Viewing of Complex Molecules of Ethidium Bromide and Plasmid DNA in Solution by Atomic Force Microscopy

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Tertiary structure changes in plasmid DNA, induced by ethidium bromide intercalation, have been observed in aqueous solutions by the use of an atomic force microscope. A relaxed closed circular pBR322 molecule became a positively supercoiled complex on the drug binding. The supercoiling always resulted in an interwound (or a plectonemic) form, but never a solenoidal (or a toroidal) form. A quantitative analysis of the compactness of such supercoiled complexes has been carried out.

Key words atomic force microscopy; plasmid DNA; ethidium bromide; interwound form of DNA duplex; plectonemic form of DNA duplex

In a continuation of our series of studies on drug–DNA interaction,[1—3] we have examined topographic images of such interacting molecules, using an atomic force microscope. Atomic force microscopy (AFM) is an emerging technique for direct observation of biological macromolecules and their assemblies.[4—9] One of the advantages of AFM, over other high-resolution microscopies (electron microscopy, for example), is that sample preparation is relatively simple. It does not involve negative staining or shadow casting with a metal coating. In addition, the sample does not need to be kept in vacuum. Through this technique, we have succeeded in viewing the tertiary structure of plasmid pBR322 DNA, kept in a proper solution, in single molecule resolution. How and to what extent is such an image of the tertiary structure altered by the binding of various drugs? This question has been the subject of our recent investigation.

Quite recently, Pope *et al*.[10] published their AFM studies of changes in the pBR322 DNA tertiary structure induced by the binding of ethidium bromide. The results of their observation are different in some respects from what we have observed of the same drug–DNA system. It would be significant, therefore, to publish our results on the ethidium–pBR322 DNA interaction here, before publishing our results on other drug–DNA interaction.

One of the main differences between the Pope *et al*. investigation and ours is in the sample morphology. In the Pope *et al*. experiments, the plasmid DNA solution was spotted on mica, and the mica was rinsed with H_2O, and blown dry with compressed N_2. In our experiments, the DNA solution was placed in a liquid cell, which was placed on mica, and the DNA sample, adsorbed on mica, was kept in the solution during the measurement without drying. It is another advantage of AFM that the imaging is permitted in buffer solution without drying the sample. This is certainly advantageous, not only because biological samples can be kept intact, but also because the results of imaging can be correlated with other experimental results in solution. On the interaction of ethidium bromide and pBR322 DNA in solution, various pieces of information were established by our previous study.[1] One molecule of ethidium bromide, when it is intercalated into a double-helical DNA, is known to unwind the DNA duplex so that the base pair to base pair angle (viewed along the helix axis) is reduced. Therefore, a relaxed closed circular pBR322 DNA molecule is predicted to change into a positively supercoiled form on an ethidium bromide binding. We can also predict an approximate number of supercoils (which is nearly equal to writhing number τ) in the plasmid molecules kept at a given concentration in an aqueous solution which contains a given amount of ethidium bromide. How do the individual molecules look in such a solution? The results of our observation regarding this question will be given below.

Experimental

Materials The sample of plasmid pBR322 DNA was purchased from Takara Shuzo Co. Topoisomerase I from calf thymus was also purchased from Takara Shuzo Co., and used without further purification. Ethidium bromide was purchased from Sigma Co.

AFM Plasmid pBR322 DNA was treated with topoisomerase I, and then subjected to an agarose gel electrophoresis test. When it was confirmed that the molecules were in a completely relaxed conformation, plasmid was dissolved into 10 mM HEPES buffer (pH 7.0) containing 1 mM $NiCl_2$.[11] The DNA concentration was always adjusted to 0.05 μg/ml. The ethidium bromide concentration was adjusted to 0, 0.25, 0.50, 0.75, 0.80, 0.90, 1.0, 2.0 or 10 μg/ml. The instrument used was Nanoscope III, Digital Instruments. Freshly cleaved mica was placed on the instrument, and then the liquid cell (an attachment provided by Digital Instruments) was set over the mica. The sample solution was placed in this liquid cell (see Fig. 1). The instrument was operated using tapping 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.

