Effects of Pressure on the DNA Minor Groove Binding of Hoechst 33258

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We investigated the pressure perturbations of DNA minor groove binding of the antibiotic drug Hoechst 33258 (2′-(4-hydroxyphenyl-5-(4-methyl-1-piperazinyl)-2,5′-bi-1*H*-benzimidazole trihydrochlolride) on fluorescent titrations and on restriction endonuclease protection assays. Significantly, pressure elevations were found to disturb the binding of Hoechst 33258 with poly(dA)·poly(dT) and poly[d(A–T)]·poly[d(A–T)] in opposite directions, which suggested different volume changes (sign and size) in their complexation reactions. The ability of Hoechst 33258 to provide the restriction endonuclease protection for plasmid DNA was notably enhanced by the application of pressure, implying that there is a large volume contraction following the EcoRI site-drug interaction. These data were explained by DNA hydration and its effects on the ligand complexation that is sequence dependent.

Hoechst 33258# (2′-(4-hydroxyphenyl-5-(4-methyl-1-piperazinyl)-2,5′-bi-1*H*-benzimidazole trihydrochlolride, Fig. 1) is a B-DNA minor groove binding drug which targets successive dA/dT base pairs (bps).[1] The minor groove binding destroys the hydration spine in the minor groove and induces moderate modifications in local DNA structures.[2] Upon the DNA binding, the fluorescence spectroscopic parameters of Hoechst 33258 undergo major changes, such as the great enlargement in quantum yields and a notable spectral shift, depending on the base composition, the binding ratio, and the microenvironment.[3] Binding of Hoechst 33258 to the narrow groove may be an obstacle to protein–DNA interactions with sequence- and/or groove-specificity. This gives Hoechst 33258 a physiological role and may be useful in the analysis of protein–DNA interactions in vitro.

Shifts of the DNA hydration equilibrium following the minor groove binding may be important for the structural dynamics and the ligand binding of DNA.[4,5] They can be experimentally demonstrated as volume changes during the binding reaction by several methods; and this can give information about the relationships of DNA hydration to sequence, structure, and environment and can suggest functional consequences.[6—8] These methods include perturbations of the ligand binding of DNA by high hydrostatic pressure.[9] High pressure has been found to increase the stability of DNA duplex and triplex structures against thermal melting via a negative volume change upon the stacking of base pairs,[10,11] while it may potentially disturb DNA interactions with protein or other ligands, probably owing to the tendency of the complex interface to be further solvated. As for the interaction of DNA with low molecular weight reagents, some pressure-related studies have been done for intercalating reagents such as ethidium bromide.[12,13] Here we have focused on the DNA-association behavior of a minor groove binding drug, Hoechst 33258. The use of density or acoustic measurements has been applied to the DNA minor groove binding of netropsin and distamycin, but not to that of Hoechst 33258, possibly because of the self-aggregation tendency of Hoechst 33258 in the high concentrations which are required by using these two methods.[6,7]

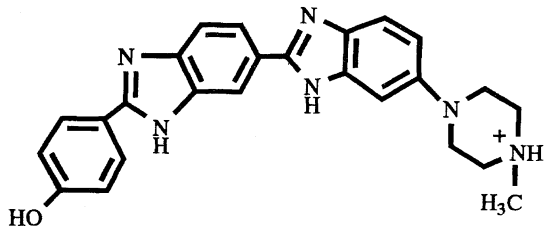

Fig. 1. Structure of Hoechst 33258.

Perturbations of high hydrostatic pressure are very useful for studying the mechanisms of interactions between biological systems.[12] Research results related to nucleic acids have shown that high pressure can modify not only enzyme catalysis efficiency but also enzyme specificity under certain conditions.[14,15] One important control factor that is responsible for pressure effects may be the hydration of DNA and its variations. This has been analyzed in previous studies of the interactions of DNA with enzymes and small ligands.[13,15] In this paper, we examine the DNA binding of Hoechst 33258 using pressure perturbations of the fluorescent titration of poly(dA)·poly(dT) and poly[d(A–T)]·poly[d(A–T)] and of the restriction endonuclease protection assay (REPA) of the pBR322 DNA. We found that the binding of Hoechst 33258 with the two synthetic polymer isomers was differently affected by the elevation of pressure, suggesting that their

base pairs, bps; 4′,6-diamidino-2-phenylindole, DAPI; 2′-(4-hydroxyphenyl-5-(4-methyl-1-piperazinyl)-2,5′-bi-1*H*-benzimidazole trihydrochlolride, Hoechst 33258; restriction endonuclease protection assay, REPA; standard molecular volume changes, ΔV_0.

respective complexations feature different volume changes. Our fluorescence titration results were affirmed by observations of the pressure-increased ability of Hoechst 33258 to protect EcoRI endonuclease cleavage. These results can be explained by DNA hydration and its effects on the ligand complexation that is sequence dependent.

