Identification of DNA – cisplatin adducts in a blind trial of *in situ* scanning tunneling microscopy

A.M.Jeffrey^{1,2}, T.W.Jing, J.A.DeRose, A.Vaught, D.Rekesh, F.-X.Lu¹ and S.M.Lindsay*

Department of Physics and Astronomy, Arizona State University, Tempe, AZ 85287, ¹Division of Environmental Sciences and ²Department of Pharmacology, Columbia University, New York, NY 10032, USA

Received September 17, 1993; Revised and Accepted November 9, 1993

ABSTRACT

Scanning tunneling microscopy (STM) reveals nanometer scale details of hydrated DNA but the interpretation of the images is controversial because of substrate artifacts and the lack of a theory for image contrast. We demonstrate that we have overcome these problems by identifying five DNA samples by their STM images alone in a blinded trial. The samples were single-stranded and double-stranded DNA with and without covalent modification by the anti-tumor drug cisplatin. The cisplatin adducts were distinguished by substantial kinking at the drug binding site. The oligomers were 20 bases in length, which was too short to permit the kinking angle to be determined with precision. However, models with a 45° kink gave a better fit to the images of the duplex adducts than models with a 90° kink. A variety of structures was observed for the single-stranded adducts.

INTRODUCTION

The great potential of the scanning tunneling microscope (STM) for imaging DNA and other biomolecules in aqueous buffers at near-atomic resolution (1,2) has proved difficult to realize. Images that appear to show the helical structure of DNA (3,4) have been called into question since they may be substrate artifacts (5,6). We have improved the reliability of STM imaging by electrochemically depositing and maintaining DNA on positively-charged gold substrates (7,8) where it is imaged in the electrolyte (9,10). The helical structure of both double-stranded and single-stranded DNA oligomers can be resolved in aqueous buffers and a simple tunneling model allows us to calculate images that are in good agreement with experiments (11). In this paper, we describe how we have tested our methods in a blinded trial in which we identified various DNA – cisplatin adducts by their STM images alone. The results demonstrate that unusual DNA structures can be identified with a high degree of reliability (and a resolution of about 1 nm) by direct imaging in aqueous buffer.

Cisplatin is an anti-neoplastic agent that binds covalently at the N7 positions of adjacent guanines (12,13). Base stacking is disrupted (14) and the adducts appear to bend into the major

groove (15,16). Oriented fibers of a DNA – cisplatin adduct have been studied by atomic force microscopy (17). Fibers made with the adduct were less oriented than fibers made with unmodified DNA. However, the resolution of the AFM was inadequate for studying single molecules. The STM has provided better resolution and we have found clear differences in the STM images of small oligomers with and without covalent modification by cisplatin. Images of the modified duplexes are consistent with the presence of a kink of less than 90°. However, the agreement between the calculated and experimental images is not as good as was found for unmodified DNA (11).

MATERIALS AND METHODS

DNA samples

The following five oligodeoxyribonucleotides were synthesized at Columbia University: d(TCTCCTTCTTGGTTCTCTTC) (S2); the complementary strand, d(GAAGAGAACCAAGAA-GGAGA) (S1); S2 reacted with cisplatin (S2Pt); S1 annealed with S2 to produce a 20 base-pair duplex (D) and S1 annealed with S2Pt to produce a duplex with cisplatin (DPt). Oligonucleotides were synthesized on a 394 DNA/RNA Synthesizer (Applied Biosystems Inc., Foster City, CA), the crude products purified on NAP-5 columns (Pharmacia LKB Biotechnology, Uppsala, Sweden) and washed with 10 ml TE buffer (10 mM Tris – HCl, 1 mM EDTA, pH 8.0). Complementary single strands were annealed in TE buffer at 60°C for 2 h, and cooled to room temperature over a period of 4 h. DNA was repurified by HPLC and desalted by dialysis against triply-distilled water. Control samples were obtained from aliquots from the HPLC column that contained no DNA.

cis-(Pt(NH₃)₂(H₂O)₂)²⁺ was prepared by reacting 3 μM cis-platinum(II)-diammine dichloride (Sigma Chemical Co., St. Louis, MO) with 6 μM AgNO₃ in 330 μl water at 37°C overnight. The precipitated AgCl was then removed by centrifuging twice (18). Strand S2 was allowed to react with 1.5 equivalents of cis-(Pt(NH₃)₂(H₂O)₂)²⁺ for 5 h in the dark at 37°C (19). The major reaction product was purified by HPLC (Gen-Pak FAX column). The yield of purified material was about 50%. S2 or S2Pt was mixed with an equal amount of S1 in a

