Detecting DNA damage at a single-molecule level by atomic force microscopy

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DNA damage can be caused daily by as many as one million molecular lesions per cell by normal metabolic activities and environmental factors. Even though these lesions only take 106 of the human genome which is approximately 109 base pairs, uncorrected lesions in critical genes such as tumor suppressor genes can block cells and prevent them from carrying out regular functions and increase the possibility of tumor formation. First, the majority of DNA damage affects the primary structure of the double helix. The lesions cause a change in DNA mechanics which can be measured easily by SMFS. Second, a variety of repair strategies have evolved to restore lost genetic information. Specific DNA repair enzymes or antibodies bind at or near the site of damage, recruiting other molecules to bind and triggering the actual repair to take place. These processes can also be visualized directly, and the lesions can be quantified at a single-molecule level by AFM imaging of these repair enzymes or antibodies. The article highlights research progress in the detection of DNA damages using AFM imaging and SMFS methods. Multiple examples are presented on visualizing and manipulating DNA at a single-molecule level.

Keywords DNA damage; Single molecule; Atomic force microscopy (AFM), Force spectroscopy, Imaging

1. Introduction

DNA is being damaged at a rate of 103 to 106 lesions per cell per day [1] due to inside metabolic processes and outside environmental factors. Even though these lesions only take 106 of the human genome which is approximately 109 base pairs, uncorrected lesions in critical genes such as tumor suppressor genes can block cells and prevent them from carrying out regular functions and increase the possibility of tumor formation. In most cases of DNA damage, the bases are chemically modified by non-native chemical bonds or bulky adducts. These chemical modifications can in turn alter the spatial configuration of the structure of the DNA double helix. Such alterations can be detected by the specific DNA enzymes. Once damage is localized, specific DNA repair enzymes bind at or near the site of damage, summoning other enzymes to bind and form a complex that triggers the following repair to take place.

Detecting DNA damage is the first step towards understanding the complex damage repair process. DNA damage detection is typically a multi-step process involving many enzymatic reactions, various labeling methodologies and the separation of DNA molecules [2]. Many methods and techniques have been developed to study DNA damage quantitatively in bulk material [3-8]. Among them, gel electrophoresis [3, 4] is a well-established bulk analysis method, it is frequently used in DNA damage studies because it allows separation of intact and damaged plasmids by its size or geometry. High sensitivity methods include immunoassays, which use specific antibodies in response to DNA damage [5] and HPLC coupled with mass spectrometry [5, 6]. Within the last ten or so years, single-cell gel electrophoresis, or the comet assay, was established as one of the standard methods for quantifying DNA damage [7, 8].

Since DNA alterations vary from one DNA molecule to another, it would be advantageous to detect various lesions in individual molecules. The last decade witnessed a rapid development of new single-molecule techniques which were useful in detecting DNA damage [9]. Atomic force microscopy (AFM) has been utilized for manipulating and imaging of individual DNA since 1989 [10-26]. It is one of the leading techniques in single molecule or nanoscale science because it can visualize individual DNA, proteins and DNA-protein complexes under physiological conditions in real time. However, AFM studies of DNA were mostly focused on the biological structure and dynamic processes of DNA, [10-17] and relatively limited application was found in DNA damage and repair research [18-26]. For example, Wang et al investigated the interaction between MutS and DNA in the mismatch repair system [18]. Chen et al used AFM cantilevers with carbon nanotube probes to visualize human 8-oxoguanine DNA glycosylase scanning DNA in the search for damage [19]. Murakami et al imaged supercoiled plasmids damaged by high doses of gamma radiation by AFM and compared the results with that obtained by gel electrophoresis of damaged DNA [24]. Pang et al quantified single-strand breaks (SSB) and double-strand breaks (DSB) in supercoiled DNA plasmid induced by high doses of various types of ionizing radiation [25, 26].

