Single-Molecule Methods for Investigating the Double-Stranded DNA Bendability

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https://doi.org/10.14348/molcells.2021.0182
www.molcells.org

The various DNA-protein interactions associated with the expression of genetic information involve double-stranded DNA (dsDNA) bending. Due to the importance of the formation of the dsDNA bending structure, dsDNA bending properties have long been investigated in the biophysics field. Conventionally, DNA bendability is characterized by innate averaging data from bulk experiments. The advent of single-molecule methods, such as atomic force microscopy, optical and magnetic tweezers, tethered particle motion, and single-molecule fluorescence resonance energy transfer measurement, has provided valuable tools to investigate not only the static structures but also the dynamic properties of bent dsDNA. Here, we reviewed the single-molecule methods that have been used for investigating dsDNA bendability and new findings related to dsDNA bending. Single-molecule approaches are promising tools for revealing the unknown properties of dsDNA related to its bending, particularly in cells.

Keywords: DNA bending, fluorescence resonance energy transfer, persistence length, polymer mechanics, single-molecule techniques

INTRODUCTION

Double-stranded DNA (dsDNA) bending is the most prevalent mechanical deformation of DNA in vivo and is observed in many biological phenomena, such as DNA-protein binding (Napoli et al., 2002; Privalov et al., 2009; Sarangi et al., 2019; Tan et al., 2016; Yoo, 2021). Transcription factors regulate transcription through DNA loop formation (Han et al., 2009; Kim et al., 2021a; Lee and Schleif, 1989; van der Vliet and Verrijzer, 1993) (Fig. 1A). To form a nucleosome, a histone octamer is wrapped (about 1.7 turns) by 147 base-pair (bp) dsDNA (Luger et al., 1997; Richmond and Davey, 2003) (Fig. 1B). DNA viruses store bent dsDNA in viral capsids under a high pressure (Ben-Shaul, 2013; Purohit et al., 2003) (Fig. 1C). The dsDNA bending process, playing pivotal roles in regulating intracellular genetic information by transcription, requires a significant energy (Garcia et al., 2007; Privalov et al., 2009).
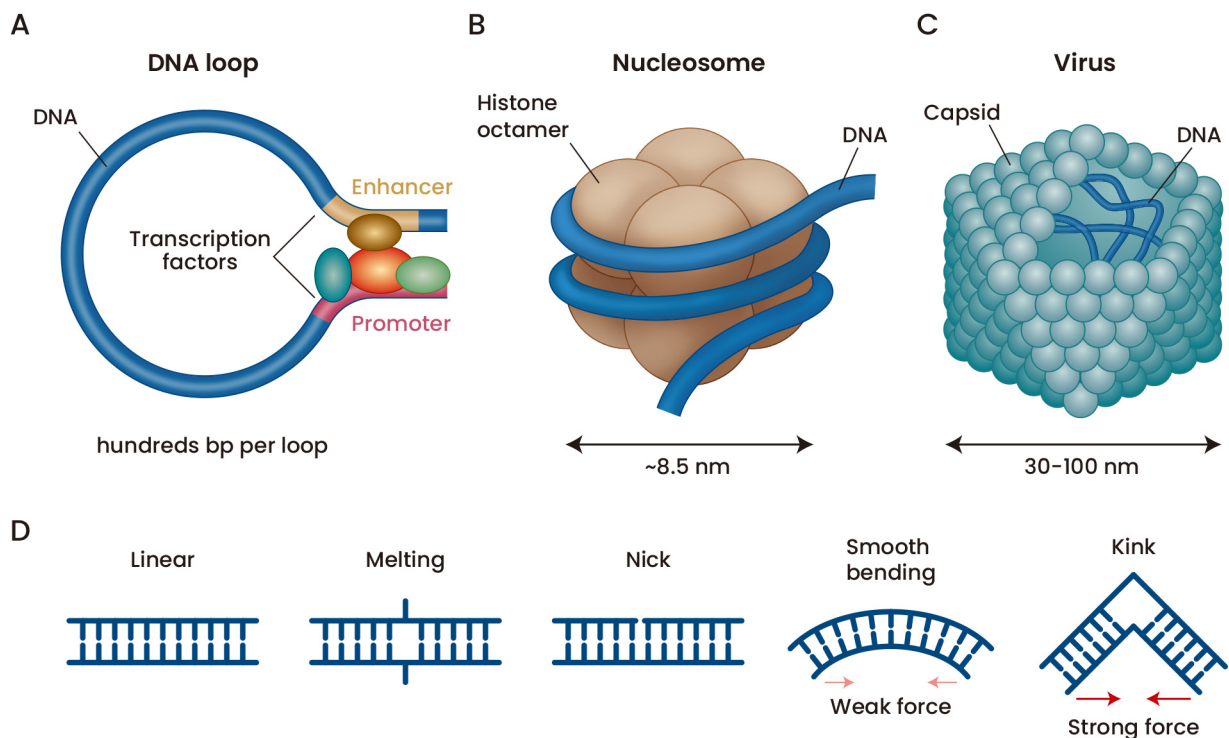
Most physical and chemical investigations of dsDNA bending have been conducted through bulk experiments, wherein many molecules were simultaneously sampled. For example, gel electrophoresis with cyclization of linear dsDNA has been commonly used at the bulk level to study dsDNA bendability (Forties et al., 2009; Geggier et al., 2011; Geggier and Vologodskii, 2010; Harrington, 1993; Nathan and Crothers, 2002; Shore et al., 1981). Dozens of base pairs at each end of the linear dsDNA are composed of complementary single-stranded DNA (ssDNA), which are annealed with a very small probability by thermal fluctuation. DNA loops with both ends annealed are covalently joined by a DNA ligase. The amounts of linear dsDNA and circular dsDNA are quantified by gel electrophoresis and used to calculate the J-factor, the ratio of the equilibrium constant for DNA cyclization and that for bi-

