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Effect of Torsion on Cisplatin-Induced DNA Condensation *

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We investigate the effect of torsion on DNA condensation with the covalently closed circular DNA as the torsion-constrained system, using an atomic force microscope. It is found that there are two stages in the DNA condensation process under torsional constraint. At the early stage, the low torsion will accelerate DNA condensation by promoting the formation of micro-loops or intersection structures; while at the later stage, the increasing torsion will slow it down by preventing the crosslinking of cisplatin and DNA since the DNA molecule becomes more rigid. Our results show the important role of torsion in DNA condensation and sheds new light on the mechanism for DNA condensation.

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Torsion is actively maintained in eukaryotic and prokaryotic chromosomes, and plays key roles in biological functions of DNA. In eukaryotic cells, chromosomes are organized by nucleosomes with eight histone proteins wrapped by 147 bp DNA in a left-handed direction, which introduces negative supercoils into the DNA.[1] In bacteria, DNA is compacted into negative supercoils by polyamines and proteins.[2] In these chromosomes, torsion generally exists. Moreover, the torsion in DNA also affects the activity of nearby genes and even induces structural transitions in DNA duplexes.[3,4] For instance, torsion is considered as a determinant for the initiation of DNA replication, as well as facilitating supercoil formation that contributes to the juxtaposition of distant DNA sites and chromosome condensation.[5–8] In previous studies, however, the effect of torsion remains unclear, especially on DNA condensation. Here we study the effect of torsion on cisplatin-induced DNA condensation.

Cisplatin is one of the most widely used clinical medicines in chemotherapy.[9,10] Its anticancer function is attributed to the formation of crosslinks between cisplatin and DNA molecules. These cisplatin-DNA adducts are able to influence the fundamental biological functions of cells that are related to DNA molecules, and finally result in the cell death.[10–12] Under physiological conditions, cisplatin that is activated by hydration can bind to the N7 atoms of guanine or adenine on DNA molecules, leading to the formation of mono-adduct or di-adduct.[13–15] The cisplatin-DNA di-adducts that are mainly from intrastrand and interstrand crosslinks can bend the DNA and induce local unwinding around the binding sites. As a result, the torsion of DNA molecules near di-adducts will be changed.[16]

Recently, single-molecule techniques including atomic force microscopy (AFM), magnetic tweezers and optical tweezers have been exploited to investigate DNA-protein or DNA-drug interactions.[17–20] Here with the aid of AFM, we directly observe the configuration transition of circular DNA molecules induced by cisplatin. Torsion-constrained circular DNA is from plasmid pBR322 vector, and torsion-unconstrained circular DNA is produced by nicking the pBR322 vector. We find that circular DNA molecules will be gradually compacted into globule state from the originally polymer state with time. Quantitative analyses from AFM images show that there are two stages in the DNA condensation process under torsional constraint.

Plasmid pBR322 from New England Biolabs is a double-stranded covalently closed circular DNA (cccDNA), 4361 base pairs in length. Nicked pBR322 is an open circular DNA (ocDNA), which is obtained from plasmid pBR322 through nicking reaction using the nicking endonuclease Nt.BsmAI. The cccDNA is regarded as a torsion-constrained system because the torsion of the DNA double helix structure cannot be released. The ocDNA is usually regarded as a torsion-unconstrained system because the torsion of DNA molecules can be released by the single-strand nicks on the DNA duplex. According to the recognition sites of Nt.BsmAI and the sequence of pBR322

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molecule, the nicking map of pBR322 vector is shown in Fig. 1(a). There are three nicks in this DNA system, which are able to release the torsion energy of ocDNA molecules.

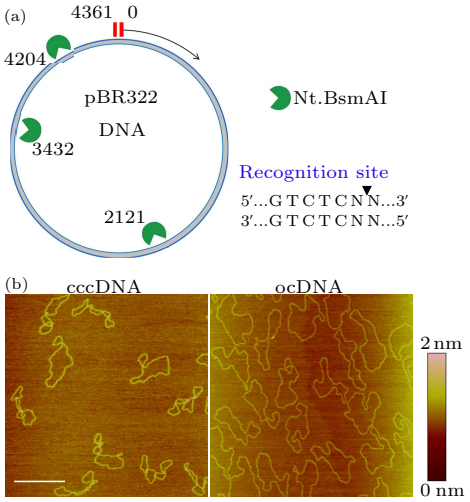

Fig. 1. (a) Recognition sites and nicking map of Nt.BsmAI on pBR322 vector. (b) AFM images of cccDNA (left) and ocDNA (right) on mica surfaces. Scale bar is 500 nm. Height range is 2 nm for all AFM images.

