Using Ethidium Bromide To Probe the Interactions between DNA and Dendrimers†

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Investigations have been carried out to probe the interactions between double-stranded DNA and amine-terminated, ethylenediamine core polyamidoamine starburst dendrimers generation 2 (G2), generation 4 (G4), and generation 7 (G7). Ethidium bromide is used as a fluorescent probe for this study. Under our experimental conditions, the results show that there is mainly one binding mode between ethidium and DNA−dendrimer complexes and that dendrimers bind to DNA sufficiently strongly that they cannot be displaced by ethidium. In the proposed binding model for DNA−dendrimer complexes, the DNA wraps around G7 dendrimers while the wrapping of DNA does not occur for G2 and G4 dendrimers. DNA−dendrimer interactions display little preference on DNA base components. The influence of pH and ionic strength is in agreement with electrostatic interactions as the major binding force for DNA−dendrimer complexes.

Introduction

Macromolecular associations between nucleic acid hosts and polycationic guests are important in many biochemical issues such as molecular recognition, biological transport, and cell repair.[1−3] Clear examples of these associations can be found in the electrostatic host−guest interactions of DNA and histones, or of DNA and spermidines, both of which serve vital roles in natural biological processes such as DNA stabilization, transcription regulation, and drug delivery.

Starburst dendrimers (SBDs) are a relatively new class of polymers possessing radially branched amidoamine units emanating from a central core, whose structure is created by the stepwise attachment of layers, termed generations.[4] Figure 1 shows the two-dimensional schematic structures of a generation 2 dendrimer (termed G2) and a generation 6 dendrimer (termed G6) with an ethylenediamine core as representatives of "earlier" and "later" generation dendrimers, respectively.[5] SBDs are highly soluble in aqueous solution and have unique well-defined three-dimensional structures for G0 to G10, ranging in size from ca. 10 Å to 130 Å. At physiological pH, amine-terminated dendrimers are partially positively charged,[6,7] which makes it possible for SBDs to interact electrostatically with biological polyanions such as nucleic acids. *In vitro* experiments have shown that dendrimers G5 to G10 can act as gene transfer vectors to chaperone DNA through cell membranes and to achieve highly efficient gene transfection with low cytotoxicity in a wide range of mammalian cell lines.[8,9] It was also found that dendrimers can assist regulation of *in vitro* gene expression by transferring antisense oligonucleotides or antisense expression plasmid DNA into cells.[10] G7 to G10 dendrimers have diameters of 80−130 Å and appear to mimic histones in both size and shape. This might account for the stability of DNA−dendrimer complexes as well as for the enhanced gene transfection observed for these high-generation dendrimers.[11]

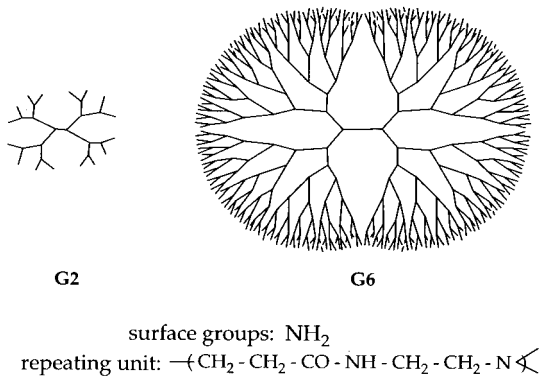

Figure 1. Two-dimensional schematic structures of dendrimer G2 and G6.

To date, there have been few investigations on DNA−dendrimer interactions. Bielinska et al. studied the interactions between plasmid DNA and polyamidoamine dendrimers by examining their electromicroscopic structures, nuclease sensitivity, and transcriptional activity.[12] In another study, researchers used potentiometric and turbidimetric titrations to measure the pH dependent

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binding of polypropyleneimine dendrimers with polyanions, including DNA.[13]

We initiated a systematic investigation of two- and three-way interactions of simple models of DNA, dendrimer, and cell membrane since such studies would provide a fundamental understanding of the gene transfection mechanism. Further, the studies would serve to improve the design of new gene transfer vectors. Beginning with a study on the structural properties of the DNA−dendrimer complexes, we seek to understand the DNA−dendrimer interaction on the molecular level in terms of DNA conformation and accessibility of DNA to probe molecules after binding to dendrimers.

Ethidium bromide (EB) is a planar aromatic dye which readily intercalates between the base pairs of DNA.[14] It displays little base pair preference, with moderate binding affinity depending on the ionic strength ($K_{EB} = 10^4-10^6$ M^{-1}).[15] Because the intercalation of EB unwinds the DNA base pairs near the intercalation site by 26°, it provides information on the conformational flexibility of DNA.[16] EB is a very convenient fluorescence spectroscopic probe of DNA conformation and is widely used to study the binding properties of other species to DNA through competition studies.[15,17] Moreover, it has been used as a probe to determine the structure of DNA inside chromatin.[1,18] By using EB as a fluorescent probe, we seek to characterize the binding interactions between DNA and dendrimers.