A

DNA loop

DNA

Enhancer

Transcription
factors

Promoter

hundreds bp per loop

B

Nucleosome

Histone
octamer

DNA

~8.5 nm

C

Virus

Capsid

DNA

30–100 nm

D

Linear   Melting   Nick   Smooth bending   Kink

Weak force   Strong force

Fig. 1. Various dsDNA bending structures in biological systems. (A) DNA loop formation by transcription factors. (B) Nucleosome consists of a histone octamer and 147 bp DNA. (C) DNA virus encapsulates its whole DNA in the small volume of the viral capsid. (D) Illustration of the various dsDNA structures.

molecular association (Harrington, 1993; Shore et al., 1981). Using this method, for example, the alternation of dsDNA bendability depending on diverse factors, such as temperature (Geggier et al., 2011), mismatches (Forties et al., 2009), sequences (Geggier and Vologodskii, 2010), and methylations (Nathan and Crothers, 2002), has been observed. However, the innate averaging nature of bulk measurements makes it difficult to obtain detailed information about the bending structure, dynamics, and variation in dsDNA bending depending on the extent of the external force.

The bending elasticity of long dsDNA has been described by the worm-like chain (WLC) model, which defines dsDNA as the chain of a continuously isotropic flexible rod (Kratky and Porod, 1949; Marko and Siggia, 1995). In this model, the bendability of dsDNA is described using the persistence length, a characteristic length value wherein the correlation between the two tangent vectors along the polymer disappears (Hagerman, 1988). Specifically, the persistence length (P) is defined as the angle between two tangent vectors (θ) over the length (L) as follows, $<cos\theta>=e^{-L/P}$. This definition indicates that the average curvature of a polymer decreases as the persistence length increases. Thus, as the persistence length increases, dsDNA becomes less bendable. The probability of cyclization in solution, i.e., the j-factor, is well matched with the WLC model prediction with a persistence length of approximately 50 nm (Du et al., 2005). However, if the persistence length is 50 nm, it is difficult to generate the bending structure of dsDNA under physiological conditions.

For example, the bending energy of nucleosomal dsDNA with 127 bp wrapped around the histone core with a radius of 4.3 nm is estimated to be approximately 58 k_BT (Prinsen and Schiessel, 2010), which is much higher than the thermal energy. This suggests that the bending structure of dsDNA in the cell may be different from that predicted by the WLC model. In fact, the looping of approximately 100 bp dsDNA, which is similar to the dsDNA length occurring under physiological conditions, tends to have a higher looping ratio than the expected ratio by the WLC model (Cloutier and Widom, 2004). Therefore, further studies on the bending mechanics of dsDNA are required to understand dsDNA bending under physiological conditions.