Cisplatin is from Sigma-Aldrich and dissolved in 10 mM $NaClO_4$ at 60℃ in the dark for 10 min before use. Nt.BsmAI is from New England Biolabs, which can cleave only one strand of the DNA. In the nicking reaction, 5 μg plasmid pBR322 vector and 50 units Nt.BsmAI are incubated together in NEBuffer 1.1 at 37℃ for 20 min. After purifying with QIAquick PCR purification Kit (Qiagen), nicked pBR322 with three nicks are obtained. Mg_2Cl and other chemicals are also from Sigma-Aldrich.

In our AFM experiments, cccDNA or ocDNA at a concentration of 5 ng/μl was incubated with 33.3 μM cisplatin in 10 mM $NaClO_4$ buffer at 37℃ in the dark. After incubating for 2, 4, 6, 10 and 14 h, 2 μl of the sample was taken out and added by 1 μl of 100 mM $MgCl_2$ and 17 μl Tris-HCl (pH 7.4). Then 10 μl of the solutions was deposited onto a freshly cleaved mica. After 15 min, the mica surface was washed with 200 μl Milli-Q filtered water three times and blown dry by a gentle stream of nitrogen gas. The imaging was performed in air using a multi-mode AFM with nanoscope Ⅲa controller (Digital Instruments, USA) in tapping-mode. Silicon probe RTESP14 from Veeco (America) and E scanner were employed. DNA tracing and other measurements were performed automatically using the custom codes in Matlab.

To study the effect of torsion on DNA condensation induced by cisplatin, we firstly make observation of the original configurations of torsion-constrained cccDNA and torsion-unconstrained ocDNA under AFM. As the DNA molecules are deposited on freshly cleaved mica surfaces using magnesium ion, the absorbability is weak and DNA molecules are equilibrated on mica surfaces.[21] As shown in Fig. 1(b), the configurations of cccDNA and ocDNA are clearly observed by AFM. For cccDNA, intersection configurations are present on each DNA molecule, consistent with the negative supercoiling of wild plasmid pBR322.[22] In comparison with the cccDNA system, most ocDNA molecules show circle configurations with no intersections, indicating that the torsion of DNA vectors is released by the nicks. These results demonstrate that the cccDNA and ocDNA molecules can be used as torsion-constrained and torsion-unconstrained systems, respectively.

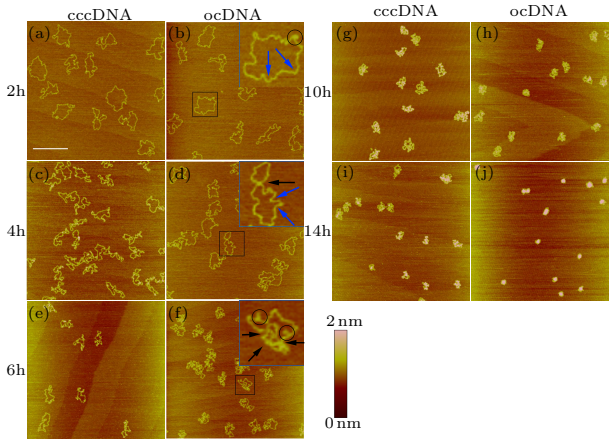

Fig. 2. AFM images of torsion-constrained cccDNA (a, c, e, g, i) and torsion-unconstrained ocDNA (b, d, f, h, j) incubated with 33.3 μmol/L cisplatin at different times. Three kinds of DNA structures are defined by their configurations. Kinks are marked by blue arrows; micro-loops by small black circles; and intersection structures by black arrows. Scale bar is 500 nm. Height range is 2 nm for all AFM images.