*To whom correspondence should be addressed

tube containing 60 μ l TE buffer. The tube was kept in a 60°C water bath for 2 h, followed by cooling to room temperature over 4 h. The annealed double-stranded oligonucleotides (D and DPt) were repurified by HPLC using a Waters Gen-Pak FAX column with 25 mM Tris-HCl, 1 mM EDTA, pH 8.0 buffer and increasing the NaCl concentration from 0.25 to 0.45 M over 40 min and a flow rate of 0.75 ml/min at room temperature. Retention times were (S2Pt) 12.7 min, (S1) 16.6 min, (D) 30.7 min, (DPt) 32.1 min. The samples were dialyzed against water to remove salt.

Microscopy and experimental protocol

The five samples were sent to Arizona State University, identified by letter ('A'-'E') only. Their identity was not revealed until after we had (correctly) identified them by microscopy alone. The samples were dissolved to a final concentration between 0.2 and 5 μ g/ml in a buffer solution ($\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.0) of between 1 and 6 mM strength. Details are given in the figure captions.

We controlled deposition of molecules onto an atomically-flat gold substrate (20) by altering the substrate charge potentiostatically. Our microscope operates in electrolyte so that the deposition process may be monitored directly (11). DNA is co-adsorbed onto a positive Au(111)- $23\times\sqrt{3}$ surface with the phosphate buffer which binds in a reversible electron transfer reaction and stabilizes the DNA (10,11). Control samples (aliquots which contained no DNA) were imaged by the same process in order to check that spurious images were not generated. Our microscope is a homemade unit designed for electrochemical applications. It has low drift when operating in liquid (around 1 Å/minute) and is hermetically sealed for environmental control. It is operated with a commercial controller (NanoScope II; Digital Instruments, Santa Barbara, CA). All images were obtained in the constant-current mode with a tip-to-substrate bias of 100 mV and tunnel currents of 20–100 pA. The scan dimensions were calibrated to within 2% before each run using features on the gold surface (11). The substrates were held at potentials of 240–260 mV (Saturated Calomel Electrode scale) for deposition and imaging.

Image interpretation

Each sample was imaged many times to ensure that features in the image were not a consequence of tip structure alone. Poor tips produced images in which nanometer scale structure could

Figure 1. Images of normal single-stranded and double-stranded oligomers. 'EX' is an STM image and 'CA' is a calculated image (11). From left to right, the pairs are a 61 base pair duplex, a 31 base duplex with 25 paired bases and 6 overhanging bases, a 20 base pair duplex, a 20 base single-stranded oligomer and an 11 base single-stranded oligomer. The single-stranded ends of the 31 base sample are not visible. Models for calculated images assumed a rise-per-base of 0.33 nm and a twist of 36° (duplexes) and 40.5° (single-stranded DNA) (11).

not be discerned. Images that showed nanometer scale structure were verified by repeating the experiment with a different tip. The images displayed here show the results of at least two such runs. Measurements of image dimensions were made using the public domain software NIH Image 1.4. It was calibrated in x,y and z dimensions using features on the clean Au(111) $23\times\sqrt{3}$ surface.

Comparison with model structures was carried out using the simple tunneling model we have outlined elsewhere (11). The model neglects local variations in barrier heights which could cause errors up to two-fold in the calculated contrast (assuming, of course, that the basic premises of the model are correct). The input parameters to this model are a trial structure, the measured STM contrast (1–3 Å) and a Gaussian broadening radius (7 Å) which is based on the measured tip radius.

We have proposed that the microscope senses only the uppermost atoms of a structure (11). Consequently, the contrast is small (2 Å) and highly variable (± 1 Å) and the appearance of molecules can vary considerably within a single scan. We therefore adjust contrast to a common level when making measurements of transverse dimensions.

Height fluctuations in the phosphate adlayer alone can be as large as 0.5 Å. Consequently, adlayers of DNA and phosphate together yield images with large (2 ± 1 Å) and small (up to 0.5 Å) heights. We have checked that these smaller features are not attributable to an underlying layer of DNA by reducing the concentration of DNA. The features with contrast in the 2 ± 1

Figure 2. Pairs of scans (raw data) obtained in different runs with different STM tips for the 20 base single-stranded oligomers d(TTCCTTCTTGTTCTCTT-C) (A) and d(GAAGAGAACCAAGAAGGAGA) (B) and the complex of d(TTCCTTCTTGTTCTCTT-C) with cisplatin (C and D). Arrows point to the beginning and end of a single molecule. Substrate orientation is indicated by single arrow. For (A) 4.5 μ g/ml of DNA in 6 mM buffer was deposited for 1 h; for (B) 3.2 μ g/ml of DNA in 5 mM buffer was deposited for 4 h; for (C) 0.5 μ g/ml of DNA in 3 mM buffer was deposited for 1 h; for (D) 4.4 μ g/ml of DNA in 6 mM buffer was deposited for 2 h.