Various single-molecule methods have been attempted recently to overcome the limitations of bulk experiments and thus obtained detailed information on the bending mechanics of dsDNA (Basu et al., 2021a; Kang et al., 2021; Zlatanova and van Holde, 2006). Single-molecule methods, such as atomic force microscopy (AFM) (Binnig et al., 1986; Peters and Maher, 2018), optical and magnetic tweezers (Ashkin, 1997; Ashkin et al., 1986; Smith et al., 1992), tethered particle motion (TPM) (Fan et al., 2018; Schafer et al., 1991), and single-molecule fluorescence resonance energy transfer (smFRET) measurement (Ha et al., 1996), have been used to investigate the static structure and dynamics of bent forms of dsDNA. In this review, we have described how these single-molecule methods have been used to elucidate the nature of dsDNA bending and new findings describing dsDNA

bendability obtained by single-molecule methods. DNA mechanics have been extensively reviewed (Basu et al., 2021a; Brahmachari and Marko, 2018; Saran et al., 2020); therefore, we focused on studies investigating dsDNA bending and deformations in dsDNA structure induced by the bending force (Fig. 1D).

DIRECT OBSERVATION OF THE DNA BENDING STRUCTURE BY ATOMIC FORCE MICROSCOPY

AFM measures the bendability of dsDNA by observing the contours of dsDNA deposition on a mica surface (Binnig et al., 1986) (Fig. 2A), whereby an AFM probe tip attached to the cantilever interacts with a mica surface and immobilized dsDNAs. The vertical displacement of an AFM probe by deposited dsDNA causes the cantilever deflection, which is de-
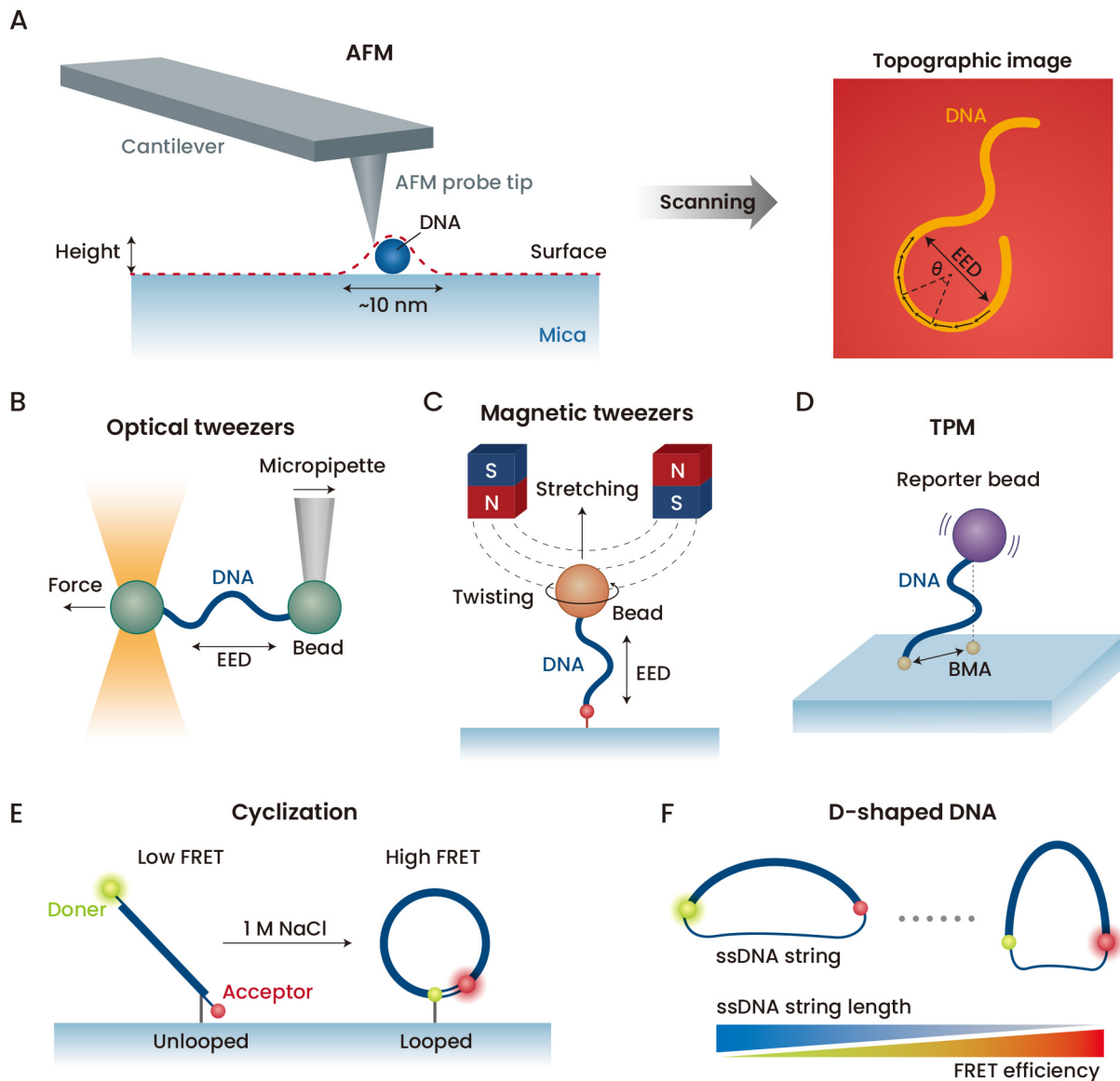
Fig. 2. Single-molecule methods for investigating the properties of dsDNA bending. (A) A schematic illustration of AFM and the topographic image of dsDNA. (B) A schematic illustration of the optical tweezers. The optical tweezers stretch the dsDNA using two beads. (C) A typical magnetic tweezers. The magnetic tweezers are able to stretch and twist the dsDNA. The optical and magnetic tweezers measure the EED of dsDNA depending on the stretching force. (D) A schematic illustration of TPM. (E) Single-molecule cyclization assay uses dual-labeled dsDNA having sticky ends. The FRET efficiency increases as dsDNA forms a loop. (F) A schematic illustration of D-shaped DNAs. As the length of ssDNA string becomes shorter, the dsDNA portion bends more. The increase in the curvature of the dsDNA portion is measured by the FRET efficiency.

