Separating DNA with Different Topologies by Atomic Force Microscopy in Comparison with Gel Electrophoresis

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Atomic force microscopy, which is normally used for DNA imaging to gain qualitative results, can also be used for quantitative DNA research, at a single-molecular level. Here, we evaluate the performance of AFM imaging specifically for quantifying supercoiled and relaxed plasmid DNA fractions within a mixture, and compare the results with the bulk material analysis method, gel electrophoresis. The advantages and shortcomings of both methods are discussed in detail. Gel electrophoresis is a quick and well-established quantification method. However, it requires a large amount of DNA, and needs to be carefully calibrated for even slightly different experimental conditions for accurate quantification. AFM imaging is accurate, in that single DNA molecules in different conformations can be seen and counted. When used carefully with necessary correction, both methods provide consistent results. Thus, AFM imaging can be used for DNA quantification, as an alternative to gel electrophoresis.

Introduction

Many methods and techniques have been developed to study DNA quantitatively in a bulk material or single molecule level.[1−4] Among them, gel electrophoresis is one of the well-established bulk analysis methods, which is often used for DNA study. This simple, fast, and accurate analytical technique can be used for DNA preparation, separation, and quantification by its size or geometry. It is frequently used in DNA damage studies because it allows separation of intact and damaged plasmids.[5,6] Gel electrophoresis can be used to analyze DNA materials from tens of nanograms to micrograms.

Atomic force microscopy (AFM) has been utilized for single molecule manipulation and imaging of individual DNA since 1989.[7−16] It is one of the leading techniques in single molecule or nanoscale science because individual DNA molecules can be visualized under physiological conditions in real time.[9,17−20] However, AFM studies of DNA were mostly focused on the biological structure and dynamic processes of DNA.[21−26] This fairly new and very promising type of microscopy has so far found a relatively limited application for DNA quantification.[27−29] For example, Pang et al. quantified strand breaks in supercoiled DNA plasmids induced by high doses of ionizing radiation.[28,29] Murakami et al. analyzed the radiation damage of supercoiled plasmid DNA by both AFM imaging and gel electrophoresis methods.[27] However, the results obtained by both methods were inconsistent: the fraction of open circular form estimated by AFM was smaller than the one obtained by gel electrophoretic analysis.

Recently, we utilized AFM imaging of plasmids to quantify DNA damage caused by UV radiation.[30,31] We compared our AFM results with the results obtained by gel electrophoresis, and found that both methods are consistent, but only if the gel results were carefully corrected to include the differences between fluorescence efficiencies of different topological forms of DNA plasmids.[30,31] Surprisingly, we found that small differences in the composition of the buffers used in various enzymatic assays for damage detection, such as the presence or absence of small amounts of Mg^{2+}, significantly affected DNA bands' intensities and individual correction factors had to be determined for various buffers. A failure to correct for these variations would lead one to erroneous conclusions as to the amount and types of DNA damage.[30] To the best of our knowledge a careful comparison of effects of different buffers on separation of DNA by gel electrophoresis has not been investigated.

Here we evaluate in detail the performance of AFM imaging to quantify plasmid DNA with different topologies in a single molecule level. As a method, we compare the quantitative results from AFM imaging with those obtained by gel electrophoresis on bulk DNA materials. The advantages and shortcomings of both techniques for DNA separation and quantification are discussed in detail. We also analyze the possible sources of errors and artifacts for both methods. The results show that AFM imaging is a viable alternative to gel electrophoresis as a DNA quantification method.

Experimental Methods

Materials. pUC18 plasmid, which has 2686 base pairs, was used as the DNA substrate. It was extracted from *E. coli* cells and purified by using the QIAfilter plasmid maxi kit from Qiagen Inc. The intact pUC18 DNA is in a supercoiled form. Linear pUC18 was obtained by *Eco*RI digestion, following the provided protocol. Because pUC18 contains a single *Eco*RI site, the digestion with this enzyme produces a uniform population of linear molecules of

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the same size. T4 endonuclease V and *E. coli* endonuclease IV were purchased from New England Biolabs.