Electrophoresis Plasmid pBR322 DNA was dissolved up to 10 μg/ml in buffer-T (35 mM Tris–HCl (pH 8.0), 72 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 5 mM spermidine, and 0.01% bovine serum albumine). The ethidium bromide concentration was adjusted to 0, 0.25, 0.50, 0.75, 1.00, 1.25, or 1.50 μg/ml. The mixture solution was incubated at 37 °C for 10 min to complete the binding reaction. Next, the proper amount (6 units for 200 μl of the mixture solution) of topoisomerase I was added, and the solution was incubated for 2 h, to cause a complete relaxation of the supercoiled plasmid (with the drug bound). Then, the drug and enzyme were re-

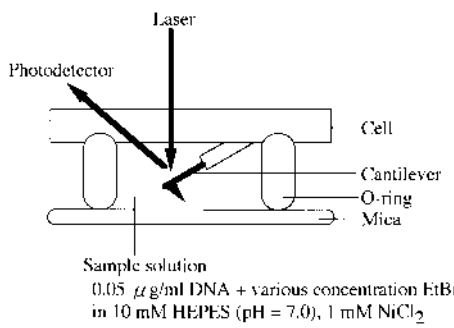

Fig. 1. Section of a Liquid Sample Cell for Atomic Force Microscope

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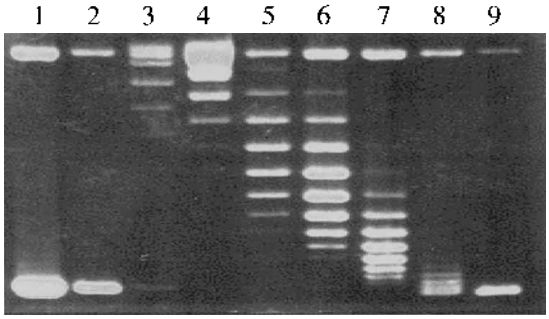

Fig. 2. Electrophoresis Analysis of Topoisomers of pBR322 DNA, Produced by an Ethidium Bromide Binding and the Topoisomerase I Relaxation, Followed by the Removal of the Drug and Enzyme

1. Native intact pBR322 DNA; 2. Native pBR322 DNA treated with incubation, phenol extract, and ethanol precipitation in buffer-T containing no topoisomerase I and no ethidium bromide; 3—9. Ethidium bromide had been added before topoisomerase was introduced. DNA concentration=10 μg/ml. Drug concentrations (μg/ml): 3, 0; 4, 0.25; 5, 0.50; 6, 0.75; 7, 1.00; 8, 1.25; 9, 1.50.

moved through phenol extraction, and the DNA was isolated by ethanol precipitation. The isolated DNA was then subjected to 1% agarose gel electrophoresis. The buffer used here was TBE (90 mM Tris–borate and 2 mM EDTA). The experiment was done by imposing 40 V, at room temperature (25 °C), for 20 h. After that, the gel was stained with ethidium bromide and subjected to photography.

Results and Discussions

Configurations Predictable from Electrophoresis In our previous study,[1] the binding of ethidium bromide was found to unwind DNA duplex. Let us start from a relaxed closed circular plasmid molecule with 4362 base pairs. Suppose that the number of the bound drug molecules increases, so that the total unwinding angle reaches 360°. Then, the number (β) of helical turns must be reduced by one, so that β becomes 436 to 435. Because the linking number (α) of this plasmid should remain unchanged, writhing number τ must increase by one, if the local molecular conformation is kept unchanged, so that a law of conservation, $\alpha=\beta+\tau$, is realized.

The number of ethidium bromide molecules bound to one pBR322 DNA molecule in a solution must be a function of the drug concentration in the solution. Therefore, we first examined the average τ value of pBR322 DNA in a solution with the same amount of ethidium bromide as that in the solution subjected to the AFM study. The τ value can be estimated by using topoisomerase I relaxation followed by gel electrophoresis.[1] The result of this examination is shown in Fig. 2. As shown in the figure, the ethidium bromide concentration 0.25 μg/ml caused an average linking number change $\Delta\alpha=2$ for plasmid, 0.50 μg/ml caused $\Delta\alpha=5$, 0.75 μg/ml caused $\Delta\alpha=7$, 1.00 μg/ml caused $\Delta\alpha=10$. Such observed $\Delta\alpha$ values were assumed to be entirely attributed to the amounts of unwinding ($\Delta\beta$) caused by the drug binding. Therefore, these must be equal to the τ values caused by the drug binding. The τ values in this assumption are listed in the second column of Table 1.

