the diffusion of the mitochondrial transcription factor TFAM (Mitochondrial Transcription Factor A). This protein has vital roles in transcription initiation, replication, and transmission of mitochondrial DNA (29, 30). TFAM has been shown previously to diffuse rapidly on B-form DNA via a 1D sliding motion, with a diffusion coefficient of ~10 × 10⁶ nm² s⁻¹ (30). It has been proposed that this sliding provides a mechanism for the protein to efficiently search for its promoter sites on the mitochondrial genome (30). Notably, the mitochondrial genome is circular (and therefore torsionally constrained) and is believed to exhibit a range of topologies in vivo, including negatively supercoiled states (31). However, the influence of supercoiling on TFAM diffusion and on protein diffusion in general is unknown. To address this, we used ODS to compare the dynamics of TFAM on a range of underwound and nonsupercoiled DNA substrates (Fig. 5A). Here, we incubated each DNA substrate in a low concentration of TFAM (fluorescently labeled with Alexa Fluor 555). Each DNA substrate was subsequently moved to a protein-free environment and 55 pN (Fig. 4).

![Figure 4](https://www.pnas.org/cgi/doi/10.1073/pnas.1908826116) King et al.
buffer channel allowing the imaging of monomers of Alexa-555-TFAM bound to the DNA (Fig. 5B). Using single-particle tracking, we then calculated the average mean-squared displacement (MSD) of TFAM monomers on each DNA substrate over time. We first examined the influence of negative supercoiling on TFAM diffusion at low DNA tensions (5 pN). As shown in Fig. 5C, the MSD of TFAM increases linearly with time in all cases, consistent with free 1D diffusion. However, the diffusion coefficient for TFAM (determined from the slopes in Fig. 5C) decreases significantly as a function of negative supercoiling. As summarized in Fig. 5D, the diffusion coefficient for TFAM on DNA at 5 pN decreases by up to ~65% as $\sigma$ is varied from 0 to ~0.69. Interestingly, a substantial decrease in diffusion requires only a relatively modest superhelical density ($\sigma \sim 0.15$). Since negatively supercoiled DNA contains small regions of base-pair melted DNA at 5 pN (Fig. 4 and SI Appendix), we hypothesize that these bubbles reduce the mobility of the protein. To confirm this, we next measured the diffusion of TFAM on overstretched end-closed unconstrained DNA, which contains extended bubble-melted structures at low ionic strength. Analysis of the corresponding MSD behavior (Fig. 5E, blue) reveals that TFAM diffusion is similar, or even slightly slower, on overstretched unconstrained DNA compared with negatively supercoiled DNA at 5 pN (Fig. 5F). As controls, we note that TFAM diffusion on nonsupercoiled DNA at 5 pN is unaffected by the presence of torsional constraint and is only minimally force dependent (Fig. 5E and F). Together, these observations indicate that TFAM mobility on DNA is highly sensitive to the presence of underwound structures—either due to negative supercoiling or overstretching.

While the mobility of TFAM on nonsupercoiled (B-form) DNA is rapid, it is still lower than the maximum limit for rotationally coupled diffusion along the DNA backbone (32). This implies that the protein is slowed down by more stable transient interactions with the DNA. The current work suggests that these interactions are either stronger and/or less transient when the DNA is underwound. This is consistent with a recent study showing that TFAM has a higher binding affinity for negatively supercoiled plasmids (33). We thus propose that TFAM interacts more strongly with local underwound structures, resulting in a slower 1D sliding on DNA. It is notable that, despite the reduced diffusion of TFAM on underwound structures, we do not observe evidence of confined diffusion on these substrates (Fig. 5C and E). Therefore, although underwound DNA structures can reduce TFAM diffusion, the protein is still capable of sliding over such structures. Taken together, this has potentially important implications for the ability of TFAM to locate and interact with promoter sites in vivo and suggests a mechanism by which supercoiling could regulate processes such as mitochondrial transcription.

**Discussion**

Here we have presented a versatile methodology to generate and study negatively supercoiled DNA at the single-molecule level. Since ODS is based on a standard dual-trap optical tweezers assay, it is compatible with a wide range of functionalities that are difficult to combine with traditional methods to control DNA linking number. Importantly, this includes the ability to image the whole DNA molecule with fluorescence microscopy (20, 34). Additionally, the supercoiled substrate can be freely moved between different microfluidic channels, allowing for fast buffer exchange. This latter feature is greatly advantageous because it facilitates sequential binding of different proteins to the substrate, and also enables fluorescence images to be recorded under background-free conditions (20). Furthermore, in ODS, the supercoiled state is generated rapidly (on the order of seconds), independent of the length of the DNA molecule. This is important because it facilitates the use of much longer substrates (such as λ-DNA), which is beneficial for many fluorescence imaging studies (20).

Exploiting the above features, we have used ODS to quantify the heterogeneous structure of underwound DNA and reveal how these structures can influence the mobility of a key mitochondrial transcription factor. These applications showcase the unique ability of ODS to interrogate long molecules of supercoiled DNA using a combination of 3D nanomanipulation and fluorescence microscopy. In this way, we demonstrate that the method is a robust and powerful approach for probing the structural properties of underwound DNA as well as the interplay between negative supercoiling and DNA-binding proteins. We propose that ODS can therefore be applied to address many open questions in
Calibrating the Extent of Supercoiling. The fixed change in DNA linking number generated using ODS can be calibrated using reference force-extension curves. Such reference curves can be obtained from literature or independently using alternative single-molecule approaches (such as magnetic or micropipette tweezers). In our case, we chose to compare our force-extension curves with those of Léger et al. (25). To this end, we plotted the DNA extension at 70 pN as a function of $\sigma$. At this force, the reference data show a near-linear relationship between the DNA extension at 70 pN and $\sigma$ (over the range of $0 < \sigma > 0.07$, see Fig. 2B). By comparing the data from our measurements at 70 pN with those of the reference data, the value of $\sigma$ generated in our method can be determined. The uncertainty in the absolute value of $\sigma$ determined using this calibration procedure is typically between 0.03 and 0.045 (SI Appendix, Fig. S1 and SI Note 1).

TFAM Diffusion Analysis. End-closed DNA molecules were incubated in a low concentration of Alexa-555-labeled TFAM (∼5 nM) such that only a few protomers were bound. The DNA molecules were then transferred to a protein-free buffer channel where fluorescence videos were recorded with a frame rate of 0.5 s. Kymographs of these videos were then analyzed with a custom-written MATLAB-based program that tracked the position of each Alexa-555-TFAM monomer on the DNA as a function of time. Only traces spanning longer than 10 s and which did not cross another were considered. The diffusion constant ($D$) was determined from MSD plots ($MSD = 2Dt + offset$) for all trajectories measured.

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