Visualization of Complexes of Hoechst 33258 and DNA Duplexes in Solution by Atomic Force Microscopy

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Tertiary structure changes in DNA duplexes, induced by Hoechst 33258 binding, have been examined by the use of atomic force microscopy. Besides minor groove binding, which is an established mode of binding for this drug, Hoechst 33258 has now been found to show another binding mode, which causes an unwinding of the duplex. When the drug concentration is as high as 0.5 μg/ml, the Hoechst 33258 molecule seems to function as a clamp for two DNA chains and forms a condensate. The condensate was found to have a toroidal shape. By surveying more than 100 microscopic images of such condensates formed in 1 μg/ml drug solution, a mechanism of toroidal condensate formation has been proposed.

Key words DNA, tertiary structure; Hoechst 33258; DNA duplex, unwinding; atomic force microscopy; DNA, toroidal condensate

Hoechst 33258 (2′-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1*H*-benzimidazole) is an interesting dye which binds to double stranded DNA. It has been useful not only as a fluorescent DNA stain, but also as a therapeutic drug having anti-cancer activity. As shown in Fig. 1, it is a thin, elongated cation in neutral aqueous solution, and should readily fit in a DNA duplex groove. Actually, this was found to be the case and the binding site has now been established to be an AT rich minor groove by X-ray and NMR studies.[2] By a fluorescence study, however, more than one binding modes for this drug to native DNA have been distinguished.[3] Recently, such multimode binding has been investigated by means of titration rotational viscometry[4] and electric linear dichroism measurements.[5,6] It has been suggested that the 2-amino group of guanine protruding in the minor groove may prevent Hoechst 33258 from getting access to the minor groove of GC sequences. If so, the second binding mode of this drug, which would occur after the first stage binding at AT rich sequences has finished, may take place at GC rich sequences. This second binding mode was further suggested to alter greatly the overall structure of the DNA duplex.[5—7]

In our previous work, we addressed this problem with Hoechst 33258.[8] By the use of a closed circular DNA duplex with a topoisomerase II reaction, followed by gel electrophoresis, we showed that this drug unwinds DNA duplexes through a binding mode other than minor groove binding. We have now attempted to visualize such an unwinding by the use of atomic force microscopy. Atomic force microscopy (AFM) is a powerful technique for direct observation of biological macromolecules and their assemblies. One of the advantages of AFM over other high-resolution microscopies, is that the imaging is permitted in aqueous solution without drying the sample. This is certainly advantageous, not only because biological samples can be kept intact,[9] but also because the results of imaging can be correlated with other experimental results in solution. With AFM in solution, we could previously visualize unwinding of the closed circular DNA duplex, pBR322, caused by ethidium binding.[10] Such an unwinding of pBR322 DNA induced by Hoechst 33258 binding has now been visualized by AFM imaging. Details are reported below.

In the course of this AFM examination, we encountered another function of Hoechst 33258, *i.e.*, compacting DNA molecules. In general, condensation of DNA into compact structures is a common process for virus genomes. An examination of condensation of DNA by multivalent cations can provide useful insights into the physical factors governing the folding and packaging of DNA *in vivo.* Condensation of DNA by spermidine has been examined in detail by means of electron microscopy[11—13] and by AFM.[14] Because Hoechst 33258 is a trivalent cation as spermidine is, the condensation of DNA by Hoechst 33258 is an interesting subject of study. The results of such a study with AFM are also reported below.

Experimental

Materials The sample of plasmid pBR322 DNA was purchased from Takara Shuzo Co., Kyoto, Japan. Topoisomerase I from calf thymus and Hind III were also purchased from Takara Shuzo Co. Hoechst 33258 was purchased from Sigma Co. These materials were used without further purification.

Sample Preparation Relaxed closed circular pBR322 DNA was prepared by topoisomerase I in buffer-T (35 mM Tris–HCl (pH 8.0), 72 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 5 mM spermidine, and 0.01% bovine serum albumin).

Linear pBR322 DNA was prepared by Hind III in buffer-M (10 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM NaCl). The reaction time was 40 min. and the temperature was 37 °C. The enzyme was then denatured by 0.6% SDS, digested with 0.05% proteinase K for 1.5 h at 37 °C, and was removed through a phenol extraction procedure. Finally, the DNA was isolated by ethanol precipitation, and then subjected to an agarose gel electrophoresis test.

