

A novel assay for drug–DNA binding mode, affinity, and exclusion number: Scanning force microscopy

(intercalation/minor groove binding)

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Communicated by M. A. El-Sayed, Georgia Institute of Technology, Atlanta, GA, August 9, 1996 (received for review March 15, 1995)

ABSTRACT Determining the mode-of-binding of a DNA ligand is not always straightforward. Here, we establish a scanning force microscopic assay for mode-of-binding that is (i) direct: lengths of individual DNA–ligand complexes are directly measured; (ii) rapid: there are no requirements for staining or elaborate sample preparation; and (iii) unambiguous: an observed increase in DNA length upon addition of a ligand is definitive evidence for an intercalative mode-of-binding. Mode-of-binding, binding affinity, and site-exclusion number are readily determined from scanning force microscopy measurements of the changes in length of individual drug–DNA complexes as a function of drug concentration. With this assay, we resolve the ambiguity surrounding the mode of binding of 2,5-bis(4-amidinophenyl)furan (APF) to DNA and show that it binds to DNA by nonintercalative modes. APF is a member of an important class of aromatic dicationic drugs that show significant activity in the treatment of *Pneumocystis carinii* pneumonia, an opportunistic infection that is the leading cause of death in AIDS patients.

Nucleic acid ligands have been discovered and successfully engineered to act as anticancer drugs (1), probes of nucleic acid damage and structure (2), and as sequence-specific binding and cleavage agents (3, 4). Nucleic acid ligands are used in the treatment of genetic, oncogenic, and viral diseases. A molecular level understanding of nucleic acid interactions is a minimum first step in the development of useful new ligands.

Ligands bind to DNA (i) by intercalation, (ii) within the major or minor grooves, (iii) by “nonclassical” modes (5), or (iv) by a combination of these. Definitive assays for mode-of-binding are three-dimensional structure determination by x-ray diffraction or NMR spectroscopy. However, these structural techniques are labor-intensive, are often precluded by lack of site-specificity, rapid exchange, or multiple binding modes, and are limited to short DNA fragments. Assays suitable to long DNA fragments involve viscometry, sedimentation, and linear and circular dichroism. These methods are reliable when ligands bind by conventional intercalative or minor groove modes (6), but can be confounded by mixed and nonclassical modes. Difficulties arise in part because these assays are indirect and inferential.

Here we establish scanning force microscopy (SFM) as a direct, rapid, and unambiguous assay for mode-of-binding conventional and nonclassical ligands to DNA. SFM has been shown previously to be a high-resolution method for imaging nucleic acids (7–10). We have demonstrated that the site of binding of intercalators can be pinpointed with SFM (11). Intercalators stack between base pairs, lengthening DNA by an amount equivalent to the van der Waals thickness of each intercalating moiety (3.4 Å) (12). A ligand that does not lengthen DNA does not intercalate. Our SFM assay directly measures the lengths of individual DNA molecules. Increases

in DNA length upon ligand binding provides direct evidence for intercalation. Binding affinity and site-exclusion number are also readily determined from SFM measurements of the changes in length of individual drug–DNA complexes as a function of drug concentration. We validate this assay with two well-characterized intercalators: ethidium and daunomycin.

To illustrate the general applicability of this assay, we have determined the mode-of-binding of 2,5-bis(4-amidinophenyl)furan (APF). APF is an unfused aromatic dicationic ligand whose mode-of-binding to DNA was heretofore ambiguous. Molecules from this class exhibit very significant activity and low toxicity in the treatment of *Pneumocystis carinii* pneumonia, an opportunistic infection that is a leading cause of death in AIDS patients (13, 14).