We recently exploited the AFM imaging of individual DNA molecules, alone as well as in complexes with a suite of DNA enzymes or antibodies, to directly quantify DNA damage caused by UV and gamma radiation [27-29]. We found that high doses of short wave ultraviolet (UVC) and medium wave ultraviolet (UVB) light not only produce
cyclobutane pyrimidine dimers (CPDs), but also cause significant DNA degradation. At very low UVB doses, the relationship between the number of CPDs and UVB dose is almost linear [27]. We also examined long-wave ultraviolet (UVA) damage to DNA at a single-molecule level and found that UVA (365 nm) produces a significant amount of abasic sites and CPDs. Our results strongly suggest that CPDs can be produced directly by UVA [28].

Besides, the result of the detection and quantification of gamma radiation damage show that the number of SSB per DNA molecule is linearly proportional to the plasmid size and inversely correlated to the DNA concentration [29]. All these results show that AFM imaging is a direct and quantitative approach to detect DNA damage. Its accuracy and reliability are validated through the comparison with traditional agarose gel electrophoresis.

A clear relationship between DNA damage and the nano mechanics of DNA double helixes by AFM single-molecule force spectroscopy (SMFS) was also established [30]. The results show that the width of the B-S transition shortens in a UV dose-dependent manner below the standard 70% of the contour length and becomes less cooperative. In λ-phage DNA the second, high-force transition is also reduced in a dose-dependent manner. Thus, SMFS can also be used to detect and investigate DNA lesions. Taken together these observations lay a foundation for the future use of SMFS in nanoscale DNA damage diagnostics.

In the article we highlight these new assays for the detection of DNA damage at a single-molecule level using AFM imaging and SMFS. We also describe in detail how different kinds of DNA damage were detected by AFM imaging and SMFS. Multiple examples are presented on visualizing and manipulating DNA at a single-molecule level.

## 2. Experimental Section

### 2.1 Materials

pUC18, 2686 base pairs (bp), was isolated from *E. coli* and purified using the QIAGEN Plasmid Maxi kit (QIAGEN Inc). λ-phage DNA (D3779) was purchased from Sigma–Aldrich Co. DNA endonucleases that might be used for UV damage detection were summarized in Table 1.

**Table 1** DNA endonucleases for damage detection.

<table>
<thead>
<tr>
<th>Enzyme or Antibody for damage detection</th>
<th>Supplier</th>
<th>Specific lesions these enzymes can detected</th>
<th>Enzyme’s activity</th>
</tr>
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<tbody>
<tr>
<td><em>E. coli</em> endonuclease IV</td>
<td>New England Biolabs</td>
<td>apurinic/apyrimidinic site base paired with adenine, 5,6-dihydrothymine</td>
<td>100%, &lt;10%</td>
</tr>
<tr>
<td>T4 endonuclease V</td>
<td>New England Biolabs, Epicentre</td>
<td>cyclobutane pyrimidine dimer, apurinic/apyrimidinic site base paired with adenine</td>
<td>100%</td>
</tr>
<tr>
<td><em>E. coli</em> endonuclease III</td>
<td>Trevigen</td>
<td>thymine glycol, apurinic/apyrimidinic site base paired with adenine, 5,6-dihydrothymine</td>
<td>100%, &lt;10%</td>
</tr>
<tr>
<td><em>E. coli</em> Fpg</td>
<td>Trevigen</td>
<td>8 oxoguanine base paired with a cytosine or guanine, apurinic/apyrimidinic site base paired with adenine, 5,6-dihydrothymine, 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine</td>
<td>100%, &lt;10%</td>
</tr>
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</table>

### 2.2 Enzyme and antibody treatment

For DNA endonuclease treatment, 10 µg/ml UV irradiated supercoiled pUC18 plasmids were incubated with different kinds of enzymes as shown in Table 1 to a total volume of 40 µl at 37 °C for 30-60 minutes in their preferred reaction buffer as suggested by the enzyme provider. After specific enzyme incubation, the complex was diluted by DNA imaging buffer containing 10 mM Tris HCl, 1 mM EDTA, and 100 mM NaCl to the final DNA concentration of 0.5-1 µg/ml and then deposited on mica for AFM imaging. For photolyase treatment, 10 µg/ml DNA was incubated with 2 µg/ml photolyase in 1x REC Buffer 14 containing 20 mM Tris-HCl pH=7.8, 1 mM EDTA, 1 mM DTT, and 50 mM NaCl at a total volume of 20 µl. The incubation was performed for 30 min at room temperature in darkness to prevent photolyase from repairing damage and disassociating from damage sites. The solution was then diluted with the addition of 10 mM Tris HCl, 1 mM EDTA, and 5-10 mM MgCl2 to a final DNA concentration of 0.5-1 µg/ml. The sample was then deposited on mica immediately for AFM imaging.
2.3 AFM imaging