tected by the laser reflected from the cantilever. This vertical displacement is scanned over the mica surface and converted into a topographic image, allowing direct observation of the immobilized dsDNA structure. The persistence length of dsDNA is then estimated from dsDNA contours in the image. The WLC model provides the persistence length from two parameters: the angle between the separated tangential vectors and the end-to-end distance (EED) of the separated length of dsDNA (Peters and Maher, 2018). As a result, the diverse dsDNA bending structures observed by AFM indicate the bendability of dsDNA. For example, the cytosine methylation decreases the average curvature of dsDNA and simultaneously increases the average EED of dsDNA contour, which indicates an increase in the persistence length (Cassina et al., 2016). Strong bending of dsDNA was observed from the supercoiled dsDNA (Pavlicek et al., 2004), and the kink form, a sharp bending, was also directly observed in a negatively supercoiled dsDNA by base-pair resolution dsDNA image analysis (Pyne et al., 2021). Interestingly, the kinks were generated in the dsDNA contours more frequently from AFM measurements than expected by the WLC model, which indicates an increase in the flexibility of dsDNA, consistent with the bulk cyclization experiments (Chen et al., 2010; Wiggins et al., 2006).

DNA-BEAD TETHERING METHODS MEASURING DYNAMICS OF A SINGLE dsDNA IN REAL TIME

Optical and magnetic tweezers
Stretching experiments of single dsDNA were performed using optical or magnetic tweezers (Figs. 2B and 2C). The optical tweezers use a laser beam to force the dielectric bead in the direction of a focused laser beam (Ashkin, 1997; Ashkin et al., 1986). A long dsDNA was attached to the two beads. One bead was optically trapped on the platform, and the other bead was attached to the micropipette. The EED of a long single dsDNA and the stretching force were measured using the two beads. The magnetic tweezers also manipulate the EED of dsDNA, which is attached to the glass surface and a superparamagnetic bead (Smith et al., 1992). Magnetic tweezers have the advantage of manipulating both the twist and stretching of a single dsDNA (Strick et al., 1996). These methods have been used to investigate the real-time dynamics of the EED of dsDNA by an external stretching force. The persistence length of dsDNA can be obtained from the stretching force-extension curve. The stretching force-extension curves of a long dsDNA obtained by both optical and magnetic tweezers are in good agreement with the prediction using the WLC model with a persistence length of 50 nm (Smith et al., 1992; 1996). These results indicate that the bending of long dsDNA by the external force is well explained by the WLC model. A structural transition involving DNA melting is observed in instances of a strong stretching force, which increases the dsDNA extension (Baumann et al., 2000; van Mameren et al., 2009). Interestingly, tweezer studies commonly reported that DNA methylation decreases the persistence length of dsDNA in contrast to other single-molecule and bulk measurements (Pongor et al., 2017; Shon et al., 2019). A possible explanation for this discrepancy is the sequence-dependent effects or the interactions between dsDNA strands.