Materials and Methods

Poly[d(A)]·poly[d(T)] and poly[d(A–T)]·poly[d(A–T)] were purchased from Sigma and Pharmacia, respectively. Their concentrations were determined through absorption measurements with their respective molar extinction coefficients per molar base pairs (bps) of 12000 $M^{-1}\,cm^{-1}$ and 13200 $M^{-1}\,cm^{-1}$ (1 M = 1 mol dm^{-3}). The pBR322 DNA was purchased from New England Biolabs. The EcoRI endonuclease was obtained from Toyobo Corp. (Osaka). Its activity was determined by the manufacturer and the DNA hydrolysis reaction was done in an H-buffer. Hoechst 33258 was obtained from Wako Chemicals Co. (Osaka). A stock solution of Hoechst 33258 in 10 mM Tris-Cl buffer (pH 7.2) was stored in the dark below 0 °C. The working solutions were freshly diluted before use and the concentration was determined by using ε = 42000 $M^{-1}\,cm^{-1}$.

The fluorescent titration of Hoechst 33258 by DNA was processed at 25 °C by the addition of concentrated solutions of poly[d(A)]·poly[d(T)] or poly[d(A–T)]·poly[d(A–T)] (0.1 μM to 1 mM) in 10 mM Tris-Cl buffer (pH 7.2) and 1 mM EDTA. Increases in the 460 nm-fluorescence intensity (λ_{ex} = 360 nm) of Hoechst 33258 upon the DNA-association in ambient and high pressure were monitored in an optical pressure vessel (Teramecs, Kyoto) that was located in the sample chamber of a RF5000 spectrofluorometer (Shimadzu, Kyoto) with a circulating temperature bath. The titration curves were used to obtain the equilibrium association parameters by analyzing the date according to the exclusion-site model:[17]

$$\frac{\nu}{[L]} = \frac{K_{app.}(1-n\nu)[(2\omega-1)(1-n\nu)+\nu-R]^{n-1}[1-(n+1)\nu+R]^2}{[2(\omega-1)(1-n\nu)]^{n-1}[2(1-n\nu)]^2}, \quad (1)$$

$$R = \left\{[1-(n+1)\nu]^2 + 4\omega\nu(1-n\nu)\right\}^{1/2}. \quad (2)$$

Here ν is the ligand binding density on the polynucleotide lattice, n (the site size) is the number of bps occluded by the further binding of a ligand, ω is the cooperativity parameter, and $K_{app.}$ is the apparent association constant of a ligand binding to a naked DNA lattice. The dilution effect following titration and the compression effect from pressure on the solution volume were corrected for. The pressure dependences of the association constants of Hoechst 33258 and the polynucleotide were used to determine the standard molar volume change (ΔV_0) according to the standard thermodynamic relationship:

$$\partial\,[\ln(K_{app.})]\,/\partial(P)_T = -\Delta V_0/RT, \quad (3)$$

were R is the gas constant (8.314 $cm^3\,MPa\,K^{-1}\,mol^{-1}$) and T is the absolute temperature (K).

The restriction endonuclease protection assay was done as described in our previous paper.[9] By assuming a simplified relationship between the drug binding to the EcoRI site and the protection efficiency,[16] we determined the apparent binding constants of Hoechst 33258 and the pBR322 DNA from the following equation:

$$(1-P)/(P-P_0) = 1/K_{app.}\,[L], \quad (4)$$

where P and P_0 are the portions of uncut DNA in the presence and in the absence of the drug, and [L] is the concentration of the drug not binding to the EcoRI site and its flanking sequences. For the practical determination of this value, it was assumed that binding of Hoechst 33258 to the non-EcoRI sites and non-flaking sequences rarely contributed to the restriction endonuclease protection, so that the [L] value can be written as $(1-P)[L_0]$. The exact situation may be simplified by this, but it does permit one to obtain a semi-quantitated estimation of the binding constants of Hoechst 33258 and DNA.[8,9]