For experiments in which the imaging buffer contains Mg²⁺, a freshly cleaved mica was used as DNA substrate directly, while the imaging buffer did not have Mg²⁺, 1-(3-Aminopropyl)aminopropyl-silatrane-functionalized mica (APS-mica) was used for the binding of DNA molecules. APS-mica was prepared as described by Shlyakhtenko et al [31]. A drop of 10-50 µl of DNA solution (0.5-1 µg/ml) was deposited on the APS-mica surface at room temperature for 3 min. The sample was rinsed and air-dried before imaging. AFM images were taken using a Nanoscope IIIa MultiMode Scanning Probe Microscope (Veeco Instruments Inc) using Tapping mode with an E scanner. RTESP probes (Veeco) were used for imaging in air. The spring constant of AFM cantilevers was 20-80 N/m and their resonance frequency was 275-316 kHz. NP-S probes (Cantilever C) were used for imaging in solution. The spring constant of the cantilever was 0.32-0.64 N/m and their resonance frequency was 56-75 kHz. The All images were collected at a scan rate of 2-3 Hz, a scan resolution of 512×512 pixels, and scan sizes of 1-5 µm.

2.4 Quantifying and verification of DNA damage from AFM images

The number of lesions was quantified by a Poisson distribution \( f(n; \lambda) = \frac{\lambda^n \exp(-\lambda)}{n!} \) [32, 33], based on percentages of supercoiled and relaxed DNA. The average number of lesions per molecule, \( \lambda \), can be obtained from \( \lambda = -\ln(f(\lambda; \theta)) \), where \( f(\lambda; \theta) \) is the fraction of supercoiled (damage \( n=0 \)) plasmids. Using this method we can directly detect and quantify damage to supercoiled DNA, by visualizing and counting individual intact and damaged plasmids captured by AFM imaging.

2.5 AFM-based SMFS

For SMFS measurements, we used nonspecific adsorption of DNA to fresh gold substrates [34] and untreated silicon nitride AFM probes (MLCT from Veeco). The cantilevers had a nominal spring constant of 10 mN/m and an actual spring constant between 10-20 mN/m, as determined by using the energy equipartition theorem [35]. DNA pulling experiments were carried out on λ-phage DNA after exposing the molecules to various doses of UV radiation. 100 ml of a DNA solution (100 µg/ml) suspended in PBS/Tris EDTA buffer was deposited onto a gold substrate, incubated for 2–3 h, and then gently rinsed 3-5 times with the buffer solution.

3. Detect DNA damage by AFM Imaging

3.1 Detect DNA SSB and DSB by AFM imaging

Supercoiled DNA is a good model for studying DNA damage because it is highly sensitive to structural modifications such as SSB and DSB [36, 37]. One SSB will relax its superhelical structure to a circular form. Similarly, one DSB will open the relaxed circular plasmid to a linear form. More DSBs will fragment the plasmid to linear duplexes of shorter lengths as compared to the original length. All these topological or length variations of DNA are easy to resolve by visualizing individual intact and damaged plasmids in AFM [20, 21, 24-26].