Tethered particle motion
TPM is a method that allows the measurement of the effective length of a tethered dsDNA in the absence of an external force (Fan et al., 2018; Schafer et al., 1991) (Fig. 2D). The reporter bead is tethered to the surface by a long dsDNA, which is used to observe the Brownian motion amplitude and the root-mean-square of the bead displacement (Fig. 2D). Because the root-mean-square of the bead displacement has a positive correlation with the persistence length of dsDNA, the persistence length of dsDNA can be obtained using TPM (Brinkers et al., 2009). Unlike typical optical or magnetic tweezers, TPM can obtain data in a high-throughput format, which is appropriate for observing a large number of single dsDNA molecules at the same time (Brunet et al., 2015a). The dsDNA persistence length which depends on the temperature and ion strength would thus be obtained quantitatively. The persistence length decreases linearly as the temperature increases (Brunet et al., 2018; Driessen et al., 2014). The persistence length decreases non-linearly with an increase in the ion strength, but the reduction efficiency differs depending on the ion type (Brunet et al., 2015b; Guilbaud et al., 2019).

These dynamic measurements of dsDNA from tethered beads with or without an external force are limited in the investigation of the bendability of short dsDNAs. The typical dynamic measurements of bead tethered to dsDNA require a very long dsDNA (> 1,000 bp), except for special methods (Shon et al., 2019), to observe a sufficient displacement of the tethered bead, which cannot completely rule out the change in the EED of dsDNA due to external effects, such as intermolecular interactions of dsDNAs (Basu et al., 2021a; Yoo et al., 2016). In addition, the persistence length measured by these methods cannot explain the formation of dsDNA local bending structure under physiological conditions. Therefore, alternative methods are required to study the bending dynamics of a short dsDNA in real time at a high resolution.

SINGLE-MOLECULE FRET MEASUREMENTS OBSERVING DNA LOOPING DYNAMICS AND THE CURVATURE OF DNA BENDING STRUCTURE

Single-molecule FRET measurement
The smFRET measurement is one of the most widely used approaches for observing the structural dynamics of biomolecules (Lerner et al., 2021). This method relies on the energy transfer between two fluorophores: a donor and an acceptor (Forster, 1946). The FRET efficiency (E) is calculated from the distance between the two fluorophores (R) as a function of $E = 1/[1 + (R/R_0)^6]$, where R_0 is the value at which half of the energy is transferred (Hellenkamp et al., 2018). The FRET efficiency is inversely proportional to the sixth power of the distance between two dyes, which allows for a very precise and sensitive measurement of the distance. Since R_0 typically has a range of 5 to 6 nm, the smFRET measurement is optimal for measuring distance variations in the range of 2-10

nm, which is the same as the length of dozens base pairs of dsDNA (Ray et al., 2014). Two methods are commonly used to measure the FRET efficiency of fluorophores labeled on a single dsDNA: one is to image tethered samples using total internal reflection (TIR) (Hildebrandt et al., 2015; Kim et al., 2021b); and the other is to measure freely diffusive samples using confocal microscopy (Kapanidis et al., 2004; Kim et al., 2015; Lee et al., 2005).