Atomic Force Microscopy Relaxed circular or linear plasmid DNA was dissolved in 10 mM HEPES buffer (pH 7.0) containing 1 mM $NiCl_2$.[15] The DNA concentration was always adjusted to 0.05 μg/ml. The Hoechst 33258 concentration was adjusted to 0, 0.1, 0.5, 0.6, 0.7, 0.8, or 1 μg/ml. The instrument used was a Nanoscope III, Digital Instruments. Freshly cleaved mica was placed on the instrument and the liquid cell (an attachment provided by Digital Instruments) was set over the mica. The sample solution was placed in this liquid cell. The instrument was operated using tapping

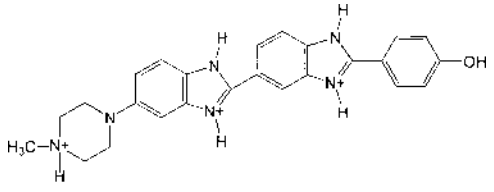

Fig. 1. Chemical Structure of Hoechst 33258 in Neutral Aqueous Solution

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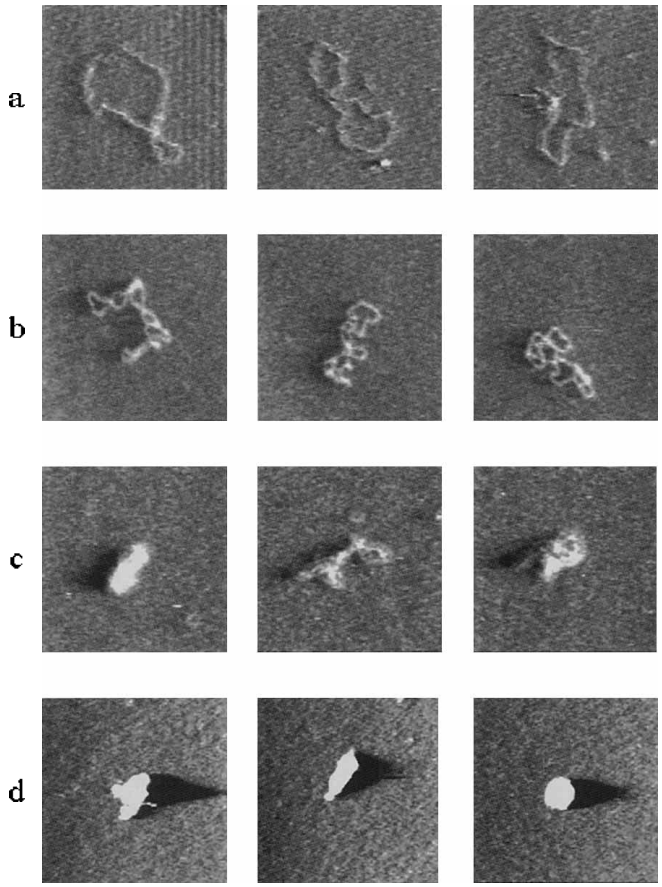

Fig. 2. AFM Images of Closed Circular pBR322 DNA in Aqueous Solutions Containing Hoechst 33258

DNA concentration: 0.05 μg/ml. Drug concentrations: (a) 0 μg/ml, (b) 0.1 μg/ml, (c) 0.5 μg/ml, (d) 1 μg/ml. Magnification: every square has a dimension of 1 μm$\times$1 μm.

mode AFM. A cantilever equipped with an oxide-sharpened silicon nitride probe was used. The scan rate was 5.09 Hz, and tapping frequency was 9 to 10 kHz.

Results and Discussion

Effects of Hoechst 33258 Binding on Relaxed Closed Circular DNA As shown in Fig. 2a, the relaxed pBR322 DNA molecules have a circular form with one, two, or three fold interwindings. This is understandable from the expected Boltzmann distribution of this DNA molecule among different topoisomers with different writhing numbers (τ). As is detailed in our previous paper,[16] a relaxed closed circular DNA duplex molecule is expected to have an average linking number (α), which is equal to its twisting number (β). Here, $\beta=N/h$, where N is the total number of base pairs involved in the closed circular duplex, and h is the number of base pairs involved in one pitch of the DNA helix. Because $\alpha=\beta+\tau$, the average writhing number (τ) must be zero ($\tau=0$). However, the free energy differences among the topoisomers are not great, so that not every topoisomer necessarily has $\tau=0$. Some may have $\tau=1$, some have $\tau=-1$, some have $\tau=2$, or $\tau=-2$, and a few have $\tau=3$ or $\tau=-3$.