Shown in Figure 1A, is a typical AFM image of pUC18 plasmid deposited on the APS-mica from a buffer solution. Neither the binding to the mica nor the AFM imaging process significantly affect the DNA structure which remains in the plectonemic supercoiled configuration. We followed the approaches which were developed earlier for electron microscopy and AFM imaging of supercoiled plasmids [36, 38, 39], and counted the number of nodes in each DNA molecule as the number of visible crossover points in AFM images [38]. For a given plasmid size, the number of supercoiled nodes typically vary somewhat among different molecules, and also strongly depends on the ionic conditions in the buffer and deposition conditions [40]. In the case of pUC18 relaxation assay, we used the same buffer with constant NaCl concentration of 100 mM in all experiments. For intact pUC18 we found the average number of nodes is eight, and this is consistent with expected value for a supercoiled pUC18 DNA [16, 36-38]. We considered the molecules with a node number greater than five as intact, whereas molecules with the node number equal to or less than five indicated relaxed DNA. By this criterion, our pUC18 DNA samples contain more than 95±1% (mean±standard deviation) of intact supercoiled DNA and this high percentage of intact DNA is important for a reliable quantification of DNA damage as shown in Figure 1D. Less than 5±1% of the plasmids show less than six nodes. We assume that these plasmids represent a background of damaged DNA in our sample of “intact” pUC18 after DNA purification processes. Figure 1B and 1C show AFM images of pUC18 plasmids, which were exposed to UVC (254 nm). At the dose of 12.5 kJ/m², almost all supercoiled molecules relaxed, as shown in figure 1B and 1E, with 95±1% of molecules having at least one SSB (Figure 1E). At the dose of 75 kJ/m², 60±5% of the plasmids became linear, indicating that these molecules developed DSBs (Figure 1C and 1F). We conclude that AFM imaging can capture even single SSB lesion in single DNA molecules.
3.2 Detect DNA base modifications by AFM imaging with assistance of DNA endonucleases

In most cases of DNA damage, only the DNA bases are chemically modified, where the topological structure of the super helix doesn’t change. As a result, supercoiled plasmid relaxation assay cannot be employed to quantify the DNA damages directly. In this case, DNA endonucleases were adopted to recognize and remove the specific DNA modifications as part of the base-excision repair pathway, (see Table 1 for the details of the endonucleases). These enzyme binds to damaged DNA and scans the DNA until it encounters specific damage site. The enzyme then cleaves the glycosyl bond of the 5’-pyrimidine of the dimer and the 3’ phosphodiester bond, which results in a SSB or DSB in the DNA [2, 41].

As shown in Figure 2, we adopt T4 Endonuclease V to incise DNA at a CPD location and to convert supercoiled pUC18 plasmids to a relaxed circular form, which should make it easy to be identified in the AFM. We show AFM images of pUC18 molecules, which were exposed to various doses of UVB (302 nm) radiation, then treated with T4 Endonuclease V for 30 min and subsequently deposited on APS-mica surface, dehydrated and AFM imaged in air. In Figure 2A, we see the molecules, which were exposed to 1.4 kJ/m² of UVB radiation and then treated with T4 Endonuclease V. By analyzing this and other images, we determined that ~89% of these plasmids converted to a linear form and ~11% relaxed to a circular form (Figure 2D). These results indicate that ~89% of the T4 Endonuclease V-treated plasmids developed at least one DSB.

In subsequent measurements, we decreased the UVB dose to see if our assay can detect a single CPD site in a single DNA molecule. In Figure 2B we show an AFM image of pUC18 molecules, which were exposed to a much lower dose of 229 J/m² of UVB, and then incubated with T4 Endonuclease V as above. We can see that at this dose, most of the DNA molecules are in a relaxed circular (~76%) and linear (~18%) form (Figure 2E). Upon further decreasing of the UVB dose to 29 J/m² (Fig. 2C, F), the AFM captured approximately ~34% in a relaxed, relaxed circular form and ~65% to be supercoiled (six or more nodes). According to a Poisson distribution, we estimate the average number of CPD lesions per DNA plasmid at the dose of 229 J/m² to be λ=2.7 and at the dose of 29 J/m² to be λ=0.40. We estimate that at the dose of 229 J/m², 18% of the relaxed DNA plasmids have just one CPD, 25% have two CPD sites, 22% have three CPDs, and 15% have four CPDs, while at the dose of 29 J/m², these numbers are 27%, 5%, 0.7%, and 0.1% respectively.