MATERIALS AND METHODS

Images were obtained on a Nanoscope II or IIIa (Digital Instruments, Santa Barbara, CA). The DNA is 10.3-kb *p*BluBacHis b (*p*BBH b; Invitrogen, V370-20) bacterially amplified, purified by CsCl gradient, and linearized by *Hind*III (New England Biolabs). *p*BBH b samples were prepared by dilution of the stock solution in buffer (200 mM ammonium acetate/5 mM MgCl₂, pH 7.00). Ethidium (CAS 1239-45-8) and daunomycin hydrochloride (CAS 23451-50-6) were used as received from Sigma–Aldrich. Nanopure water (18.3 MΩ-cm) was used throughout. All operations with DNA ligands were carried out in subdued light. SFM substrates were freshly cleaved green muscovite (New York Mica, New York) disks. Disks were placed on top of a droplet of DNA or DNA/ligand solution (0.1 μg DNA per ml) and allowed to stand for 10–60 min. Each DNA-laden disk was dipped sequentially in water, 50:50 ethanol/water, and twice in anhydrous ethanol. Excess liquid was wicked away with a Kimwipe, and the disk was blown dry with clean compressed chlorofluorocarbon gas (Tech Spray, Amarillo, TX) directed normal to the disk surface. Disks were stored overnight under anhydrous conditions prior to imaging. Samples were imaged under a minimum constant force in the repulsive-contact regime as indicated by force-distance curves obtained frequently during the imaging procedure. Total forces encountered were typically <10 nN. Cantilevers with a force constant of 0.10 N/m and oxide-etched (i.e., sharpened) pyramidal Si₃N₄ probe tips of radius 20–40 nm (sharpened microlevers; Park Scientific, Sunnyvale, CA) were utilized. Images were obtained under a N₂ atmosphere (15). Contour lengths of DNA molecules were measured using the *x*–*y* distance measurement feature in the top view mode within the microscope’s off-line analysis software. DNA molecules with ambiguous topology were excluded.

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Abbreviations: SFM, scanning force microscopy, APF, 2,5-bis(4-amidinophenyl)furan; *p*BBH b, *p*BluBacHis b.
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RESULTS AND DISCUSSION

Clean SFM images of individual DNA molecules are afforded by our sample preparation technique. A representative image of a linear restriction fragment of ligand-free DNA is shown in Fig. 1A. The DNA topology is unambiguous and the length is easily measured. The measured lengths of 145 molecules are given in Fig. 1B. The rise per residue is nearly 3.4 Å, indicating that the molecules are predominantly in B-conformation (16). The small dispersion of measured DNA lengths indicates that intrinsic variation in length and random errors in length measurements are small.

The lengths of intercalated DNA molecules are clearly greater than those of ligand-free DNA molecules. A representative image of an ethidium-DNA complex is shown in Fig. 1C. Plots of DNA length versus ethidium concentration and versus daunomycin concentration are shown in Fig. 2. For both drugs, the DNA length increases with intercalator concentration until the DNA molecule becomes saturated with intercalator. Control experiments show that well-characterized minor groove binding drugs (e.g., distamycin) do not increase DNA length.

Exclusion numbers for ethidium and daunomycin are apparent from inspection of Fig. 2. Under saturating conditions, an exclusion number of 1 would lead to occupation of every potential intercalation site (at every dinucleotide step), doubling the length of B-form DNA. However, as ethidium concentration increases, DNA length asymptotically approaches 5250 nm, a 50% increase in length. This limiting increase indicates maximum occupation of 50% of potential intercalation sites, consistent with an exclusion number of 2. In contrast, as daunomycin concentration increases, DNA length asymptotically approaches 4670 nm, a 33% increase in length, consistent with an exclusion number of 3. Both of these SFM-determined exclusion numbers conform to previous crystallographic (17) and solution (18, 19) results.

Further, our SFM assay provides estimates of the binding affinity, K . The binding affinity of an intercalator for DNA can be described by the expression:

$$K = \frac{[\text{occupied intercalation sites}]}{[\text{unoccupied intercalation sites}][\text{free drug}]} \quad [1]$$