In correlating such results of observation with a prediction for the sample solutions of our present AFM study, we must take the difference in the solvents into account. The above τ values were determined in buffer-T, which was proper for the topoisomerase I reaction, whereas present AFM sample solutions were prepared in 10 mM HEPES buffer containing 1 mM $NiCl_2$. The change from Tris to HEPES was made because DNA in HEPES was found to be adsorbed on mica better than in Tris.[12] Also, bovine serum albumin involved in buffer-T may cause a disturbing image in AFM examination, and should be avoided. A transition metal ion, such as Ni^{2+} is known to be indispensable in a solvent for solution AFM observation.[11] Thus, it was found to be impossible to do the electrophoresis and AFM experiments in the same solvent in our present stage of experimental conditions. Therefore, we did not attempt any direct correlation. Instead, we used the τ derived from the elctrophoresis experiment as a tentative reference.

Table 1. Writhing Number (τ) of the Complex Molecule of pBR322 DNA and Ethidium Bromide, Estimated from Electrophoresis and Spectroscopic Experiments

	Experiment			
	Electrophoresis (see Fig. 2)	Absorption spectroscopy		
Solvent	Buffer-T	HEPES+1 mM $NiCl_2$		
DNA concentration	10 μg/ml	0.05 μg/ml		
	$\tau^{a)}$ (from $\Delta\alpha$)	$m^{c)}$	$\tau^{d)}$ ($\phi=15°$)	$\tau^{d)}$ ($\phi=3°$)
Ethidium bromide concentration (μg/ml)				
0	0±2	0	0	0
0.25	2±2	421	18	4
0.50	5±2	622	26	5
0.75	7±3	745	31	6
0.80	(*ca.* 8)$^{b)}$	765	32	6
0.90	(*ca.* 9)$^{b)}$	800	33	7
1.00	10±3	831	35	7
1.25	15±5	895	37	7
1.50	>15	945	39	8
2.00	(>15)	1020	43	9
10.0	(≫15)	1332	56	11

a) τ is assumed to be equal to the change of linking number, $\Delta\alpha$, in the electrophoresis experiment. *b*) Estimated by an interpolation. *c*) The number of drug molecules bound to one pBR322 DNA molecule. *d*) τ is assumed to be equal to ($m\times\phi/360$), where ϕ is the angle of unwinding in the DNA duplex caused by one drug molecule bound to it.

Configurations Predictable from Absorption Spectroscopic Measurement Ethidium bromide shows an absorption peak at 480 nm, whereas ethidium bromide/DNA complex is at 520 nm. By the use of this optical property, we can estimate the equilibrium constant (K) of this drug–DNA system in various solvents, as was detailed previously.[1] In our AFM solvent, *i.e.* 10 mM HEPES buffer (pH 7.0)+1 mM $NiCl_2$, it was found that $K=2.5\times10^5$ M^{-1} and $n=2.6$, through a Scatchard plot[13] and a McGhee–von Hippel analysis.[14] Here, n is the number of the base pairs occupied by one drug molecule. From these K and n values, the number (m) of drug molecules bound to one pBR322 DNA molecule is calculated for every pair of DNA concentration (0.05 μg/ml) and drug concentration. The result is listed in the third column of Table 1. If the angle (ϕ) of unwinding of the DNA duplex is assumed to the 15°,[1] τ is given by $\tau=\phi\times m/360$. This is given in the forth column of Table 1.

Conformation Predictable for Each τ Value For a given value of τ, two kinds of supercoiled conformations are predictable. One is an interwound form (or a plectonemically

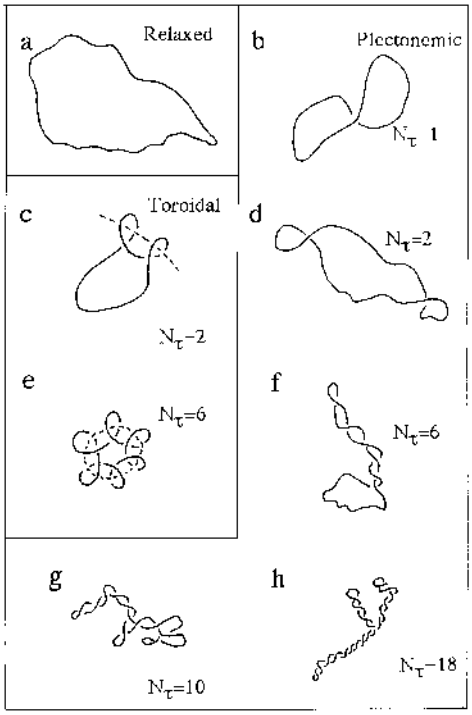

Fig. 3. Diagrammatic Illustrations of Possible Tertiary Structures of Plasmid pBR322 DNA Molecules