Our results indicate that after subtracting pre-existing background damage, 229 J/m² of UVB radiation causes 2.7 CPD lesions per pUC18 plasmids, which is equivalent to ~1000 CPDs per one million base pairs, while 29 J/m² of UVB radiation causes 0.40 CPD lesion per pUC18 plasmids, which is equivalent to ~150 CPDs per one million base pairs. Thus, at low UVB doses, the relationship between the number of CPDs and UVB dose is almost linear and 4.4 CPDs...
are produced per million base pairs (Mbp) per J/m$^2$ of UVB radiation. The treatment of irradiated plasmids with T4 Endonuclease V or other endonucleases increases the sensitivity of damage detection by more than 500 times. We conclude that with the assistance of T4 Endonuclease V, AFM imaging can even capture single base modification in single DNA molecules.

![AFM images of pUC18 molecules](image)

**Figure 2.** AFM images of pUC18 molecules irradiated with different doses of UVB radiation and incubated with T4 Endonuclease V. (A) 1.4 kJ/m$^2$ (B) 229 J/m$^2$ (C) 29 J/m$^2$. All scan sizes are 1×1 µm$^2$. (D-F) Histograms of the occurrence of various configurations of pUC18 plasmids determined from AFM images such as these shown in (A-C). Color code and error bar: same as in Figure 1. (Adapt and modified from reference [27], with permission)

### 3.3 In situ Detect DNA damage by AFM imaging

One of the most unmatched advantages of AFM is that it can image biomolecules in solution under near in vivo conditions [12, 13, 31, 42-47] and can capture dynamic processes that cannot be visualized by electronic microscope. We decided to test whether it is possible to capture the dynamic relaxation process of UV-damaged supercoiled plasmids by AFM after treating them with T4 Endonucleases V. Although various movements of DNA molecules on surfaces were previously captured by AFM [12, 31, 47-49], it was not clear whether the relaxation process that requires some parts of the DNA to untwist freely can occur on a surface, and if yes, whether we can visualize it in an AFM.

In Figure 3 we show a series of real-time AFM images (A-F) acquired in buffer and it is clear that we can capture the dynamic relaxation process of UV-damaged pUC18 plasmids in the present of T4 Endonucleases V. In contrast, UV-irradiated but untreated by T4 Endonucleases V plasmids remained supercoiled even after a prolonged scanning by AFM tip under solution (Data not shown). This observation suggests that it will be possible to examine with AFM various mechanistic aspects of the action of T4 Endonucleases V and possibly other DNA enzymes participating in the DNA damage and repair.
Figure 3. (A). Real-time AFM imaging in solution captures the relaxation of UV-damaged pUC18 plasmids by T4 Endonuclease V. pUC18 plasmids were irradiated at 1.4 KJ/m² of UVB and mixed on ice with T4 Endonuclease V. The sample was transferred to the AFM instrument and imaged immediately in solution, at room temperature. The images (A-F) were captured at 27, 32, 41, 59, 63, and 68 minutes after the AFM tip had been engaged and the first image had been acquired.

3.4 Sensitivity, accuracy and advantages of AFM assay

Based on this supercoiled plasmid relaxation assay, we can detect single lesion (SSB, DSB or base modifications) per DNA molecule. Since one SSB is able to relax a whole supercoiled plasmid, increasing the size of the plasmid should also increase the sensitivity of the damage detection. To test this hypothesis we exposed pNEBR-R1 supercoiled plasmids (10,338 bp) with 29 J/m² of UVB radiation and treated them with T4 Endonuclease V. After imaging these plasmids in the AFM, we find that 55±3% of pNEBR-R1 plasmids are in the relaxed circular form (at least one pyrimidine dimer) and 14±3%, are already in the linear form (at least 2 damage sites in close proximity on the opposite strands). From the percentage of supercoiled molecules, we estimate that pNEBR-R1 plasmids developed at least 1.12±0.035 CPD/plasmid (λ=1.12), i.e. 108±13 CPD/Mbp. Thus, increasing the size of the supercoiled plasmid by 3.8-fold resulted in a >2.8-fold (2.8=1.12/0.40) increase in damage detection sensitivity, which is consistent with our predictions.