The fractional increase in DNA length at a given ligand concentration indicates the fraction of intercalation sites occupied. The above concentrations can be explicitly related to measured lengths by the expression:

$$K = \frac{\left(\frac{L - L_0}{a}\right)[DNA]}{\left\{\left(\frac{[DNA]B}{n}\right) - \left(\frac{L - L_0}{a}\right)[DNA]\right\}\left(I_0 - \left(\frac{L - L_0}{a}\right)[DNA]\right)}, \quad [2]$$

where L = length of the intercalated DNA molecule, L_0 = length of the unintercalated DNA molecule, I_0 = total intercalator concentration, n = exclusion number, B = number of base pairs per DNA molecule, and a = lengthening per intercalation event. When this equation was fit to the ethidium lengthening data (Fig. 2A), the binding affinity was calculated to be $6.6 (\pm 1.9) \times 10^4 \text{ M}^{-1}$ with an exclusion number of 2.01. When this equation was fit to the daunomycin lengthening data (Fig. 2B), the binding affinity was calculated to be $1.2 (\pm 0.1) \times 10^5 \text{ M}^{-1}$ with an exclusion number of 2.80. These binding affinities are consistent with previous results from other assays. The exclusion number for daunomycin is noninteger. Correia and Chaires (20) suggest that noninteger exclusion numbers indicate sequence-specific binding affinity. For both data sets, the lengthening per intercalation event was 3.4 Å.

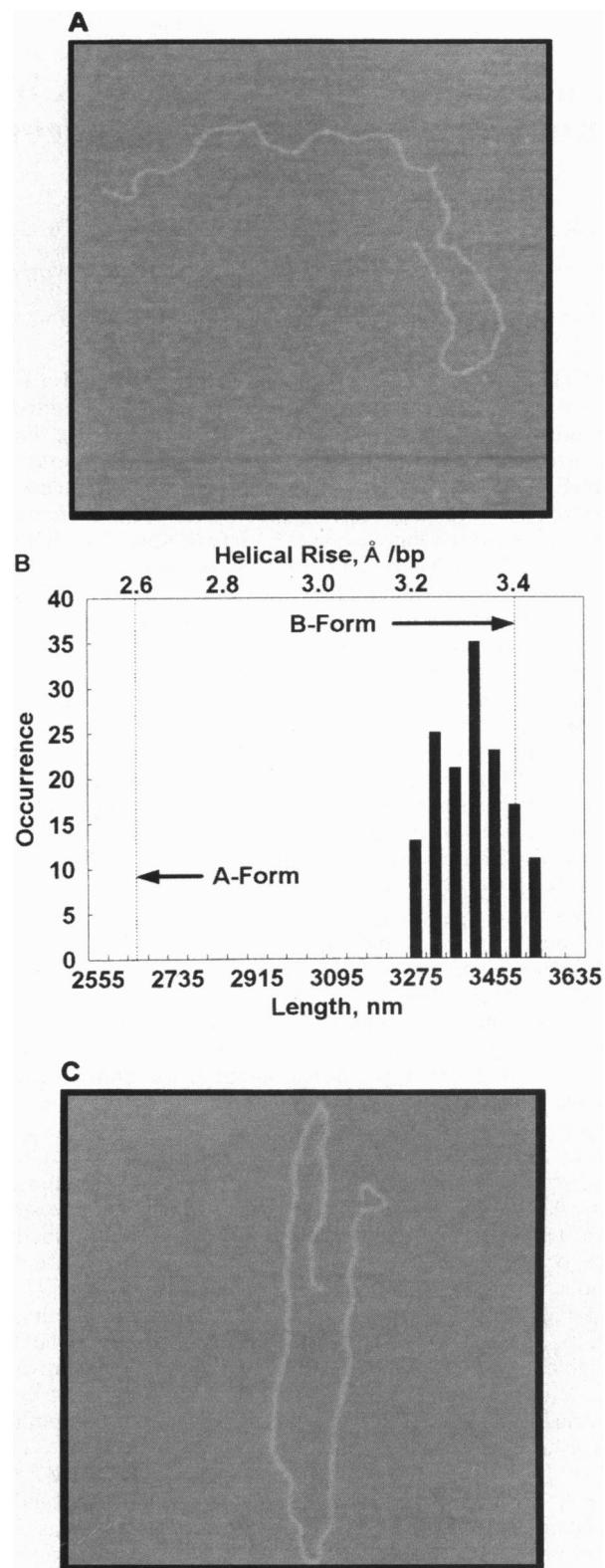


FIG. 1. (A) SFM image of ligand-free DNA. (B) Distribution of lengths of 145 individual, ligand-free duplex DNA molecules. (C) SFM image of ethidium-DNA complex. Image scales for both A and C are 2500 nm.