To induce DNA condensation, cccDNA and ocDNA are incubated with 33.3 μmol/L cisplatin at 37℃. The configurations of the two kinds of DNA systems are observed by AFM at different incubation times with cisplatin. As shown in Fig. 2, both cccDNA and ocDNA molecules are gradually condensed with time. The configuration changes of the two DNA systems can be obviously distinguished. At an incubation time of 2 h, two kinds of structures, kinks and micro-loops, are observed in the DNA molecules, which are marked by blue arrows and black rings, respectively, in Fig. 2(b). Kink is a type of defect which appears as a local rigid bending on DNA strand.[23] Previous studies have revealed that kinks are induced by formations of cisplatin-DNA di-adducts.[24] Kinks can destroy the double-helix structure of DNA, leading to the decrease of stiffness on local segments of DNA molecules.[25] Meanwhile, the micro-loop structure is defined as a small ring on local DNA segments. It appears as a tiny protuberance, of which the height is higher than the average diameter of DNA molecules on mica. It is suggested that micro-loop structures are formed by the binding of a monoadduct to a distant DNA base.[24]

At 4 and 6 h, in addition to the kink and micro-loop structures, intersection structures are observed (Figs. 2(c)–2(f)), as indicated by black arrows. An

intersection structure denotes the cross of two DNA segments, which is formed through crosslinking with longer distance than micro-loop. The more complex structures of DNA as well as the intersection structure indicate that more cisplatin-DNA adducts are formed with the increase of treatment time. At 10 h, the difference can be observed between the cccDNA and ocDNA systems as shown in Figs. 2(g) and 2(h). Both kinds of DNA molecules begin to form semi-globular structures, while the configuration of the ocDNA system is more compact. It indicates that the torsion-unconstrained DNA is easier to condense into globular structure than the torsion-constrained DNA. Finally, at 14 h, all torsion-unconstrained ocDNA molecules are condensed into compact globular structures (Fig. 2(j)); whereas over half of the torsion-constrained cccDNA molecules still remain in the semi-globular structure (Fig. 2(i)). These results on the configurational difference between torsion-constrained and torsion-unconstrained systems demonstrate that DNA condensation is obviously affected by torsion in DNA.

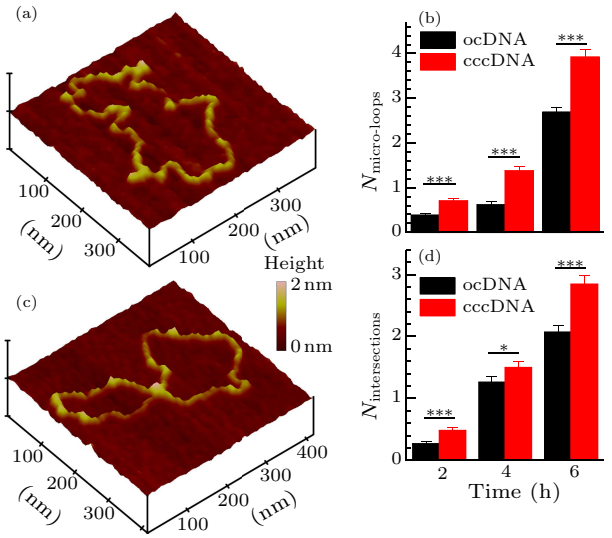

Fig. 3. An illustration of the definition of micro-loop (a) and intersection structure (c). Average number of micro-loops (b) and intersection structures (d) on a DNA molecule. Numbers of ocDNA: 114 (2 h), 121 (4 h), 175 (6 h); numbers of cccDNA: 117 (2 h), 172 (4 h), 102 (6 h). $^*p < 0.05$, $^{***}p < 0.001$ (two-sample t-Test). Error bars indicate the SEM.

To further quantify the configuration transitions affected by DNA torsion in the condensation, the numbers of micro-loops and intersection structures are analyzed. Typical three-dimension images of micro-loop and intersection structure observed by AFM are shown in Figs. 3(a) and 3(c), respectively. It is noticed that the heights of both micro-loop and intersection structures (∼1 nm) are twice that of DNA molecules (∼0.5 nm) on mica surfaces, in agreement with the previous results.[26,27] It is suggested that the two kinds of structures, micro-loop and intersection, are formed by overlapping or twining of the DNA double helixes. The average numbers of micro-loops and intersection structures in a DNA molecule increase with time (Figs. 3(b) and 3(d)). In addition, more micro-loops and intersection structures are formed in cccDNA, which indicates that the torsion in cccDNA molecules induced by cisplatin significantly promotes the formation of micro-loops and intersection structures from 2 to 6 h, in comparison with torsion-unconstrained ocDNA molecules with torsion released. For the naked DNA, the numbers of micro-loops in both torsion-constrained and unconstrained DNA are zero. We note that the average number of intersection structures in naked torsion-unconstrained ocDNA is nearly zero, whereas that in naked torsion-constrained cccDNA is 2.16 (Fig. 1(b)). The observed intersection structures in naked cccDNA originate from the negative supercoiling of wild plasmid pBR322.[22] As will be shown and discussed in the following, the number will immediately reduce to zero several minutes after incubation with cisplatin. Two-sample t-tests are used to analyze the difference between cccDNA and ocDNA, showing statistically the significant difference (p is less than 0.05).