Results

Fluorescent Titrations of Hoechst 33258 with Polynucleotides: Figure 2 shows the fluorescent titration of Hoechst 33258 with poly(dA)·poly(dT) and poly[d(A–T)]·poly[d(A–T)], respectively, in 10 mM Tris-Cl buffer including 100 mM NaCl under pressure. As expected, binding to dA·dT polymers greatly enhanced the Hoechst 33258 fluorescence and caused notable shifts in the fluorescent excitation and emission spectra to longer and shorter wavelengths, respectively. With the same NaCl concentration, the peak wavelength of fluorescence emission spectrum of the drug-homopolymer complex was shorter than that of the drug-copolymer by about 5 nm, suggesting a smaller polarity around the drug binding sites in the homopolymer. Under

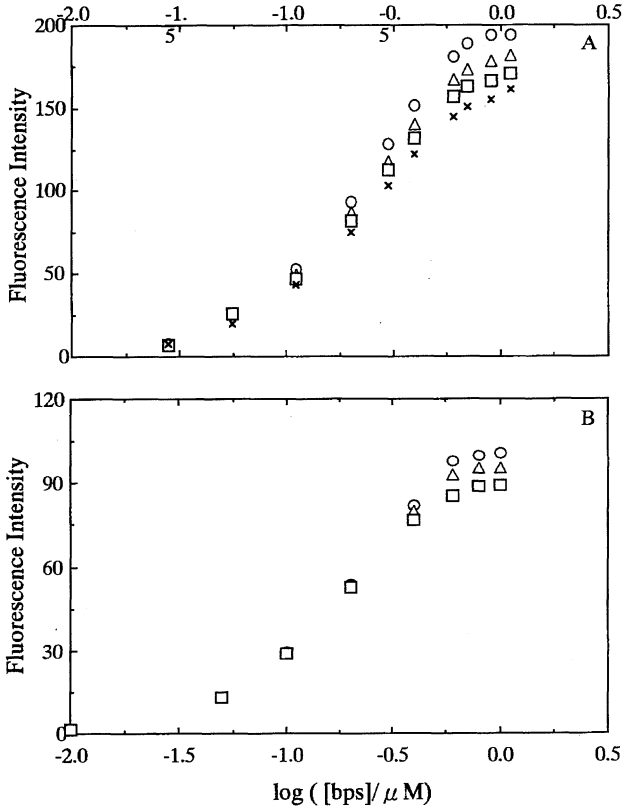

Fig. 2. Typical fluorescence titrations of Hoechst 33258 (100 nM) with poly(dA)·poly(dT) (A) and poly[d(A–T)]·poly[d(A–T)] (B) in 10 mM Tris-Cl buffer (pH 7.2, containing 100 mM NaCl and 1 mM EDTA) in 25 °C. Pressure: 0.1 (○), 80 (△), 160 (□), and 240 (×) MPa in A; 0.1 (○), 100 (△), and 200 (□) MPa in B, respectively.

high pressure, the fluorescence intensity of Hoechst 33258 saturated by DNA was changed slightly and the emission peak wavelength was slightly red-shifted as compared to the values under atmospheric pressure, possibly reflecting an additional pressure effect besides the shift in the complexation equilibrium. Pressure-induced changes in the fluorescence of the Hoechst–DNA complex were reversible. No major changes in the fluorescence spectroscopy of Hoechst 33258 free in the solution was found. Although the DNA duplex structure is pressure-stabilized and the compressibility of DNA is minimal, we may not completely exclude the possibility of pressure-induced fluctuations in certain DNA conformational parameters.

Our analysis of the pressure dependence of binding isotherms of the two dA·dT polymers that was simulated according to the exclusion model showed different pressure effects on the groove binding of Hoechst 33258; elevated pressure appeared to increase the association affinity of Hoechst 33258 for the copolymer, while it appeared to reduce its affinity for the homopolymer under the same conditions (Figs. 3A and 3B). According to previous studies, increases in the ratio of Hoechst 33258 to the dA·dT copolymer led to the formation of DNA–drug complexes with multiple stoichiometries.[18] When this drug to DNA ratio was lowered, homogenous complexes mediated by sequence specificity were formed. In fact, in those titration domains with low DNA/drug ratios, the apparent drug–DNA binding constants showed minimal variations with pressure increases. The present binding constant obtained for the copolymer in 0.1 MPa is at the same level as that found by Loontiens et al.[18] Based on the pressure dependence of binding constants, volume changes were determined for the binding of Hoechst 33258 with the two dA·dT polymers (insets in Figs. 3A and 3B). Corresponding to their opposite pressure dependences, a positive volume change ($+6.1\pm1.2$ cm^3 mol^{-1}) was determined for the binding of Hoechst 33258 with the dA·dT homopolymer, while a negative volume change (-3.4 ± 0.3 cm^3 mol^{-1}) was calculated for the binding with the copolymer (Table 1). Significant differences in volume changes between the two DNA polymers upon their binding with netropsin have also been observed by other researchers;[6,7,19] and this seems to be connected to structural and hydration features of specific DNA. Since the minor groove binding leads to repulsion of the spine of hydration from the DNA minor groove and results in a positive volume change, the present results from the copolymer indicate the presence of additional volume factors that can reverse the consequence of repulsing the hydration spine.