Our data can also be satisfactorily fit to Scatchard models (21) and to more sophisticated models incorporating cooperativity (ω) and site-exclusion (22) and heterogeneous binding constants (23). Nonlinear least squares fitting of the data in Table 1 to the McGhee and von Hippel model (22) including

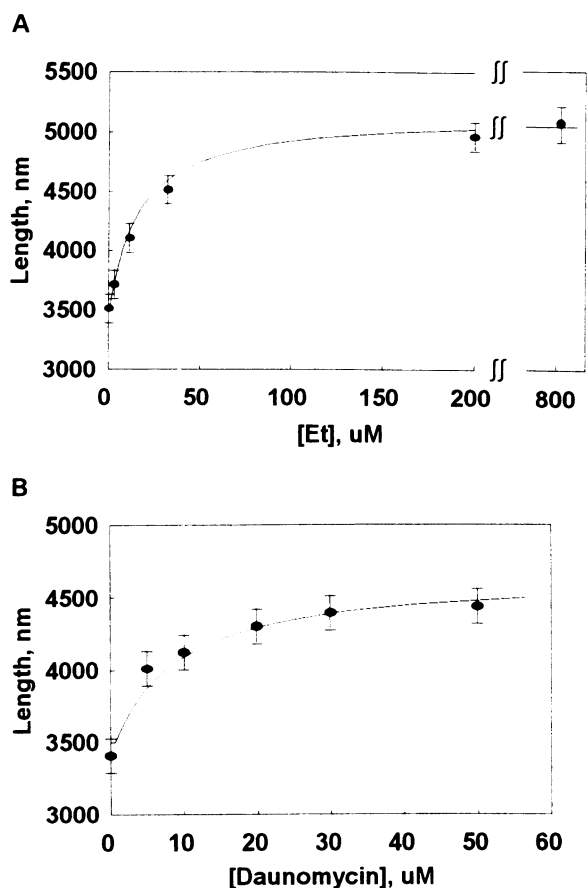


FIG. 2. Plots of DNA length versus intercalator concentration for ethidium (A) and daunomycin (B). Error bars represent $\pm 1\sigma$ about the mean length. Solid lines are the result of a least squares fit of Eq. 2.

size exclusion and cooperativity gave $K = 3.6 (\pm 0.5) \times 10^4 \text{ M}^{-1}$, $n = 2.01$, and $\omega = 1.05$ for ethidium and $K = 6.6 (\pm 2.4) \times 10^4 \text{ M}^{-1}$, $n = 3.04$, and $\omega = 2.17$ for daunomycin. Correia and Chaires (20) have shown that nonlinear fitting of models with an ω term may not be statistically justified because of the large covariance in n and ω . Thus, the mode and extent of binding of two well-characterized intercalators to DNA has been used to validate our SFM assay.

The assay is also applicable to ligands whose mode of interaction is not known or is ambiguous. On the basis of NMR

Table 1. Fraction of intercalation sites occupied at varying intercalator concentrations

Intercalator	Concentration, μM	Fraction of sites occupied*
Ethidium	0	0
	0.8	0.04
	3.5	0.10
	12	0.21
	33	0.32
	200	0.45
	800	0.52
Daunomycin	0	0
	5.0	0.17
	10	0.20
	20	0.25
	30	0.28
	50	0.29

Data are based on 10,300 bp for pBBH b and 3.4 Å lengthening per intercalation event.

*[pBBH b] = $1.62 \times 10^{-11} \text{ M}$.