Gel Electrophoresis. DNA solution was separated on 1% agarose gel matrix in TBE buffer (89 mM Tris borate, 2 mM EDTA, pH 8.3), with ethidium bromide concentration of 10 μg/mL. For DNA quantification, gel matrixes were imaged by using an 8-bit charge-coupled device (CCD) camera (Gel Doc EQ system, Bio-Rad Laboratories, Inc.). DNA was quantified with use of Quantity One software (Bio-Rad Laboratories, Inc.). For further AFM imaging, DNA molecules in each gel band were extracted and purified back into solution with a QIAquick gel extraction kit (Qiagen Inc.) with the provided protocol.

AFM Imaging. A drop of 5−50 μL of DNA solution with concentration of 0.5−1 μg/mL was deposited on an 1-(3-aminopropyl)silatrane-functionalyzed mica (APS-mica)[32] surface at room temperature for 1−3 min. The sample was rinsed with DI water and air-dried before AFM imaging. Images were taken with a Nanoscope IIIa MultiMode scanning probe microscope (Veeco Instruments Inc., Santa Barbara, CA), using Tapping mode, with an E scanner. RTESP probes (Veeco) were used with the spring constant of 20−80 N/m and the resonance frequency of 275−316 kHz. All images were collected at a scan rate of 2.0−3.0 Hz, a scan resolution of 512 × 512 pixels, and scan size of 1000 to 2000 nm. The raw images were only modified by Veeco AFM software before use. For each quantification experiment, 27−36 AFM images on 3−5 mica samples were captured and 500−1000 DNA molecules were counted to determine the fractions of supercoiled, circular, and linear molecules. The results are expressed as the mean ± standard deviation for each fraction. To remove bias and verify the accuracy of damage quantification by the AFM imaging method, numerous *t* and blind tests were performed in which the person who analyzed the AFM images did not know which sample they originated from.

Results and Discussion

Plasmid DNA is broadly used as a model DNA in DNA damage studies because it has three different conformations: supercoiled, relaxed, and linear forms depending on structural modification such as single strand breaks (SSB) and double strand breaks (DSB).[31,33,34] The numbers of SSB and DSB, which represent the level of DNA damage, can be deduced by the ratio of different conformations.[35,36] However, these three forms have the same sequence, i.e., molecular weight, which makes it difficult to separate them by normal methods. Gel electrophoresis is considered one of the best methods to separate these plasmids because different topological structures have different radii of gyration and electrophoretic mobilities, allowing them to separate by running on a gel. Recent research, however, showed that these conformations can also be identified easily by using AFM imaging,[23,24,27−29,32,37,38] and the ratio of different forms can be counted directly at a single molecule level.[31,39]

Identify Supercoiled and Linear Plasmids by Gel Electrophoresis and AFM: Calibration. Before comparing the accuracy of AFM imaging with gel electrophoresis, we need to calibrate both methods by working on a mixture with known composition. Here we use a mixture of supercoiled and linear pUC18 with a ratio of 1:1 as the reference sample.

First, we deposited a small amount of the DNA mixture on APS-mica (2.5−50 ng) for AFM imaging. From the image in Figure 1A, we can identify both supercoiled and linear forms clearly. Supercoiled DNA (labeled by letter "S" in Figure 1A) has a plectonemic configuration, while linear DNA (labeled by letter "L") clearly has two ends. By counting the numbers of DNA in different forms in this image and more similar images, we obtain the actual ratio of supercoiled and linear DNA on the mica surface. The result is summarized in Table 1. Supercoiled and linear DNA percentages were 50.1% and 48.1%, respectively, a ratio of linear/supercoiled DNA of 0.96 (the true vaule is 1 here). This means the error caused by AFM imaging is quite small, with no calibration necessary, and it