Pressure Perturbations of REPA: REPA is a very useful method for studying the ligand interactions of nucleic acids.[21,22] The use of REPA is primarily based on the competition between the binding elements that share the same DNA sequences and/or structural motifs. Previously, we showed that REPA can be used generally for analyses of the pressure perturbations of minor groove binding with drug dose dependence.[9] In this paper, we show that elevated pressure increased the EcoRI protection ability of Hoechst 33258 for

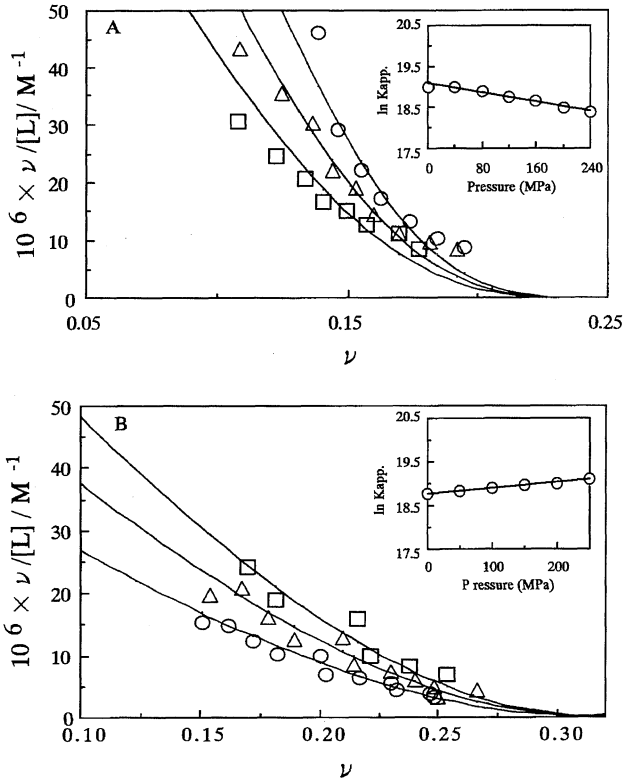

Fig. 3. Typical binding isotherms for Hoechst 33258 binding with poly(dA)·poly(dT) (A) and poly[d(A–T)]·poly[d(A–T)] (B). Experimental data were simulated according the exclusion-site model (Eqs. 1 and 2). Fitting parameters in A are: $n = 4.0$, $\omega = 0.7$, and $K_{app.}$ ($\times 10^8$ M^{-1}) = 2.3 (0.1 MPa, ○), 1.7 (120 MPa, △), and 1.3 (240 MPa, □), respectively. Fitting parameters in B are: $n = 3.4$, $\omega = 0.5$, and $K_{app.}$ ($\times 10^8$ M^{-1}) = 1.4 (0.1 MPa, ○), 1.6 (100 MPa, △), and 2.0 (200 MPa, □), respectively.
Inset: The relationship between pressure and the apparent association constant of Hoechst 33258 with poly(dA)·poly(dT) (A) or poly[d(A–T)]·poly[d(A–T)] (B).

the intact pBR322 DNA (Fig. 4). The drug concentrations required to produce 50% protection (PC_{50}) decreased notably from about 56 μM at 0.1 MPa to 11 μM at 150 MPa. The observed pressure dependence of enzyme protection efficiency led us to determine the molecular volume changes of the drug–Plasmid DNA association to be nearly -26 cm^3 mol^{-1} (inset in Fig. 4). Comparisons between the results here and those of other binding drugs (i.e., distamycin and DAPI) are summarized in Table 1.