Å range become less frequent as the DNA concentration is reduced and disappear when DNA is not present.

RESULTS AND DISCUSSION

Unmodified oligomers

We summarize our earlier findings using unmodified oligomers in Figure 1. This shows STM images of single-stranded and double-stranded oligonucleotides ('EX'), alongside images calculated using the simple tunneling model (11) ('CA'). The appearance of the images is strongly affected by the azimuthal orientation of the molecules. This orientation has been determined

for unmodified DNA on charged mica substrates by Rhodes and Klug (21). We found that single-stranded oligomers preferred what they called 'setting I' while duplexes adopted 'setting II'. The images were calculated from a B-DNA model with a rise per base of 0.33 nm and a twist of 36° (double helices) and 40.5° (single helices).

Figure 3. Scans (raw data) of the normal 20 base pair duplexes (A and B, different tips in different runs) and the corresponding cisplatin adducts (C and D, different tips in different runs). Arrows point to the beginning and end of a single molecule. For (A) 4 µg/ml of DNA in 5 mM buffer was deposited for 1.5 h; for (B) 0.24 µg/ml of DNA in 1.6 mM buffer was deposited for 2 h; for (C) 2.8 µg/ml of DNA in 5 mM buffer was deposited for 2.7 h; for (D) 2.8 µg/ml of DNA in 5 mM buffer was deposited for 2.5 h.

Table I. Dimensions of the molecules (defined in Fig. 4) obtained from STM images

Sample	L (nm)	L_1/L_2	P (nm)	W (nm)
S1 (47)	7.8 ± 0.9		2.3 ± 0.3	2.3 ± 0.4
S2 (44)	7.9 ± 0.9		2.6 ± 0.6	2.2 ± 0.5
S2Pta (14)	8.6 ± 0.8	1.7 ± 0.2		1.9 ± 0.3
$\theta = 146 \pm 5^\circ$				
S2Ptb (14)	8.6 ± 1.4	1.6 ± 0.3		2.3 ± 0.2
$\theta = 89 \pm 9^\circ$				
S2Ptc (14)	9 ± 0.7		3.4 ± 0.5	2.5 ± 0.4
D (69)	6.9 ± 0.8	1.0 ± 0.1	3.9 ± 0.5	3.2 ± 0.6
DPt (53)	7.6 ± 1.0	1.7 ± 0.4	4.1 ± 0.5	$2.2 \pm 0.5(W1)$ $3.7 \pm 0.6(W2)$

Data are listed separately for the three forms of S2Pt. The number of molecules analyzed is listed in parenthesis in the first column. Errors are $\pm$ one standard deviation.

Figure 4. Selection of STM images from different runs for (l to r) d(GAAGA-GAACCAAGAAGGAGA) (S1), d(TCTCCTTCTTGGTTCTCTTC) (S2), three forms of the single-stranded DNA-cisplatin adduct (S2Pta, S2Ptb, S2Ptc), D: the normal duplex and DPt, the double-stranded cisplatin adduct. Measurements summarized in Table I are illustrated on selected images.

Figure 5. Showing (bottom) models of a duplex cisplatin adduct with a 90° kink (A1 and A2) and models with a 45° kink (B1 and B2). Models are shown for the two arrangements in which the molecule is flat on the substrate. The calculated STM image is shown above each model. A random selection of measured images is shown in the top row for comparison.

The blind trial

Scans of S1 and S2 are shown in Figs 2(A) and 2(B) respectively. Here, and in the next figure, we show pairs of images from different runs with different tips in order to demonstrate that the internal structure is not strongly dependent upon the scanning tip. The molecules appear as small cylinders of about 7.8 nm length and 2.3 nm width with their long axis aligned along the [110] direction on the gold surface (the substrate orientation was determined by imaging the surface prior to DNA deposition). They have internal structure with a period of about 2.4 nm as was previously observed for single-stranded DNA. On this basis, we correctly identified these samples as the unmodified single-stranded DNA. At this level of resolution the images do not appear to be sensitive to the chemical composition (base sequence) of the oligomers.