The line here represents the axis of double-helical DNA chain. N_τ is the number of apparent intersection points, or the number of apparent nodes. This is considered to be nearly equal to the writhing number τ, which can be determined by electrophoresis experiment. a) Completely relaxed form. b) Interwound form where the number (N_τ) of supercoil is 1. c) Toroidally supercoiled form with N_τ=2. d) Plectonemically supercoiled form with N_τ=2. e) Toroidally supercoiled form with N_τ=6. f) Plectonemically supercoiled form with N_τ=6. g) Plectonemically supercoiled form with N_τ=10. h) Plectonemically supercoiled form with N_τ=18.

supercoiled form). The other is a solenoidal form (or a toroidally supercoiled form), in which the axis of the DNA duplex lies on an imaginary torus. Some of these kinds of superhelical forms are illustrated in Fig. 3. Each of these eight figures shows a possible projection of a plasmid molecule on a two-dimensional plane. The line here represents an axis of a double-helical DNA chain. The number (N_τ) of the intersection points of the line within each figure is supposed to be the number of supercoilings of the plasmid molecule. This number N_τ can be correlated with the writhing number τ, but these two are not exactly equal to each other. In general τ is not an integer, whereas N_τ is an integer. It would be reasonable, however, to assume that N_τ is nearly equal to τ with an ambiguity of 1.0.

AFM Images Figure 4 is a collection of topographic images recorded for plasmid pBR322 DNA molecules kept in solutions of 0.05 μg/ml. Here, the plasmid was completely relaxed by the topoisomerase I reaction., and this was confirmed by electrophoresis. Without ethidium bromide, some of each of the DNA molecules certainly looked circular with no supercoil (Fig. 4a). Many of the chains, however, had 1—3 intersection points. This is partly attributable to the solvent (10 mM HEPES+1 mM $NiCl_2$) and temperature (25 °C), in which the AFM experiment was made. These are different from the conditions (buffer-T at 37 °C) in which the plasmid was relaxed. When ethidium bromide was added to the solution, apparently interwound forms were found to appear (Fig. 4b). In the range of ethidium concentration from 0.25 to