We noticed that the value of volume changes upon binding this drug to natural DNA was larger than that upon binding to synthetic sequences; the value was determined by fluorescent titrations. This discrepancy may be due to inherent differences between the two methods and different systems. It is worthy of note that the volume changes determined from REPA and from the fluorescent titration with the copolymer share the same sign, probably owing to similarities between the copolymer and the natural DNA in the thermodynamics of hydration and ligand interactions.[6,19]

Table 1. Standard Volume Changes upon the DNA Minor Groove Binding of Hoechst 33258 Compared with the Reported Values of Other Drugs

DNA	Method	ΔV_0 (cm^3mol^{-1})			
		Hoechst 33258	Netropsin	Distamycin	DAPI
Poly[d(A–T)]·poly[d(A–T)]	Fluorescence	−3[a]			
	Densimetry		−1[b]		
	Acoustics		0[c]		
Poly(dA)·poly(dT)	Fluorescence	+6[a]			
	Densimetry		+68[b]		
	Acoustics		+5[c]		
pBR322 DNA	REPA	−26[a]		−55[d]	−10[e]
Chicken erythrocyte DNA	Circular Dichroism		−45[f]		

a) This work. b) From Ref. 6, 116 mM NaCl. c) From Ref. 7, 116 mM NaCl. d) From Ref. 9. e) Roughly estimated from data in Ref. 9. f) Estimated from the changing number of water molecules in Ref. 8 for a netropsin analog, 100 mM NaCl.

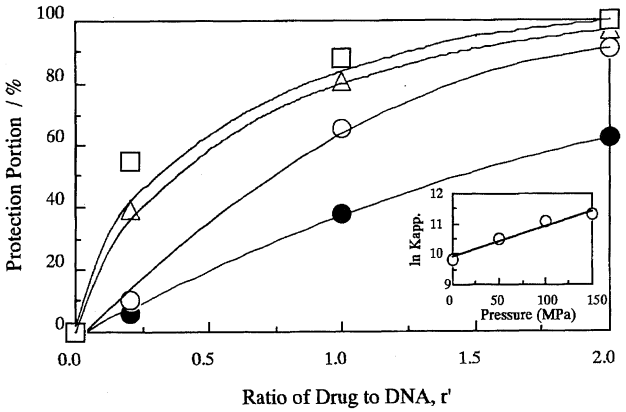

Fig. 4. Hoechst 33258 does-dependent protection curves for the EcoRI endonuclease cleavage of the pBR322 DNA at 0.1 MPa (●), 500 MPa (○), 100 MPa (△), and 150 MPa (□) in 37 °C. DNA: 39 μM bps; EcoRI: 0.1 U/μL; time for cleavage: 2 h.
Inset. The relationship between pressure and the apparent association constant of Hoechst 33258 with the EcoRI site as estimated from the data by using Eq. 4.

Discussion

Fluorescent Titration: It is known that the hydration spine lying in the minor groove of B-DNA sequences is replaced by a hydrogen-bonding network that is newly formed between the minor groove binding drug and the bases facing the groove.[23] Both the spine of hydration and the subsequent minor groove binding reagent contribute to the stability of the DNA helix structure.[24,25] Due to the electrostriction effects of solvated water, releasing the bound water from DNA into the bulk solution during the minor groove binding produces negative volume changes. A great reduction in the volume parameters have been observed in the minor groove binding to poly(dA)·poly(dT), which is largely hydrated and has an abnormal B-type structure.[24] Dickerson's group has suggested that the overall loss of water which originally resided in DNA and in netropsin was as great as 17 molecules;[23] such a loss was assumed to be responsible for a large entropy increase in the netropsin-homogenous sequences reaction.[19,23] However, binding of several drugs such as netropsin and distamycin with heterogeneous sequences are found to have small positive and eventually negative volume changes and to be enthalpy-driven in thermodynamics.[6–9,13,19,20] Besides different helix conformations of these DNA duplexes, the existences of additional volume changes is considered to reverse the early volume changes by destroying the spine of hydration during the minor groove binding. It is likely that the coexistence of these factors leads to divergent pressure effects on the binding of Hoechst 33258 to the two DNA polymer isomers.