On adding 0.1 μg/ml of Hoechst 33258, the number of interwindings increases to 5 to 10 (see Fig. 2b). Namely $\tau=5$—10. This change is taken as indicating that an unwinding has been caused in the DNA duplex by Hoechst 33258 binding. Thus, unwinding should cause a greater h, and therefore a smaller β, which must cause an increase of τ. In our previous work, a relatively weak binding reaction of this drug was found in buffer T2, which caused an unwinding of the DNA duplex.[8] The amount of unwinding was about 1 degree per one drug molecule. Buffer T2 contained 50 mM Tris–HCl (pH 8.0), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, and 0.5 mM dithiothreitol and its ionic strength was much higher than the AFM buffer used in the present study. Therefore, the number of bound drug molecules must be greater, so that the amount of unwinding must be greater in the present experiment. By taking this difference into account, the observed number of unwindings in Fig. 2b is consistent with our previous work.[8]

On elevating the drug concentration to 0.5 μg/ml, spherical or ellipsoidal clusters start to appear (Fig. 2c), and at a drug concentration of 1 μg/ml, almost all objects became such compact lumps (Fig. 2d). Each of these lumps is considered to be a product in which a trivalent cation (Hoechst 33258) clamps two or more DNA duplex chains together. Such a clamping function for this drug molecule should work not only in a closed circular DNA duplex, but also with a linear DNA duplex. We examined next whether this is the case.

Effect of Hoechst 33258 Binding on Linear DNA Duplexes The linear DNA duplex with no drug added, appears in AFM as a gently bent string (Fig. 3a). On adding Hoechst 33258 at 0.5 μg/ml, a number of intra-string contacts begin to appear (Fig. 3b). These contacts are considered to be caused by a clamping function of Hoechst 33258 cation. With a higher drug concentration (0.6 μg/ml), not only intra-string clamping, but also inter-string clamping seems to occur (Fig. 3c). When the drug concentration is as high as 0.8 μg/ml, clusters composed of a few "subunits" appear. It is noticeable that in the central frame of Fig. 3d, there are three ring-like subunits. Each of these has a diameter of about 100 nm, and has a clearly recognizable central hole. This may correspond to what was observed by previous investigators, and was called "toroid" by them.[17,18] Toroid means an object having the shape of a solid generated by a circular motion of a circle about an axis outside itself. In a word, this may be called a "doughnut" shape. At a drug concentration of 1 μg/ml, the condensation progresses even further and an ellipsoidal lump was observed (Fig. 3e). This is similar to what is observed for circular pBR322 DNA+Hoechst 33258 (1 μg/ml) (Fig. 2d).

Mechanism of Condensation of DNA Duplexes by Hoechst 33258 Further studies of the condensates have been made by examining many AFM images. The focus of interest was placed on the toroidal shape, because this was assumed to result from a specific mode of condensation, rather than due to a random assembly of the DNA duplex chains.

First, it was confirmed that the toroidal condensate is in an entirely different category from what has been called the "toroidally supercoiled form".[10] The latter is a product of a toroidal winding of DNA duplex, in which the axis of the DNA helix lies on an imaginary torus, and involves only one DNA duplex molecule. The toroid observed in this work should have a number of DNA duplex chains which seem to assemble before they form a toroid.

There are at least two models for the structure of toroidal condensate. In both the DNA duplex chains are wound