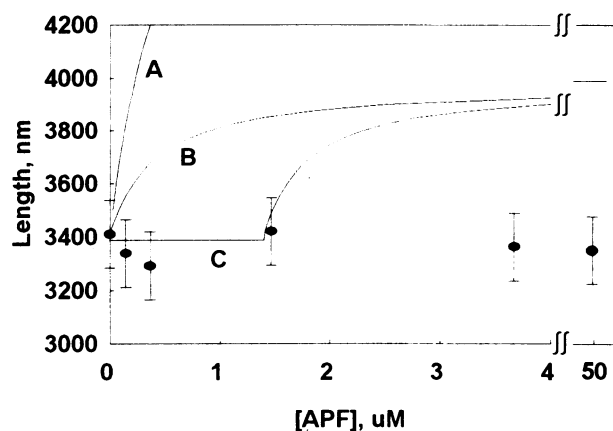


FIG. 3. Plot of DNA length versus concentration of APF. Trace A shows the expected lengthening if APF intercalates nonspecifically assuming an exclusion number of 2 and an affinity constant of $2.48 \times 10^6 \text{ M}^{-1}$. Trace B shows the expected lengthening if APF intercalates first at GC sequences and then binds in the minor groove at AT sequences with an affinity of $1.4 \times 10^7 \text{ M}^{-1}$. Trace C shows the expected lengthening if the occupancy of intercalation sites is maintained at zero until minor groove bound sites are 95% occupied.

chemical shift data, Boykin and coworkers (24) conclude that APF intercalates at CG sequences but binds in the minor groove of AT sequences. On the basis of linear dichroism data, Norden and coworkers (25) conclude that APF binds exclusively by nonintercalative modes. Samples of APF–DNA complexes were prepared and imaged in the manner employed for ethidium–DNA and daunomycin–DNA complexes. No DNA lengthening was observed for APF concentrations up to 50 μM (Fig. 3). This concentration range represents ratios of APF/DNA up to 3,400,000. Trace A in Fig. 3 represents the expected lengthening as a function of ligand concentration if APF intercalates nonspecifically. In calculating the expected lengthening, we assumed an exclusion number of two and used an affinity constant of $2.48 \times 10^6 \text{ M}^{-1}$ (24). Trace B is the expected lengthening if APF intercalates first at GC sequences and then binds in the minor groove at AT sequences with an affinity of $1.4 \times 10^7 \text{ M}^{-1}$ (24). Trace C is the expected lengthening if the occupancy of intercalation sites remains zero until minor groove bound sites are 95% occupied, a deliberate underestimate of DNA lengthening. Traces B and C predict that, at saturation, the DNA length will increase by 568 nm. Since no lengthening is observed, we conclude that APF does not intercalate.

To confirm the high affinity of APF and rule out the possibility that our experimental conditions inhibit APF binding, we performed competitive binding experiments. The results indicated that APF indeed binds to DNA under the conditions of our experiment and that binding of APF sterically encumbers intercalation of ethidium. When APF was incubated with DNA for 20 min. prior to addition of ethidium [at 2:1 or 1:1 (ethidium/APF) ratios], no lengthening of DNA was observed. When the order of addition was reversed, no lengthening was observed following extension of the incubation period to 3 hr. These observations suggest that APF binds in the minor groove. The minor groove of DNA is blocked by the ethyl and phenyl groups of ethidium, whereas the major groove is sterically unencumbered (26). Additional SFM competition experiments with major groove-blocking intercalators (in progress) should resolve the mode of APF binding.

CONCLUSIONS

The lengthening of DNA resulting from intercalation is readily quantified with SFM. By determining lengths of individual DNA–drug complexes, fraction of sites occupied, binding

affinities, exclusion numbers, and cooperativity parameters can be determined. This new assay is rapid, direct, and especially applicable to DNA fragments longer than 300 bp. Using this assay we show that APF does not intercalate into DNA. The mode-of-binding of other problematic ligands can now be easily addressed. Indeed, SFM experiments to resolve a mode-of-binding ambiguity relating to tris(*o*-phenanthroline)Ru(II) and other important DNA binding ligands are currently in progress in our laboratories.

We are indebted to Profs. W. D. Wilson, S. May, and J. B. Chaires for helpful comments and suggestions. We gratefully acknowledge partial support of this research by the Office of Naval Research-sponsored Georgia Institute of Technology Molecular Design Institute and Georgia Institute of Technology's Polymer Education and Research Center. Support of this work was provided by a grant from the American Cancer Society (NP-912 to L.D.W.), by the Bristol-Myers Squibb Company through a Research Opportunity Award presented by Research Corporation to L.A.B., and by a Fellowship from the Analytical Division of the American Chemical Society sponsored by Eli Lilly Corporation to J.E.C.

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