Table 2 Comparison of DNA detection sensitivity by gel electrophoresis and by AFM

<table>
<thead>
<tr>
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<th>Gel Electrophoresis</th>
<th>AFM</th>
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<tbody>
<tr>
<td>Minimum amount of DNA</td>
<td>~ 400 pg per lane [50]</td>
<td>~ 1 pg (total)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>~ 25 pg per band [50], (equivalent to 8.5×106 pUC18 molecules)</td>
<td>Single molecules</td>
</tr>
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</table>

To test the accuracy of the AFM assay, we compared our results with those obtained by well-established methods such as gel electrophoresis assay. The quantitative results by AFM imaging and gel electrophoresis on same sample are consistent, which indicates that AFM is an accurate tool to quantify DNA damages. It is also interesting to compare our results with the results obtained by traditional methods by other research group. We can estimate the number of CPDs/Mbp obtained by other researchers by extrapolating their results to our dose of 229 J/m² of UVB. For example, Setlow and Carrier [51], who extracted radio-labeled DNA from H. influenzae and irradiated it at 280 nm and 4 kJ/m², estimated the number of CPDs/100bp to be 2.06. Assuming that the number of CPDs scales linearly also between 229
J/m² and 4000 J/m², we find that the Setlow and Carrier’s data correspond to 1180 CPDs/Mbp at 229 J/m², which is similar to our own result (~1000 CPDs/Mbp).

Over the last ten years or so AFM has been making steady progress in demonstrating its unique advantages in nanoscale biology research. In our opinion the two unique features of AFM that make it particularly suitable for examining DNA damage and repair are: a) the ability to examine individual DNA molecules and DNA protein complexes under nearly in vivo conditions, b) extremely small amounts of DNA and protein material needed for the observation. To test this second feature we coated 0.1 µl of a pUC18 plasmid solution with the DNA concentration of 10 pg/µl on the mica surface and imaged the surface at a big scan size of 5×5 µm². We can still collect enough DNA molecules for DNA damage quantification. Thus, our measurements can be performed on the amount of total DNA of 1pg, which is ~400 times less than what is presently needed for the most sensitive gel electrophoresis assays [50] and what amounts to ~1/5th of the DNA in a single cell. It may be advantageous to combine the power of gel electrophoresis with AFM imaging together in some studies under extreme conditions. Gel electrophoresis would separate the damaged DNA into discrete bands and the DNA extracted from a particular band would provide enough material to be further examined by high resolution AFM imaging.

3.5 Detect DNA damage by AFM imaging using repair enzyme or antibodies binding assay

![AFM images showing photolyases binding to the CPD sites of pUC18 molecules](image)

Figure 4. AFM images show photolyases binding to the CPD sites of pUC18 molecules (A) with 229 J/m² UVB radiation (B) no UV as control. (C) and (D) Histograms show the distribution of photolyases on each pUC18 molecules as shown in (A) and (B). (Adapted from reference [27], with permission)

So far we have demonstrated DNA damage detection using the relaxation assay of supercoiled DNA with and without the assistance of DNA endonuclease. In an alternative approach, we can exploit the ability of DNA repair enzymes such as photolyase or DNA antibodies such as anti-CPP to recognize DNA lesions. For example, photolyase is a 54 kDa enzyme specialized in DNA photo reactivation [52], which can be directly visualized by AFM imaging [53]. In Figure 4A, we show an AFM image obtained on pUC18 plasmids irradiated with UVB (229 J/m²) and incubated with photolyase, and in Figure 4B we show the control image of untreated DNA after the incubation with photolyase. We can detect photolyases accurately as the bright dots on DNA with an elevated height in Figures 4A and 4B. Figure 4C and 4D show that on average 2.5 photolyases are located on the irradiated pUC18 molecule and 0.7 on the untreated DNA, which is nonspecific binding. Thus, using the photolyase binding assay, and after subtracting the number of nonspecifically-bound enzymes, we estimate 1.8 photolyase-sensitive sites per plasmid at 229 J/m². The number of photolyase-sensitive sites is somewhat lower than the number of T4 Endonuclease V sensitive sites (2.7 CPDs/plasmid). The difference probably reflects a dynamic equilibrium between the bound and unbound photolyase in solution, which
leaves some CPD sites on DNA unoccupied. In addition, while T4 Endonuclease V makes an incision at all CPD sites, photolyase that we used detects a subset of all CPDs, namely the cis-syn CPD [2].