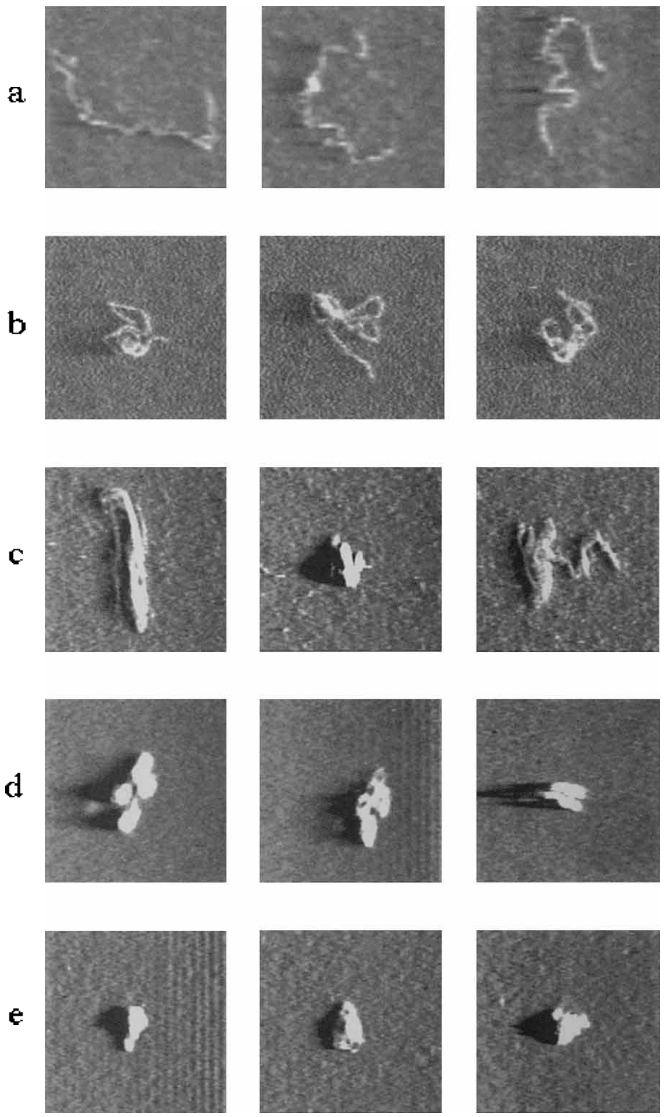

Fig. 3. AFM Images of Linear pBR322 DNA in Aqueous Solutions Containing Hoechst 33258

DNA concentration: 0.05 μg/ml. Drug concentrations: (a) 0 μg/ml, (b) 0.5 μg/ml, (c) 0.6 μg/ml, (d) 0.8 μg/ml, (e) 1 μg/ml. Magnification: every square has a dimension of 1 μm×1 μm.

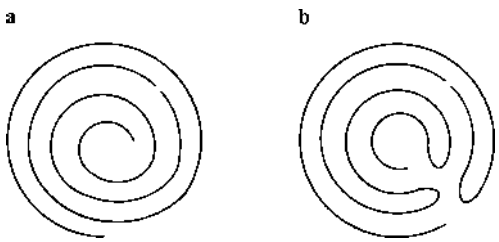

Fig. 4. Schematic Illustration of Two Models of Toroidal Condensate of DNA Duplexes

(a) Model I, (b) Model II.

around a common axis which is located at the center of the toroid perpendicular to the toroid plane (Fig. 4). In one of them, the DNA duplex chains are wound without any gap like the threads in a spool (model I, Fig. 4a).[19] In the other, every DNA duplex chain forms a loop at a vertical section to leave a plane gap which is parallel to the toroid axis (model II, Fig. 4b).[20,21]

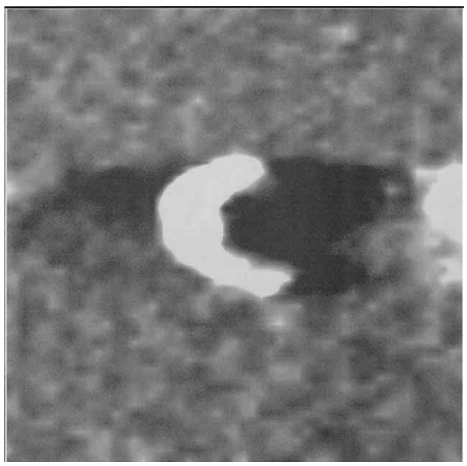

Fig. 5. AFM Image of Halfway Bent Rod Structure, Prepared from Closed Circular pBR322 DNA and Hoechst 33258

DNA concentration: 0.05 μg/ml. Drug concentration: 1 μg/ml. Magnification: the square has a dimension of 500 nm×500 nm.

At first glance, our present experimental results suggest the model II. In fact, we could observe several AFM images of a halfway bent rod (Fig. 5), suggesting an intermediate between the rod and toroid. In addition, sometimes the toroid was found to have a handle (Fig. 6b). This may be called the "racket form", but rather it gives the impression of an incompletely formed ring, like the letter 6. These observations suggest that the mechanism of at least some of the toroidal condensate formation by Hoechst 33258 is similar to that predicted by Dunlap *et al.* for PEI,[20] and also by Arscott *et al.* for hexammine cobalt(III).[21] Thus, DNA duplexes are apt to align through Hoechst 33258 parallel to one another to form a thin and long thread (left most frame of Fig. 3c). This may be folded into a shorter and thicker rod. The rod may be bent into a bow shape, into a toroidal shape, or into a racket (or letter-6-) form.