It is generally believed that DNA molecules on the mica surface are under equilibrium state, thus the area defined by the contour of a circular DNA molecule could reflect the state of DNA condensation. Moreover, the radius of gyration is usually used to estimate the flexibility of circular DNA.[28] Therefore, we next analyze the areas and gyration radii of cccDNA and ocDNA to study the effect of torsion on DNA condensation (Figs. 4(a)–4(d)). The area and the gyration radius are calculated from the DNA contour traced by our custom algorithm, as shown in Fig. S1, which is described in the previous work.[28] The gyration radius of each single polymer is calculated by

$$R_{\text{g}}^2 = \frac{1}{N}\sum_{i=1}^{N}(\boldsymbol{R}_i - \boldsymbol{R}_{\text{mean}})^2, \tag{1}$$

where R_{g} is the radius of gyration, $\boldsymbol{R}_{\text{mean}}$ is the mean position of a single polymer, $\boldsymbol{R}_i$ is the position of each segment of a single polymer, and N is the segment number on a single polymer. Figures 4(b) and 4(d) show that both mean areas and gyration radii of DNA molecules decrease with the incubation time. Note that there are some differences between the torsion-constrained cccDNA and torsion-unconstrained ocDNA. At the early stage (before 6 h), the areas of cccDNA are smaller than those of ocDNA at each time point, indicating that cccDNA compacts more tightly than ocDNA. However, at the late stage (over 10 h), the areas of cccDNA molecules are larger than those of ocDNA, suggesting that the ocDNA molecules are in a more compact state, consistent with the images presented in Figs. 2(g)–2(j). The results for the gyration radius show a similar trend as the area. These results indicate that there are two different influences of torsion on DNA condensation.

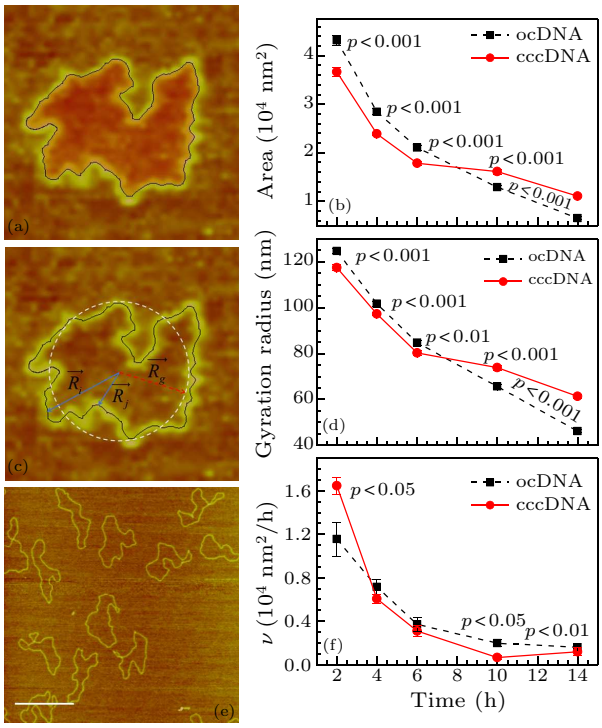

Fig. 4. Areas, gyration radii and average condensation rates of cccDNA and ocDNA plotted against the incubation time. (a) A sketch of an extracted DNA molecule configuration profile. (b) Average areas of cccDNA and ocDNA incubated with cisplatin. (c) A sketch showing the calculation of gyration radius. (d) Aerage gyration radii of cccDNA and ocDNA incubated with cisplatin. (e) AFM image of cccDNA incubated with 33.3 μmol/L cisplatin for 5 min. (f) Average DNA condensation rates of cccDNA and ocDNA incubated with cisplatin. Numbers of ocDNA: 114 (2 h), 121 (4 h), 175 (6 h), 123 (10 h), 109 (14 h); numbers of cccDNA: 117 (2 h), 172 (4 h), 102 (6 h), 108 (10 h), 100 (14 h). Error bars indicate the SEM.