DNA cyclization assay

Single dsDNA cyclization assay uses TIR microscopy to measure the FRET efficiency of the tethered dsDNA fragment (Vafabakhsh and Ha, 2012) (Fig. 2E). This assay measures the FRET efficiency of two fluorophores labeled at both ends of each dsDNA fragment with complementary ssDNA overhangs. The tethered dsDNA fragment with a low salt concentration exists as an unlooped form with a low FRET efficiency, but it has a loop form at a high salt concentration (1 M NaCl) with a high FRET efficiency. The looping dynamics is obtained by measuring the fraction of looped molecules as a function of time after the addition of 1 M NaCl. The cyclization assay can be applied to measure the bending dynamics of short dsDNA (< 100 bp) and dsDNA bendability under various conditions. For example, modification of cytosine changes the bendability of dsDNA: 5-methylcytosine decreases dsDNA bendability, while 5-hydroxymethylcytosine and 5-formylcytosine increase dsDNA bendability (Ngo et al., 2016). A mismatch in short dsDNA is known to increase bendability and thus destabilize the dsDNA loop form depending on its position (Jeong and Kim, 2019; 2020). Another advantage of the cyclization assay is that it measures the bendability of dsDNA in a high-throughput format, a loop-seq assay (Basu et al., 2021b). The cyclization assay confirmed that dsDNAs shorter than 100 bp have a much higher looping ratio than that predicted by the WLC model, indicating an increment in bendability, consistent with the bulk experiments (Vafabakhsh and Ha, 2012). The conventional WLC model cannot explain the increase in the bendability observed by the cyclization assay. Thus, the kink, a sharp bending DNA structure, was proposed. Based on this proposition, the kinkable-WLC model has been newly conceived, which assumes that the kink can be formed on a strongly bent dsDNA structure to release the bending energy. This model has described the increase in the bendability of short dsDNA (Le and Kim, 2014; Vologodskii and Frank-Kamenetskii, 2013).

The increase in bendability in short dsDNA is observed repeatedly in bulk assays, AFM, and single-molecule cyclization assays. This is interesting that the persistence length of long-length dsDNA has been reported to be 50 nm. If the persistence length of dsDNA is 50 nm, it would be very difficult to bend short dsDNA (< 100 bp). However, bending of dsDNA on a short length scale is often observed in cells (Fig. 1). Thus, kink formation, which increases bendability dsDNA, may be a key factor to reconcile the seemingly contradictory observations. In fact, it has been reported that the kink plays an important role in protein-DNA interactions (Bhairosing-Kok et al., 2019). Therefore, it is necessary to investigate the detailed mechanism of the formation and mechanical properties of the kink in order to elucidate the dsDNA bend-ing formation in cells.

D-shaped DNA nanostructures

Investigating the structures and mechanical properties of dsDNA kinks using bulk and single-molecule methods introduced so far is difficult owing to several limitations. The existence of the kink can be conceived by the cyclization assay, but direct observation of the kink is not feasible. The kink can be induced by using a nick, the discontinuity of a dsDNA molecule without a phosphodiester bond (Protozanova et al., 2004; Yakovchuk et al., 2006), or by using mismatched and melted base pairs (Jeong and Kim, 2019; Kim et al., 2015; Satange et al., 2018) (Fig. 1D). However, these tricks do not reflect the conditions for kink formation by intact dsDNA bending. Therefore, it is difficult to quantitatively study the positional effects of sequences, epigenetics, and ion strength on kinks. The immobilization of dsDNA on the surface has the probability of affecting bending and kink formation. The constant collision between dsDNA and solvent molecules in Brownian motion may induce bending and looping of dsDNA (Basu et al., 2021a). However, if a part of the polymer is tethered on the surface and thus restricts its movement, the bending property may not be identical for the entire region of the polymer and affect the formation and position of the kink (Slutsky, 2005; Waters and Kim, 2013). In addition, it is difficult to bend short dsDNA with the external compressive force using conventional single-molecule methods. Thus, it is inevitable to rely on random movements of the dsDNA polymer, making it difficult to form kinks that require a strong bending force.