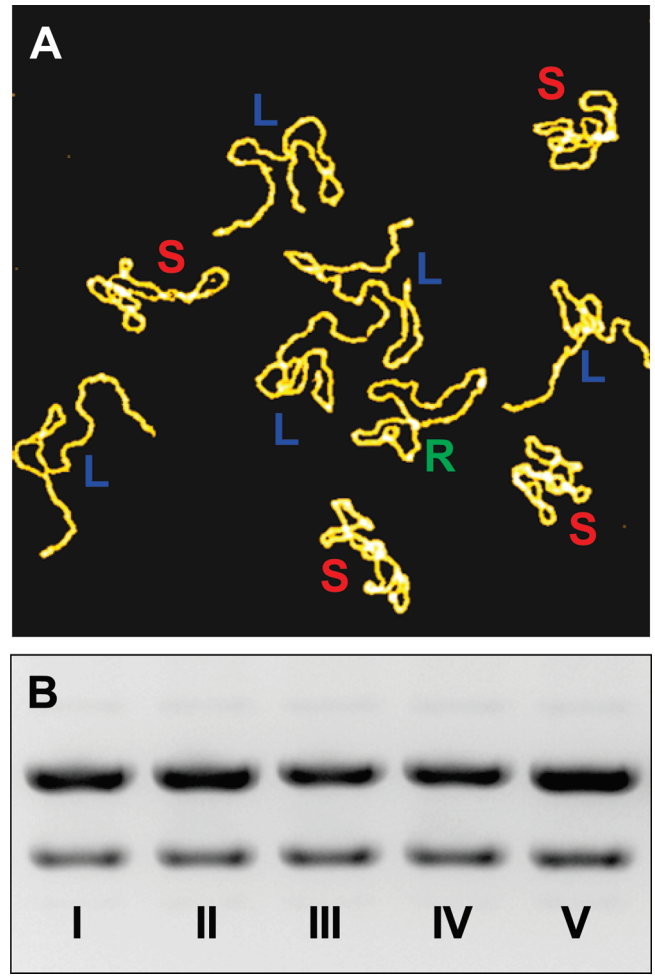

Figure 1. (A) An AFM image showing the mixture of supercoiled and linear pUC18 DNA with the same weight ratio. Scan size is 1 × 1 μm^2 and color codes are the same as given in Figure 2. (B) A photograph of the agarose gel showing the separated supercoiled and linear pUC18 bands on the same sample as shown in part A, with the same weight ratio. The gel was run with (I) *E. coli* Endonuclease IV reaction buffer, (II) *E. coli* Endonuclease IV enzyme and its reaction buffer, (III) *E. coli* Endonuclease V reaction buffer, (IV) *E. coli* Endonuclease V enzyme and its reaction buffer, (V) 10 mM Tris−HCl, 1 mM EDTA, and 100 mM NaCl buffer. The top bands in all lanes contain linear pUC18 while the bottom bands represent supercoiled DNA.

TABLE 1: Correction Factor Obtained from AFM and Gel Electrophoresis Methods Shown in Figure 1B

		GEL		
band intensity	AFM	Tris, EDTA, and NaCl	endo-IV enzyme and its buffer	endo-V enzyme and its buffer
supercoiled %	50.1	32.6	32.2	38.4
relaxed %	1.8	x[a]	x[a]	x[a]
linear %	48.1	67.4	67.8	61.6
correction factor	0.96	2.07	2.10	1.60

[a] x indicates undetectable.