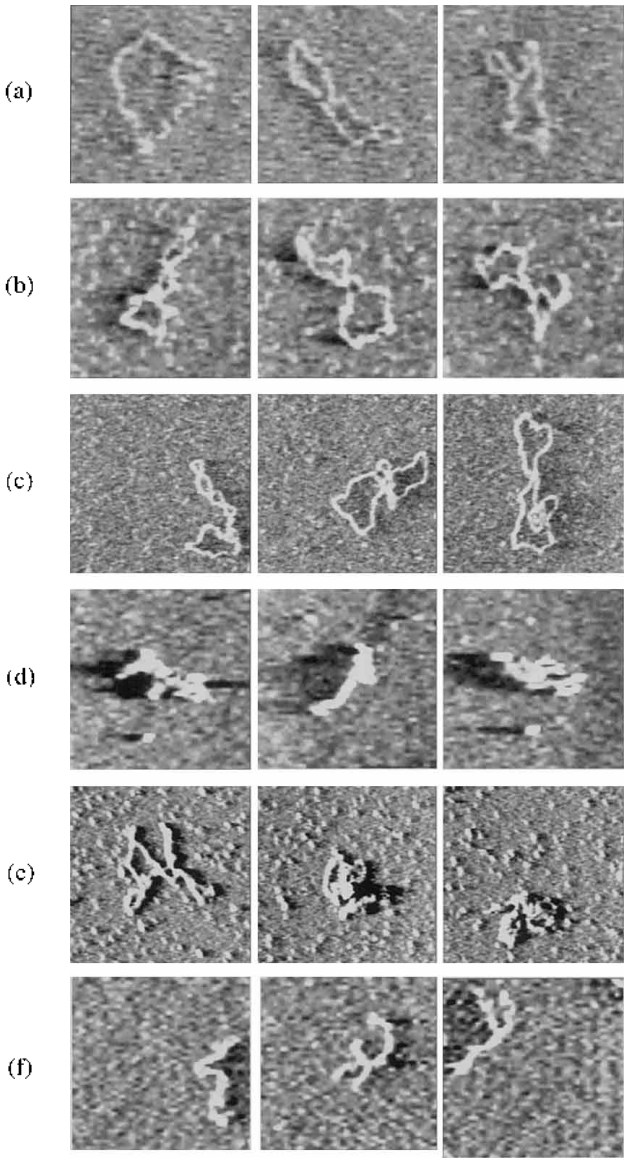

Fig. 4. AFM Images of Plasmid pBR322 DNA in Aqueous Solutions, Containing Ethidium Bromide

DNA concentration: 0.05 μg/ml. Drug concentrations (μg/ml): a) 0, b) 0.75, c) 0.80, d) 1.0, e) 2.0, f) 10. Magnification: every square here has a dimension of 1 μm×1 μm.

0.75 μg/ml, however, the observed N_τ values were found to be mostly 2 or 3 only, except for unresolvable images. When the concentration of ethidium bromide reached 0.80 μg/ml, plectonemically supercoiled forms with 6 or 7 were observed. However, many of the loops in the supercoiled forms could not be found resolved; they were observed as thick rods. Such a rod is interpreted as a condensed plectonemic supercoil (see Fig. 3g, for example). In the solution with 1.0 μg/ml ethidium bromide, more of such thick rods appeared (Figs. 4d, e). Still, one or two small loops were found in each molecule. In the solution where the ethidium bromide concentration was as high as 10 μg/ml, every plasmid molecule appeared as a thick rod. Some molecules were found to be branched rods (Fig. 4f).

In surveying the observed AFM images, a general trend of increasing of the τ value with the ethidium bromide concentration was certainly confirmed. The upward gradient of τ, however, is much smaller than what is expected from the

Table 2. Extent of Image of Plasmid DNA as a Function of the Amount of Ethidium Bromide in the Solution

DNA extent ($\times 10^4$ nm^2)	Ethidium bromide concentration (μg/ml)								
	0	0.25	0.50	0.75	0.80	0.90	1.0	2.0	10
1—5	2 (2)	1 (1)	0 (0)	1 (1)	3 (2)	6 (4)	11 (8)	35 (22)	39 (31)
6—10	4 (3)	12 (8)	9 (6)	10 (7)	22 (23)	35 (24)	55 (40)	96 (61)	63 (50)
11—15	33 (25)	39 (27)	41 (26)	41 (30)	56 (40)	47 (33)	44 (32)	18 (11)	14 (11)
16—20	43 (33)	50 (35)	61 (39)	44 (32)	26 (19)	29 (20)	16 (12)	5 (3)	3 (2)
21—25	20 (15)	26 (18)	28 (18)	23 (17)	9 (6)	16 (11)	1 (1)	1 (1)	0 (0)
26—30	16 (12)	7 (5)	11 (7)	9 (7)	7 (5)	4 (3)	5 (4)	1 (1)	1 (1)
31—	13 (10)	7 (5)	8 (5)	9 (7)	7 (5)	7 (5)	6 (4)	1 (1)	6 (5)
Total	131 (100)	142 (99)	158 (101)	137 (101)	140 (100)	144 (100)	138 (101)	157 (100)	126 (100)

Percentage is shown in parenthesis.

equilibrium constant ($K=2.5\times10^5$ M^{-1}) and unwinding angle ($\phi=15°$) (see forth column of Table 1). At an ethidium bromide concentration of 0.80 μg/ml, for example, N_τ was found to be only 6 or 7 in AFM, whereas $\tau=31$ was expected from the assumption of $K=2.5\times10^5$ M^{-1} and $\phi=15°$. For such an inconsistency, two factors may be taken into consideration.

First, the effective ϕ value may be smaller than 15°. Because a bending of the DNA duplex would increase its torsional rigidity,[15] an effective ϕ may become smaller as the number of bound drug molecules increases. At the ethidium bromide concentration=0.80 μg/ml, for example, nearly half of the drug sites on pBR322 DNA (having 4362 bp) are occupied ($m=765$ bp and $2.6\times m=1989$ bp). If $\phi=3°$, however, τ can be only 6 ($=765\times3°/360°$). This notion is not sufficient to explain what we observed in our AFM experiment. Thus, as shown in the last column of Table 1, the expected τ values are much smaller than what were actually observed.