The oligonucleotide S2Pt gave a variety of images, most of which showed kinked molecules. Examples are shown in Figs 2(C) and 2(D) (different runs with different tips). The width of the images is about 2 nm and their contour length ($L_1 + L_2$; see Fig. 4) is about 8.6 nm. The molecules in Fig. 2(C) appear to be kinked with an average kink angle (θ) of $146 \pm 5^\circ$. Those shown in Fig. 2(D) have $\theta = 89 \pm 9^\circ$. About a third of the molecules did not appear kinked so they seem to be similar to the unmodified samples. However, they are significantly longer (and the distances between the 'blobs' is larger; see Table I). We have labeled these three structures S2Pta (150° kink), S2Ptb (90° kink), and S2Ptc (extended linear form). On the basis of these images, we correctly identified this sample as the single-stranded DNA–cisplatin adduct.

Differences between the two double-stranded samples were less striking, but significant and reproducible. Images of the duplex D are shown in Figs 3(A) and 3(B) (different runs with different tips) and of the modified duplex, DPt, in Figs 3(C) and 3(D) (different runs with different tips). The unmodified duplex gave images that consisted of a pair of 'blobs' with a dyad axis in the middle (as previously observed for a 20 base pair duplex; see Fig. 1). Images of DPt lacked the dyad symmetry. One 'blob' was usually longer and narrower and the other shorter and wider (dimensions are defined in Fig. 4 and measurements summarized in Table I). These results led us to the correct identification of both the unmodified duplex and the duplex–cisplatin adduct.

Figure 4 shows a selection of images cut from a number of different scans for each sample and oriented so as to show the common features (black to white is 0.25 nm). The three structures for the single-stranded adduct are shown under columns S2Pta, b and c. Measurements are summarized in Table I. The fluctuations in the data are substantial, but there are significant differences between the distinct samples. These experiments demonstrate clearly that we can distinguish normal and modified DNA molecules on the basis of their STM images alone.

Comparison with model structures

We used two models for the double-stranded adduct. One was based only on the local interpretation of NMR data given by Kozelka *et al.* (13). The adjacent guanines at the binding site are nearly perpendicular, adopting a left handed helicoidal geometry (called 'L1') in single-stranded DNA and a right handed helicoidal geometry (called 'R2') in double-stranded DNA (13). We adjusted local backbone rotations in a B-like single or double helix (rise = 0.33 nm, twist = 36°) to accommodate the adduct while satisfying bond-length constraints. The structure was not

otherwise optimized and was kinked by about 90° . The second structure was based on the low-salt, energy minimized model proposed by Kozelka *et al.* (22). In this case, the helix is kinked by about 45° . We built our sequence using the coordinates for the energy minimized structure found by Kozelka *et al.* and then minimized the new structure using the program AMBER (23).

In this case we have no independent determination of the azimuthal orientation of the molecules on the substrate. We therefore computed the total separation between the phosphates and a substrate for all orientations of the two models. We then plotted the total separation as a function of the two independent angles that described the orientation of the models with respect to the substrate plane. Two nearly degenerate minima were found in each case corresponding to the two ('up' and 'down') flattest arrangements on the substrate. These pairs are shown in gray-scale images in the bottom of Fig. 5. A1 and A2 are the two 'flattest' orientations for the 90° kink model and B1 and B2 are the two flattest orientations for the 45° kink model. The corresponding calculated STM images are shown above each model. We have used a height of 3 Å and a Gaussian broadening of 7 Å, consistent with our calculations for the unmodified oligomers (11).

The overall agreement with experimental images (displayed above the calculated images to the same scale) is quite good. For example, for model A1, we find $W_1 = 2.2$ nm, $W_2 = 3.8$ nm, $L = 6$ nm and $L_1/L_2 = 1.33$, in reasonable agreement with experiment (Table I). Similar agreement is found for the less kinked structures. The less kinked structures (B1 and B2 in Fig. 5) also appear to fit the experimental images somewhat better although there are a number of important differences. For example, if oriented so that the small blob is at the top (like most of the experimental images) the kinking direction of the calculated image would be quite wrong. None of the single strand models resembled the observed structures, although the overall contour length of the models was in good agreement with the overall contour length of the images.

CONCLUSIONS

In conclusion, we have verified the reliability of our method for *in situ* STM imaging of small DNA molecules and shown that calculated and measured images are similar. It is clear that modified DNA structures can be distinguished from normal structures with a resolution of about 1 nm by direct imaging in aqueous solution. Single-stranded adducts, while quite different from unmodified single-stranded DNA, adopted a number of different structures, including an unkinked (but extended) structure. The double-stranded adducts were more homogeneous and the images are consistent with a kink of about 45° .