Next, we compare the condensation rates between torsion-constrained cccDNA and unconstrained ocDNA. We notice that the untreated cccDNA has supercoils that are different from the untreated ocDNA (Fig. 1(b)). However, it should be noted that, at the initial experiment by cisplatin treatment (5 min), cccDNA quickly transform into circular configurations by cisplatin (Fig. 4(e)), similar to the untreated ocDNA (Fig. 1(b)). Therefore, the torsion-constrained and unconstrained DNA molecules have almost the same initial areas in the condensation process, and we could compare the condensation level from the DNA areas. The transition of cccDNA configuration by cisplatin can be explained by the topological theory: $L_k = W_r + T_w$, where the linking number (L_k) is the number of crosses that one single strand crosses over another, the twisting number (T_w) is the number of twists of the double helix, and the writhing number (W_r) is the superhelical twists. Here cccDNA is originally in the negatively supercoiled structure. The cisplatin-DNA crosslinks will bend and unwind DNA, reducing T_w of cccDNA. As a result, W_r increases from negative to zero, just as the circle-like structure of cccDNA shown in Fig. 4(e). We calculate the condensation rates from the decreasing rate of DNA areas. Figure 4(f) shows that the condensation rates for both DNA systems decrease with the incubation time with cisplatin. This is because the binding sites will be saturated with increasing formation of cisplatin-DNA crosslinks, making the DNA condensation slow down simultaneously. We notice that the condensation rate of cccDNA is significantly higher than that of ocDNA before 4 h, which is consistent with the comparisons of micro-loops, intersection structures and areas between these two types of DNA, as described above (Figs. 3 and 4). Our present result is also consistent with previous ones.[29] After 10 h of incubation, the condensation rate of cccDNA is lower than that of ocDNA, which is opposite to the early stage of condensation.

Based on the above configuration transition and quantitative results, we present a physics model for the diverse condensations by cisplatin, for the torsion-constrained and unconstrained DNA. At the early stage, for the torsion-constrained cccDNA molecules, their writhes increase in a few minutes due to the formation of cisplatin-DNA di-adducts. The writhes could contribute to the formation of plectonemic coils and promote the crosslinking of guanines or adenines over distance along the DNA, which facilitates the formation of micro-loops or intersection structures. These structures will then make the torsion-constrained DNA condense faster. In contrast, for torsion-unconstrained ocDNA molecules, the twist can be released by the nicks, thus writhes will not be introduced and less micro-loops and intersection structures will be formed. At the later stage, cisplatin continues to unwind and reduce the twist of DNA, leading to the increase of DNA torsion in local segments. For the torsion-constrained cccDNA system, the accumulation of torsion will gradually make the DNA more rigid, hindering the DNA condensation lastly.[30] In contrast, for the torsion-unconstrained system, the DNA is relatively flexible and may be condensed into a more compact state. Thus it is concluded that at the early stage, under the condition of lower cisplatin/DNA ratio, torsion of DNA will accelerate DNA condensation; at the later stage, after the formation of cisplatin-DNA di-adducts at saturated condition, the torsion of DNA will slow down the DNA condensation process.

In this study, the DNA condensation induced by cisplatin is a slow process taking hours. We also notice that multivalent cations and cationic proteins, such as spermine and spermidine, could promote fast DNA condensation in several minutes.[31–34] Condensed DNA molecules in coiled or flower patterns, and even globular or toroid structures are reported.[35–38] In our case, we also have observed globular structures (Fig. 2). The fast DNA condensation by cations and proteins is through the like-charge attraction between charge inversion and DNA, resulting in DNA compaction and condensation.[31] However, the slow cisplatin-induced DNA condensation is mainly through the covalent bonds with

DNA molecules, which is similar to the absorption-wrapping compaction.[33] Moreover, the DNA condensation by cations and proteins is reversible by force[39] or salt.[40] In contrast, cisplatin results in the deformation of double-helix structure and the change of DNA torsion,[41] which is irreversible.[24] Therefore, the cisplatin-DNA complex, especially intersection and micro-loop structures, can potentially block some proteins in DNA repairation and can induce cell death.[10]