Secondly, in low ionic strength, the linking number deficit $\Delta\alpha$ is not totally partitioned to $\Delta\tau$, but partly to $\Delta\beta$.[8,16] The AFM solvent we used has only 1 mM $NiCl_2$, and probably because of the PO_2^-–PO_2^- repulsion, τ may be smaller than what is expected on the assumption that $\Delta\alpha=\Delta\tau$. Unfortunately, the amount of this difference cannot be estimated using our present knowledge.

Compactness of the Complex Molecule A survey of the images shown in Figs. 4a to 4e gave us a feeling that the complex molecule became more and more compact as the amount of the bound drug molecules increased. To fix this notion on a more firm basis, let us define an "extent of image" as the product of its length along the long axis and the length along the axis perpendicular to the long axis. We measured such an "extent of image" for every recorded image of plasmid DNA, and the results are summarized in Table 2. It became clear that the extent was lowered as the drug concentration in the solution was elevated. A slightly more detailed survey, however, indicates that the lowering of the extent is small when going from an ethidium concentration of 0 to 0.75 μg/ml, and that more prominent lowering starts at about 0.80 μg/ml and continues to 10 μg/ml. An interpretation of this phenomenon is yet to be done.

Particular Shapes In a microscopic study, it is important not only to deduce a general trend from a survey of many images, but also to observe individual images in detail. A special shape of a particular molecule could possibly be significant.

The image in the left-most frame of Fig. 4c is for a plasmid pBR322 DNA molecule observed at an ethidium bromide concentration=0.80 μg/ml. This is a plectonemically and positively supercoiled molecule with the number of supercoilings at 6 (compare with the illustration given in Fig. 3f). The image in the central frame of Fig. 4c has probably the same number of ethidium bromide molecules and the same number of coilings. Its shape, however, is quite different; its two interwound small loops are crowded in the central portion, and its two large loops are placed outside.

What is shown in the left-most frame of Fig. 4d is an image of a plasmid molecule observed at an ethidium bromide concentration=1.0 μg/ml. This is a plectonemically supercoiled with a τ value of about 10. Its interpretation is given by an illustration in Fig. 3g. In the right-most frame of Fig. 4f is an image for a branched plectoneme. Here, the number of supercoils must be greater than 15. Its duplex chain axis is considered to be folded as shown in Fig. 3h.

It is interesting that, contrary to the Pope *et al*. observation, we could not find any image that was assignable to a toroidally supercoiled form.

A Comparison with the Results of a Previous Study In the above mentioned AFM study, Pope *et al*.[10] recorded topographic images from DNA complexes of various ethidium bromide/base pair stoichiometries, ranging from 1/10 to 8/1. They observed a toroidally supercoiled form in a very dilute ethidium bromide concentration, for a drug/base pair stoichiometry of 1/5 to 1/1. On the other hand, we did not observe this form in the whole concentration range examined (0—10 μg/ml).

In explaining such a difference between the images observed by Pope *et al*. and by us, three factors may be taken into consideration. First, Pope *et al*. used a natural, heavily supercoiled pBR322 DNA, whereas we used a relaxed closed circular pBR322 DNA. Thus, in the Pope *et al*. experiment, pBR322 DNA had a high $\Delta\alpha$ value even with a very small number of drug molecules bound. Secondly, the effective equilibrium constant in the Pope *et al*. system is extraordinarily high. Unfortunately, Pope *et al*. ignored our detailed study[1] on the ethidium bromide+pBR322 system, which estimated the equilibrium constant values in six different solvents. Instead, they estimated the equilibrium constant by the method described by Coury *et al*.[17] The estimated value was 1.4×10^6 M^{-1}. This is just what is expected in a solvent with no salt,[1] and is probably higher than that in our system with 1 mM $NiCl_2$ by one order of magnitude. Third, some unknown reactions may occur in the process of "spotted on 3-aminopropyltriethoxy silanized mica (AP-mica), gently rinsed with 20 ml H_2O, and immediately blown dry with

compressed N_2," which was involved in the Pope *et al*. sampling, and which was not involved in our sampling.

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