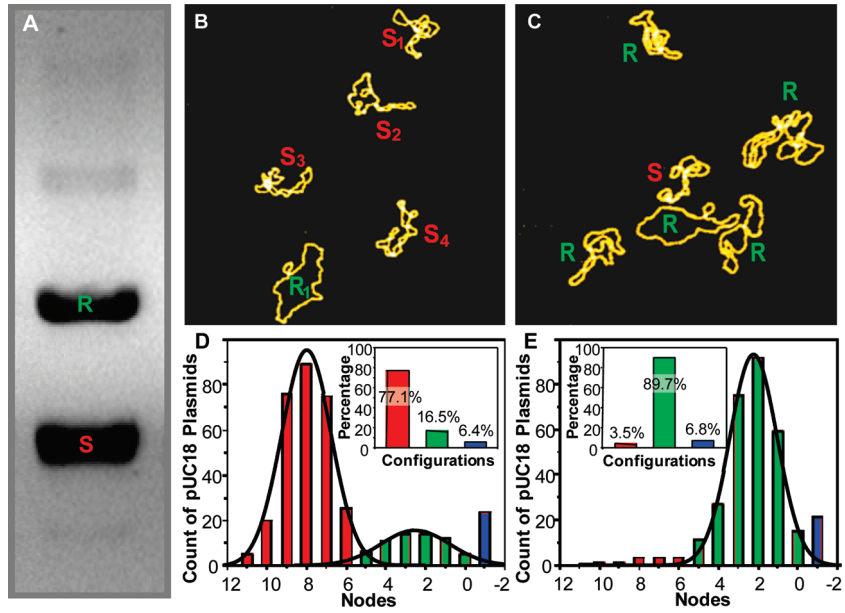

Figure 2. (A) A photograph of the agarose gel showing separated supercoiled and relaxed pUC18 bands after supercoiled pUC18 DNA was subjected to 1.5 MJ/m^2 UVA radiation and T4 Endonuclease V treatment. (B and C) AFM images of pUC18 DNAs that were extracted from agarose gel, (B) supercoiled band, and (C) relaxed band, as shown in part A. Scan size in both images is 1 × 1 μm^2. (D and E) Histograms showing the distributions of nodes of pUC18 plasmids counted from the AFM images such as those shown in parts B and C. The curves in the histogram show the Gaussian distribution fits. Inset figures show the histograms of the percentages of various conformations of pUC18 plasmids determined from the 6 nodes criterion. Color code: red, supercoiled DNA (S); green, relaxed circular plasmids (R); blue, linear DNA (L).

also means the topological structures cannot be affected by DNA binding to mica during the sample preparation process and AFM scanning process by sharp tips.

Next, we performed the gel electrophoresis assay on the same DNA mixtures as used for AFM imaging. The relative amounts of DNA in various bands were quantified by measuring the intensities of each band in the gel photo determined by a CCD camera. The results in Figure 1B and Table 1 indicate a nonlinear relationship between the relative amounts and the band intensities of DNA in supercoiled versus linear forms. Supercoiled DNA is less fluorescent compared to circular and linear forms.[40,41] Therefore, quantification of supercoiled DNA by band intensity is slightly more complicated, compared to that of relaxed or linear forms.

A fairly large correcting factor has to be determined when quantifying the relative amounts of DNA in supercoiled and relaxed or linear forms based on the band intensities. This correction factor is multiplied with the supercoiled band intensity on the gel of the reference sample. Importantly, this correction factor was found to be dependent on many experimental conditions, including plasmid type, dye concentration, and agarose and buffer type and concentration.[30,40] Table 1 lists the different correction factors for different conditions. Therefore, the correction factor needs to be determined for each specific gel experiment before a proper quantification of supercoiled versus linear plasmids can be completed.

The Errors Caused by Both AFM and Gel Electrophoresis Methods. To further test whether AFM imaging allows accurate quantitative determination of various topological fractions of DNA, we use the exact same DNA sample extracted from an agarose gel for AFM imaging. We chose a mixture of supercoiled and relaxed pUC18 plasmids as model DNA. The mixture was obtained by irradiating supercoiled pUC18 with ~1.5 MJ/m^2 UVA to produce cyclobutane pyrimidine dimers (CPDs), and then incubating with T4 endonuclease V enzyme to incise DNA at the CPD sites, forming SSB.[30,31] As a result of these treatments, the plasmids which had CPDs switched over to a relaxed circular form while intact molecules remained supercoiled.