4. Detect DNA damage by AFM-based SMFS

Besides AFM imaging, SMFS can also be used to detect DNA damage. Single DNA molecule was picked up by the AFM tip and stretched vertically in solution to determine it force-extension relationship. Figure 5 shows the result of six independent SMFS measurements on λ phage DNA after different doses of UV radiation (from 0 to 52.5 kJ/m²). The red trace in Figure 5 shows the typical force-extension curve of intact double stranded DNA (no UV radiation). The elastic properties of DNA have two unique features [34, 54, 55]. At a critical force of about 60-90 pN, a long plateau amounting to 70% of the original contour length appears in the force-extension relationship; a second plateau appears at higher forces of 150-450 pN; relaxing traces reveal patterns which are characterized by different degree of hysteresis.

The force spectrograms of UV-treated individual DNA molecules in Figure 5 show significant differences in the elasticity as compared to the untreated DNA (red line). The B-S transition was shortened by UV treatment below 70% of the original contour length measured for the untreated DNA. Moreover, the shortening of the plateau is dose-dependent. Interestingly, the second transition is also decreased in the dose-dependent manner. The shortening of the B-S transition originates from the extensive formation of CPDs and 6-4 lesions, which results in a partial loss of the double helical structure along the DNA backbone. cis-syn CPDs are known to induce bending of 20-30° and unwinding of regular B-DNA by 10° [56, 57] and 6-4 lesions cause much more severe bends or kinks of 44° [58]. These bending and unwinding caused by DNA damages weakened the base stacking and base pairing structure and DNA double helix was partially melted [59]. This effect could be responsible for the observed shortening of the overstretching transition in the UV-irradiated DNA. In addition, the normalized initial contour lengths before the onset of the B-S transition increase in a dose dependent manner, which supports our hypothesis of UV-induced unwinding of the double helix. Similarly, the same effects could also cause a shortening of the width of the second transition. The B-S transition of UV irradiated DNA at the dose of 10.5 kJ/m² (green line with square trace) is ~ 9% shorter compared to that of intact DNA. Douki et al. reported that the number of CPD is 30/10,000 bases at the dose of 10.5 kJ/m² [60]. It means ~0.3 % of all nucleotides form CPDs. As exposure time is increased, B-S transition length shortens, suggesting an increase in the number of pyrimidine dimers. Strikingly, the shortening of the width of the B-S transition saturates at the dose of ~40 kJ/m² (purple line with circle trace) which is exactly the same dose at which Douki et al. observed the saturation of
the number of UV-induced lesions. This coincidence proves that the shortening of the B-S transition is indeed caused by the formation of CPD lesions.

We established the relationships between UV damage and the mechanics of DNA double helices at a single-molecule level. The width of the B-S transition shortens in a dose-dependent manner below the standard 70% of the contour length and becomes less cooperative. The results show that SMFS, which provided many insights into the mechanics of intact DNA, can also be used to study DNA lesions. This research is the first direct measurement that determines the relationship between DNA nanomechanics and damage, and lays a foundation for the future use of SMFS in DNA damage and repair.

5. Summary

AFM, as one of the leading techniques in single molecule or nanoscale science, can be used to detect DNA damage quantitatively and accurately. Different from the traditional techniques, this damage detection method based on AFM imaging is performed at a single-molecule level with higher sensitivity and consumes less DNA materials than bulk methods. Besides, SMFS can also be used to investigate DNA damages by measuring the DNA nanomechanic change caused by damage, which provide unique method in DNA diagnostics.

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