Recently, Yoshikawa *et al.* performed a Monte Carlo simulation on the coil–globule transition for a neutral stiff polymer chain.[22,23] They showed that the chain must eventually go into a stable toroidal form, which is essentially model I above, and they showed a number of snapshots on the linear to toroid process of the polymer chain. At some stages of the simulation, there appeared a ring with one tail and one loop. In our AFM examination images with a similar form were sometimes seen. In the rightmost frame of Fig. 3c, for example, a cluster with one tail and one loop attached is seen. In the Monte Carlo simulation, two folded spheres connected by a linear chain were also predicted to appear under appropriate conditions. Actually, in the rightmost frame of Fig. 3c, an AFM image with similar shape was observed. Therefore, at least some of the DNA duplex molecules may be considered to follow a process similar to what Yoshikawa *et al.* predicted, rather than the simple bending rod process described above.

We could reach another finding relevant to the problem in question: different behavior for closed circular DNA duplexes compared to linear duplexes in the formation of toroidal condensates. Surveying about 50 AFM images of condensate products (with 1 μg/ml Hoechst 33258) from lin-

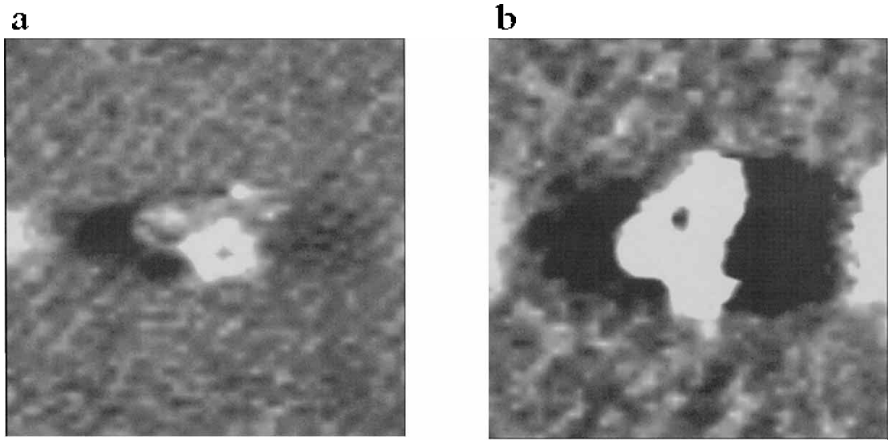

Fig. 6. AFM Images of Toroid Structure (a) Prepared from Linear pBR322 DNA, (b) Prepared from Closed Circular pBR322 DNA
DNA concentration: 0.05 μg/ml. Drug concentration: 1 μg/ml. Magnification: every square has a dimension of 500 nm×500 nm.

ear DNA duplexes revealed that 19% of them had a toroidal shape, whereas only 4% of 50 AFM images of the condensates from closed circular DNA duplexes showed a toroidal shape. This difference is similar to the difference found by Arscott *et al.* for toroid formation with hexamine cobalt.[21] This fact supports the mechanisms proposed above, because it is understandable that linear duplexes would require less free energy for the parallel association relative to closed circular DNA duplexes. We found, in addition, that toroid from closed circular DNA duplexes was about twice as large as the toroid from linear duplexes (Fig. 6). This may be taken as indicating that intermediate condensates consisting of closed circular duplexes are harder to bend compared to the intermediate condensates consisting of linear DNA duplexes. This is understandable both for intermediates for model I and those for model II, because closed circular DNA duplexes already have a number of bends within the molecules, before they are bent to fit a toroidal shape.

Conclusions

The present observations with AFM have led to the following conclusions.

(1) When the concentration of Hoechst 33258 is somewhat higher than that causing groove binding, somewhere around 0.1 μg/ml for example, it causes an unwinding of DNA duplexes.

(2) Even higher concentrations of Hoechst 33258 (1 μg/ml, or so) cause condensation of DNA duplexes, as spermidine and hexammine cobalt (III) also do, and involve toroid formation.

(3) With a closed circular DNA duplex, Hoechst 33258 causes a toroid condensate less frequently than with a linear DNA duplex, and on average the former toroid is larger than the latter.

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