Recently, using the controllable self-assembly behaviors of ssDNA strands, various nanostructures have been easily manufactured with decent stability (Wang et al., 2021). For example, DNA nanostructures have been used to study DNA mechanics. The persistence length and intrinsic curvature of the A-tract were observed in real time using fluorophore-labeled DNA nunchucks (Cai et al., 2020). Compressive force can be applied to short dsDNA using a nanostructure called D-shaped DNA (Kim et al., 2015; Lee et al., 2016; Qu et al., 2011; Yeou and Lee, 2021). D-shaped DNA is formed by annealing the ssDNA ring and partially complementary linear ssDNA, which consists of a rigid dsDNA portion and a flexible ssDNA string (Fig. 2F). The flexible ssDNA string connects both ends of the dsDNA portion and causes entropic compressive forces that have a negative correlation with the contour length of the ssDNA string (Shroff et al., 2005; 2008). As a result, the ssDNA string reduces the EED of the dsDNA portion (Kim et al., 2015; Yeou and Lee, 2021). The induced entropic stretching force generated by the ssDNA string is strong enough to bend the dsDNA portion due to high flexibility of ssDNA string (Kang et al., 2014; Murphy et al., 2004; Roth et al., 2018). Therefore, it is possible to control the bending force applied to the dsDNA portion by modulating the size of the ssDNA ring. A donor and an acceptor dyes were labeled at a distance of approximately 8-10 nm on the dsDNA portion. Then, the curvature of the freely diffusive dsDNA portion can be measured by using confocal microscopy. Theoretically, the persistence length can be calculated from the curvature of the dsDNA depending on the bending

force and the size of the ssDNA ring (Lee et al., 2016; Yeou and Lee, 2021). This method is useful for measuring the bendability of short dsDNA at various compressive forces. In addition, the kink can be easily observed by adjusting the ssDNA string length, which can be used to investigate the effects of various factors on kink formation (Fields et al., 2013; Qu et al., 2011).

Using a D-shaped DNA nanostructure in combination with smFRET measurements, the curvature of the bent dsDNA and kink formation has been observed. The D-shaped DNA with short ssDNA string (< 16 nt) and a 30 bp dsDNA portion compresses the dsDNA portion to induce the melting of the base pairs at the center of the dsDNA portion (i.e., kink) or base-pair melting at the ends of the dsDNA portion (Kim et al., 2015; Lee et al., 2016). These two conformations of D-shaped DNA are interconverted dynamically within a millisecond time scale. The FRET efficiency of the kink conformer is similar to that of the dsDNA portion with three mismatches positioned at the center of dsDNA, confirming kink formation at the dsDNA portion with base-pair melting by strong compressive force. The D-shaped DNA with a long ssDNA string (> 16 nt) exhibits smooth bending on the dsDNA portion. The FRET efficiency of smoothly bent dsDNA is increased by the DNA nick (Yeou and Lee, 2021). The nick is known to induce kink under a bending force, indicating that kinks directly increase dsDNA bendability (Protozanova et al., 2004; Yakovchuk et al., 2006).

CONCLUSIONS AND PERSPECTIVES

Here, we discuss various single-molecule methods for investigating the dsDNA bending mechanics. Understanding how the dsDNA bending structure is formed by various biological processes in cells is a key question in molecular biology. Transcription, nucleosome formation, and DNA packaging in the viral capsid may induce dsDNA bending structures of various sizes that may involve dsDNA deformation, such as a kink. However, measuring the bendability of dsDNA using bulk experiments is not suitable for investigating the detailed bending mechanics of dsDNA. Single-molecule methods have been widely used to study the bending mechanics of dsDNA. AFM provides images of static dsDNA bending structures. The optical or magnetic tweezers and TPM can observe the bending dynamics of relatively long dsDNA in real time. The cyclization assay based on smFRET is able to observe the bending dynamics of short dsDNA and thus verify the high flexibility of short dsDNA. Curvature analysis using D-shaped DNA nanostructures in combination with smFRET confirms the generation of a kink by strong compressive forces. We can broaden our knowledge about the bending structure, mechanics, and dynamics of dsDNA under various external forces by using these single-molecule methods.

Many aspects of dsDNA bending remain unknown. The bending properties depending on the sequences, the effects of methylation, the atomic structure of a kink form, and how the bending stress is compromised in the highly bent form in nucleosomes, remain unresolved. A method to control the compressive force and kink formation, such as using a D-shaped DNA nanostructure, could lead to a deep study

of the properties of a kink and other properties. Ultimately, as stated above, it is necessary to connect dsDNA bendability to biological processes in cells, such as the formation of nucleosomes. Advances in biophysical technology, such as high-throughput loop-seq assay (Basu et al., 2021b), are expected to be able to determine the connectivity between DNA bendability measured by single-molecule methods and genetic expression in living cells.

AUTHOR CONTRIBUTIONS
S.Y. and N.K.L. wrote the manuscript.

CONFLICT OF INTEREST
The authors have no potential conflicts of interest to disclose.

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