The basis for our model of contrast is, at this stage, almost purely speculative. Its relative success with unmodified oligomers might be coincidental. We cannot, therefore, ascribe the differences between calculated and measured images to real structural differences between the models and the molecules. The range of possible structures would be much better constrained by measurements on longer oligomers.

ACKNOWLEDGEMENTS

We are grateful to Stephen Lippard for supplying us with coordinates for the 'low-salt' model. We thank Jack Larsen for help in the lab. AMBER 4.0 was supplied by the University of

California, San Francisco. We profited from discussions with Rodney Harrington, Yuri Lyubchenko, Lyuda Shlyakhtenko, Michael Hogan and Gregory Petsko. Support was received from the NSF (grant DIR 89-20053 to SML), ONR (grant N00014-90-J-1455 to SML) and NIH (grant CA21111 to AMJ).

REFERENCES

1. Sonnenfeld, R. and P.K. Hansma (1986) *Science* **232**: 211–213.
2. Lindsay, S.M. (1993) *Biological Applications of the Scanning Probe Microscope*, in *Scanning Tunneling Microscopy: Theory, Techniques and Applications*, D. Bonnell, Editor. VCH Press: 335–408.
3. Beebe, T.P., T.E. Wilson, D.F. Ogletree, J.E. Katz, R. Balhorn, M.B. Salmeron and W.J. Siekhaus (1989) *Science* **243**: 370–372.
4. Driscoll, R.J., M.G. Youngquist and J.D. Baldeschweiler (1990) *Nature* **346**: 294–296.
5. Clemmer, C.R. and T.P. Beebe (1991) *Science* **251**: 640–642.
6. Heckl, W.M. and G. Binnig (1992) *Ultramicroscopy* **42-44**: 1073–1078.
7. Lindsay, S.M. and Barris (1988) *J. Vac. Sci. Technol. A* **6**: 544–547.
8. Lindsay, S.M., T. Thundat, L.A. Nagahara, U. Knipping and R.L. Rill (1989) *Science* **244**: 1063–1064.
9. Tao, N.J., J.A. DeRose, P.I. Oden and S.M. Lindsay (1991) in *Proc. 49th Ann. Mtg. EMSA*. San Francisco Press, San Francisco: pp 376–377.
10. Lindsay, S.M., N.J. Tao, J.A. DeRose, P.I. Oden, Y.L. Lyubchenko, R.E. Harrington and L. Shlyakhtenko (1992) *Biophys. J.* **61**: 1570–1584.
11. Jing, T., A.M. Jeffrey, J.A. DeRose, Y.L. Lyubchenko, L.S. Shlyakhtenko, R.E. Harrington, E. Appella, J. Larsen, A. Vaught, D. Rekes, F.X. Lu and S.M. Lindsay (1993) *Proc. Natl. Acad. Sci. (USA)* **90**, 8934–8938.
12. Kozelka, J., S. Archer, G.A. Petsko, S.J. Lippard and G.J. Quigley (1987) *Biopolymers* **26**: 1245–1271.
13. Kozelka, J., M.H. Fouchet and J.C. Chottard (1992) *Eur. J. Biochem.* **205**: 895–906.
14. Brabec, V., J. Reedijk and M. Leng (1992) *Biochemistry* **31**: 12397–12402.
15. Rice, J.A., D.M. Crothers, A.L. Pinto and S.J. Lippard (1988) *Proc. Natl. Acad. Sci. (USA)* **85**: 4158–4161.
16. Kozelka, J. and J.C. Chottard (1990) *Biophys. Chem.* **35**: 165–178.
17. Rampino, N.J. (1992) *Biochem. Biophys. Res. Commun.* **182**: 201–207.
18. Lippert, B., C.J.L. Lock, B. Rosenberg and M. Zvagulis (1977) *Inorg. Chem.* **16**: 1525–1529.
19. Pinto, A.L., L.J. Naser, J.M. Essigmann and S.J. Lippard (1986) *J. Am. Chem. Soc.* **108**: 7405–7407.
20. DeRose, J.A., D.B. Lampner and S.M. Lindsay (1993) *J. Vac. Sci. Technol.* **A11**: 776–780.
21. Rhodes, D. and A. Klug (1980) *Nature* **286**: 573–578.
22. Kozelka, J., G.A. Petsko, G.J. Quigley and S.J. Lippard (1986) *Inorg. Chem.* **25**: 1075–1077.
23. Pearlman, D.A., D.A. Case, J.C. Caldwell, G.L. Seibel, U.C. Singh, P. Weiner and P.A. Kollman (1991) *AMBER 4.0*. University of California: San Francisco.