In conclusion, we have built torsion-constrained and unconstrained DNA systems, and for the first time, we have studied the effect of torsion on cisplatin-induced DNA condensation by AFM. Three kinds of DNA configurations have been observed in ccc/ocDNA condensations under cisplatin treatment, including kinks, micro-loops and intersection structures. To quantify the effect of torsion on DNA condensation, we analyze the micro-structures of micro-loops and intersections, and the morphological parameters of contour areas, gyration radii and condensation rates. We discover two stages in the time course of the torsion-constrained DNA condensation. At the early stage, the condensation rate as well as the formation of micro-loops and intersection structures in torsion-constrained DNA molecules are higher than that of torsion-unconstrained DNA, revealing that moderate torsion could promote the condensation of DNA molecules by formation of distant crosslinking structures. At the later stage, condensation rate of torsion-constrained DNA is lower than that of torsion-unconstrained DNA, indicating that excessive DNA torsion could hinder the condensation. A model is also provided to explain the effect of torsion on DNA condensation. The degree of torsion may regulate some biological functions of DNA and proteins *in vivo* as well. For instance, DNA torsion could regulate the histone stabilization by stabilizing the outer turn of the nucleosome and repressing the transcription, and torsion could also promote the transcription elongation by destabilizing the nucleosome inner turn.[42] As torsion commonly exists in cellular DNA molecules, our results shed new light on the understanding of many physiological processes involving DNA condensation. More importantly, the cisplatin-induced DNA condensation under torsion is closer to the real physiological condition, which may help the design of new DNA-targeted drugs in the future.

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Supplementary Material: Effect of Torsion on Cisplatin-Induced DNA Condensation

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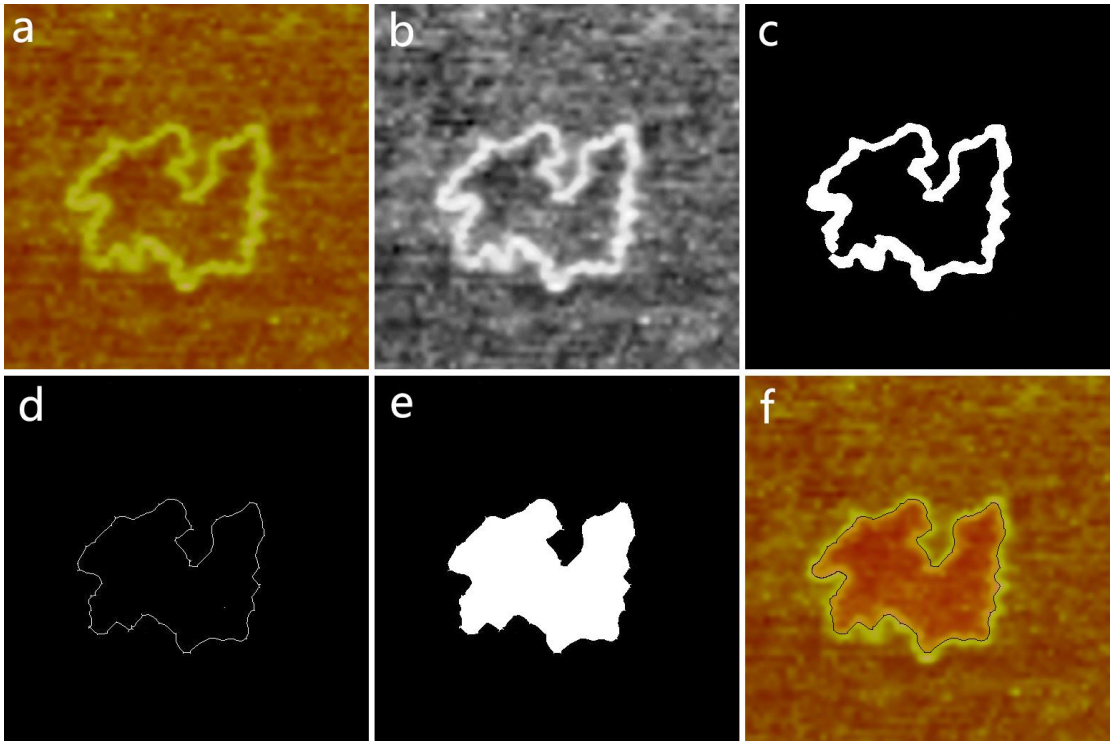

Fig. S1. (a) The original image was captured by AFM. (b) The grey image was obtained by the original image (a). (c) The backbone of DNA molecule was recognized depending on the distinction of grey value between DNA molecule and background. (d) The skeleton of DNA molecule was recognized as centre of DNA backbone in image (c). (e) The area surrounded by DNA skeleton was obtained and the pixels within the skeleton were marked by white value. (f) The quantity of pixels in the white area was recognized and calculated.