Here we report our study on DNA−dendrimer interactions probed by the fluorescence of EB. In order to rationalize the dependence of the gene transfection efficiency on dendrimer generations, dendrimers G2, G4, and G7 were investigated as representatives of "earlier", "middle", and "later" generation dendrimers, respectively.[5] Synthetic, double-stranded poly(deoxyguanylic-deoxycytidylic acid) ([poly(dG-dC)]$_2$) and poly(deoxyadenylic-thymidylic acid) ([poly(dA-dT)]$_2$) were compared with calf thymus DNA (ctDNA) to clarify the dependence of DNA−dendrimer interactions on DNA base components. Ionic strength dependence and pH dependence were tested to investigate the electrostatic interactions between DNA and dendrimers. Our study shows that dendrimers bind to DNA sufficiently strongly that they cannot be displaced by EB. A binding model is proposed here according to the experimental results.

Experimental Section

Materials. The synthesis and characterization of starburst dendrimers employed in this study have been previously described in detail.[4] The core of the dendrimers is ethylenediamine, and the surface groups are primary amines (Figure 1). The dendrimer samples were stored in methanol solution. EB was purchased from Aldrich and used as received. CtDNA (Sigma) was purified by standard methods.[19] Synthetic double-stranded [poly(dG-dC)]$_2$ and [poly(dA-dT)]$_2$ (Pharmacia Biotech), in which the purine and pyrimidine bases alternate on both strands, were suspended in PBS and gently stirred for 1 h. The determination of the concentrations of EB and DNAs was based on their UV−vis absorbance. Concentrations of DNA and dendrimers are given in terms of nucleotides and surface amine groups, respectively.[8]

Experiments were performed in PBS (10 mM phosphate, 100 mM NaCl, pH 7.5) except those mentioned specifically with different pH values or different NaCl concentration.

Methods. UV−vis absorbances were measured with a Hewlett-Packard 8452A diode array spectrometer. Steady-state fluorescence experiments were performed with a SPEX FluroMax-2 spectrometer.

Fluorescence titrations of EB with DNA in the presence of various amounts of dendrimers were performed as following: Samples typically consisted of 10 μM DNA in 2 mL of PBS and were titrated with 5 or 10 μL aliquots of 200 μM EB. Emission spectra were collected from 540 to 750 nm with 510 nm excitation at 25 °C. The integrated fluorescence spectral area was used for the data analysis. The data were corrected for the volume changes during titration and then analyzed according to the method of LePecq and Paoletti to obtain the bound (C_b) and free (C_f) concentrations of EB.[15] Scatchard plots were made by plotting r/C_f vs r (where $r = C_b/[DNA]$).

Another type of fluorescence titration was also carried out by adding dendrimers into a premixed solution of EB and DNA for comparison.

Results and Discussion

Fluorescence Titration of EB to CtDNA−Dendrimer Complex. The binding of dendrimers to ctDNA was investigated by the fluorescence titration of EB with a premixed solution of DNA and various amounts of dendrimers. In the presence of DNA, the fluorescence emission of EB is enhanced relative to that in water as a result of the intercalation of EB between DNA base pairs.[15] The addition of dendrimers to DNA prior to EB titrations results in DNA−dendrimer binding and hinders access of EB to a certain portion of the binding sites available on the DNA helix. The competition of dendrimers with EB for binding sites on ctDNA was studied for G2, G4, and G7 dendrimers. Scatchard plots of EB titration with ctDNA−dendrimer complexes were constructed, as shown in Figure 2. The data plotted in Figure 2 were fitted to the theoretical expressions for the McGhee and von Hippel conditional probability model of excluded site binding according to eq 1.[20] Fitting data to this equation

$$\frac{r}{C_f} = 0.5K_{EB}(1 - 2sr)\left[\frac{1 - 2sr}{1 - 2(s - 1)r}\right]^{(s-1)} \qquad (1)$$

yielded a binding constant for the binding of EB on DNA (K_{EB}) and the average size of binding sites in terms of DNA base pairs (s). From the s value we can obtain the number of binding sites available to EB per DNA nucleotide (r_{max}), which equals 1/2s. Standard errors were within 5% from our data sets, and the parameters K_{EB} and r_{max} are listed in Table 1.