The results of negative volume changes upon minor groove binding experimentally obtained before and here contradict the notion of destruction and replacement of the spine of hydration in the minor groove which has been well established. It seems that changes of hydration may take place on other sites of DNA and may mediate the connections to the binding drug. For example, subsequent to the minor groove binding, the solvent accessibility to Hoechst 33258 is created by a new hydration network formed around the terminal NH_3 of this drug.[2] In some cases, water molecules are involved in the direct hydrogen bonding to bridge the drug and base pairs in the minor groove.[5] However, the factors that contribute to the final differences in volume changes between the two types of DNA polymers are not clearly defined. They seem to include the original hydration differences between the free molecules of homopolymer and copolymer in solution and in fiber. Following the minor groove binding, earlier discrepancies in the DNA structure and hydration between the two polymers were supposed to be alleviated.[26] The observed differences in overall volume changes upon the drug reactions thus seemed to be mainly due to their original hydration states rather than due to the final complexes of drug molecules.[6,13] This assumption has been disputed because the netropsin–dA·dT copolymer complex was actually more hydrated than the netropsin-homopolymer complex.[7] Given a previous study indicating a connection of the fluorescent stokes shift of Hoechst 33258 to the polarity around its binding site,[27] the present findings of a more blue shift in the fluorescence emission spectra of Hoechst 33258 complexed with poly(dA)·poly(dT) relative to that complexed with poly[d-

(A–T)]·poly[d(A–T)] suggest reversed hydration differences between the two dA·dT polymers during their individual reactions with Hoechst 33258. Since the minor groove width of the copolymer is larger than that of the homopolymer, there may be more exposure of the organic groups of Hoechst 33258 in its copolymer complex to the solvent, which might lead to a structural rearrangement in the hydration water covering the minor groove. Another volume contribution may come from the binding-induced release of counterions from the DNA lattice.[7] The re-solvation of free counterions induces negative volume changes that counteract the effects of releasing bound water into the bulk solution from the DNA and the drug molecules. Furthermore, the possibility of attracting additional water into the complex of heterogeneous sequences has been strongly suggested by other researchers through their investigations by titration calorimetry and circular dichroism spectroscopy.[8,20,28]

Principally, our present data indicate that the two DNA polymers could be discriminated by the minor groove binding such as that of Hoechst 33258 with distinct hydration changes of opposite signs and a difference value of 9.5 $cm^3\ mol^{-1}$ at 100 mM NaCl. From the discussion above, we conclude that different volume changes reflect the original structural and hydration properties and the subsequent reorganizations of hydration structure upon the ligand binding. It may be impractical to obtain crystal structures of Hoechst 33258 with sequences of pure dA·dT base pairs. Part of the structural information of such complexes may be reached by study of the Hoechst 33258 complexes of $d(CGCGAATTCGCG)_2$ and $d(CGCGATATCGCG)_2$. Binding of this drug behaves similarily for the two sequences; however, the accurate binding sites have a 3′-direction shift by one base pair.[5] It is likely that the orientations of Hoechst 33258 along the minor groove are opposite in the two complexes. The numbers of hydrogen bondings from a set of amide NH hydrogens bridging between adenine N3 and thymine O2 acceptor on opposite strands are 1 and 3 in the complex of AATT sequence, compared to 1 and 2 in the complex of ATAT sequence. It is believed that Hoechst 33258 undergoes multiple conformational changes even bound to the minor groove, so that the crystal structures are averaged among disordered states.[5]

REPA: The observation that elevated pressure enhanced the endonuclease protection ability of Hoechst 33258 was in accordance with the fluorescent titration data of Hoechst 33258 binding to the copolymer. The apparent binding constant for Hoechst 33258 with the EcoRI site (GAATTC) that was measured by REPA was much lower than that for the two dA·dT polymers measured by the fluorescent titration. This is likely due to the shielding effect of a pool of nonspecific sites and the competition effect of other potential binding sites. It is not unusual that the measured binding constants of small chemical reagents and natural DNA molecules are relatively low when compared with those values of DNA samples containing simple or short sequences. The limitation inherent in the method of REPA is another factor which prevents one from exactly quantifying the binding constant. When we turn to calculate volume changes upon the ligand–DNA complexation, however, these limitations can be effectively alleviated by resorting to the relative values of apparent binding constants. As was shown previously, the pressure effect can be explained by a net negative volume change upon the minor grooving binding event rather than by the inhibition of enzyme activity.[9,15] This can be seen as due to the minimal effects of increasing pressure up to 150 MPa on keeping the pBR322 DNA intact in the presence of EcoRI endonuclease. Since the copolymer acts more like normal DNA while the homopolymer acts abnormally,[4,19] the enhancement of endonuclease protection ability is in line with fluorescent titration results for the copolymer. A previous study where the application of osmotic stress inhibited the binding of a netropsin analog with natural DNA also gives credence to the present data.[8] The additional uptake of a large number of water molecules into the complex was thus suggested both by the positive effects of high pressure here and by the negative effects of osmotic stress[8] on the minor groove drug–DNA complexation.

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