Next, the mixture was loaded on an agarose gel. After gel electrophoresis, two bands which contain supercoiled and relaxed DNA were separated as shown in Figure 2A. Supercoiled DNA is still the same as that shown in Figure 1A, while relaxed DNA (labeled by letter "R") looks like an open circle. As the result of supercoiling, the radius of gyration of DNA in the supercoiled band is smaller than those in the relaxed band, and DNA mobility is greater than that of the relaxed circular DNA. The DNA molecules in the supercoiled and relaxed DNA gel bands were extracted and purified back into solution individually for further AFM imaging. Parts B and C of Figure 2 show the AFM images of those DNA obtained from supercoiled and relaxed DNA bands, respectively. Comparing parts B and C of Figure 2, it is clear that the conformations of DNA molecules are quite different. The DNA molecules obtained from the supercoiled band have significantly more nodes (the number of visible crossover points in AFM images) than those from the relaxed band. For example, the DNAs marked with letter "S4" and "R1" in Figure 2B have 8 and 1 nodes, respectively.

Further, we counted the nodes of each DNA molecule and drew their distribution histogram as shown in parts D and E of Figure 2. As observed from this quantitative study, supercoiled and relaxed DNAs have clearly different node distributions. Most of the plasmid molecules in the supercoiled band have 6−11 nodes, while those in the relaxed band only have 0−5 nodes.[31] Thus, supercoiled and relaxed DNA molecules can be identified simply by counting their nodes from AFM images. We fitted their node count histograms with Gaussian distributions and found 8.0 ± 13 nodes (mean ± standard deviation) for DNA in the supercoiled band and 2.2 ± 1.2 nodes for DNA in the relaxed band. On the basis of these Gaussian distributions, we confirm the previous theoretical node distribution for pure supercoiled and relaxed DNA, where DNA molecules with 6 or more nodes can be considered supercoiled, and those with less than 6 nodes can be considered to be relaxed.[31]

According to this criterion, DNA molecules were identified as either supercoiled or relaxed plasmids, which were marked

by letter "S" as supercoiled and "R" as relaxed DNA in Figure 2B,C. The distribution of percentages of various conformations of pUC18 plasmids can also be found by counting the numbers of DNA molecules in different conformations, as shown as insets in parts D and E of Figure 2.

However, from the AFM results shown in Figure 2, we found some inconsistencies between AFM imaging and gel electrophoresis results. Only 77.1% of DNA molecules extracted from the supercoiled band in the gel were actually supercoiled plasmids as shown in Figure 2B,D. There are some relaxed (16.5%) and even linear (6.4%) molecules among the DNA extracted from the supercoiled band. These discrepancies may be caused by supercoiled DNA being damaged by the later extraction and purification processes, and as a result turning into relaxed or linear forms.

More significantly, from the AFM imaging results shown in Figure 2C,E, we can identify small amounts (3.5%) of supercoiled molecules in our AFM images of the DNA extracted from the relaxed band. This number most likely under represents the actual amount of supercoiled DNA in the relaxed band, since some DNA is possibly damaged and relaxed during purification. This finding is direct evidence that gel electrophoresis cannot separate DNA entirely. Some molecules will move to the "wrong" band, possibly due to molecular entanglement.

It should be noted that in this paper, all the DNAs (supercoiled, relaxed, and linear forms) have the same sequences, i.e., same length or molecular weight. If we deal with the DNA samples with different sizes, we should be aware that smaller DNA molecules have a smaller diffusion coefficient than larger molecules, and as the result of this smaller molecules will bind to the surface faster (or in higher numbers) than larger molecules. In this case, we need to take this factor into consideration.

Conclusions

In conclusion, we evaluated AFM imaging and gel electrophoresis as methods in the application of supercoiled and relaxed DNA quantification. Both methods have their merits and drawbacks. Gel electrophoresis is fast and well established. However, calibration needs to be done for every different experimental condition. AFM imaging does not require calibration, and requires less DNA, but is a slower process. The results obtained from both AFM imaging and gel electrophoresis methods are consistent and the average deviation between the two methods is only 3.3% if they are used correctly, with gels being properly calibrated for each different experimental condition. Atomic force microscopy is proven to be an accurate method in the identification and quantification of the plasmid with different topological structures, since these measurements are based on single-molecular observations. Thus, atomic force microscopy is a viable alternative to gel electrophoresis in the quantification of different DNA structures.

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