There are several significant features of the competitive binding of EB and dendrimers to DNA. First, the fact that all of the experimental data fit very well with eq 1 is consistent with only one major binding mode of EB to DNA even when DNA is bound to dendrimers. This conclusion can be rationalized by assuming that the DNA−dendrimer complexes consist of "tightly bound DNA" regions for which the intimate contact is made between DNA and the dendrimer surface and "linker DNA" regions located between the "tightly bound DNA" regions. Because of the distortion of DNA caused by EB intercalation and the restricted mobility of DNA in the "tightly bound DNA" regions, EB can only bind to the more flexible "linker DNA" regions of the DNA−dendrimer complexes. This form of binding has been proposed in the binding of EB to

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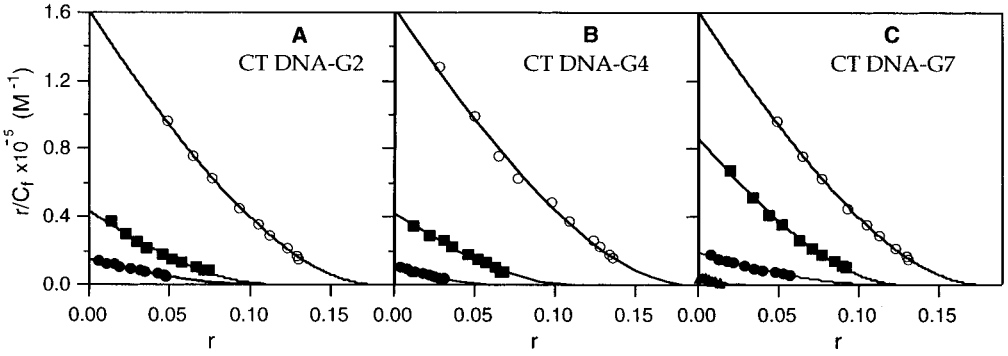

Figure 2. Scatchard plots of EB titration with ctDNA in the presence of various amounts of G2 (A), G4 (B), and G7 (C) dendrimers. Experimental data points at different [DNA]:[dendrimer] ratios: DNA alone (○), 1:0.5 (■), 1:1 (●), 2:1 (▲). The best fit lines according to eq 1 are superimposed on the experimental data points. Values of K_{EB}' and r_{max} are listed in Table 1.

Table 1. Binding of EB to ctDNA in the Presence of Various Amount of Dendrimers

	G2		G4		G7	
[DNA]:[G*n*]	K_{EB}' (M^{-1})	r_{max}	K_{EB}' (M^{-1})	r_{max}	K_{EB}' (M^{-1})	r_{max}
1:0	32×10^4	0.18	32×10^4	0.19	32×10^4	0.18
1:0.5	8.6×10^4	0.13	8.4×10^4	0.12	17×10^4	0.14
1:1	3.0×10^4	0.11	2.2×10^4	0.08	3.8×10^4	0.12
1:2	2.9×10^4	0.11	2.0×10^4	0.08	0.90×10^4	0.03

Table 2. Calculated Length of Tightly Bound DNA Region per Dendrimer Molecule at the Saturation Point

					length of "tightly bound" region	
G*n*	diameter Å	no. of surface groups	saturation [DNA]:[G*n*]	accessibility of DNA (%)	bp	Å
G2	29	16	1:1	61	3.2	11
G4	45	64	1:1	40	13	44
G7	81	512	1:2	17	106	360

nucleosomes (DNA−histones complex), where EB preferentially binds to the "linker DNA" regions of the nucleosomes (100 times stronger than binding to the "core DNA" regions where DNA has intimate contacts with histones).[21]

Second, the number of binding sites per nucleotide r_{max} for EB (Table 1) decreases with increasing amount of dendrimers, which clearly indicates that dendrimer binding on DNA is sufficiently strong that dendrimers cannot be displaced by added EB. This conclusion is supported by another type of titration experiments which showed that the fluorescence intensity from a premixed solution of EB and DNA dropped when adding increasing amounts of dendrimers into the solution. Since dendrimers were shown not to quench the fluorescence of EB, this phenomenon can be explained by noting that the originally bound EB on DNA was displaced from DNA by added dendrimers. In the case of the binding of EB to nucleosomes, the Scatchard plot was characterized by a positive initial slope, a plateau around $r = 0.05$, and then a negative slope.[15,21] The rationale is that the initial binding of EB to nucleosomes is a positively cooperative process, and a high level of EB intercalation causes the progressive dissociation of DNA from histones, so the binding at a later stage is a negatively cooperative process typical for dye binding to free DNA. On the contrary, in the case of EB binding to DNA−dendrimer complexes, the Scatchard plots are characterized as a negatively cooperative binding process and the dissociation of DNA from dendrimers does not happen at a high level of EB intercalation. This might be due to the stronger ionic interactions between DNA and dendrimers and/or to the DNA conformation being not as constrained for dendrimer binding as it is for histone binding.

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Third, the apparent binding constant K_{EB}' also decreases with increasing amount of dendrimers, which suggests that the binding of EB to DNA is weakened as dendrimer is added (Table 1). Two factors may have contributions to this phenomenon: the conformational flexibility of DNA decreases after it binds to dendrimers; and the local neutralization of negative charges on the DNA backbone will decrease the ionic attractive forces between EB and DNA.

A final significant feature is the existence of a saturation dendrimer concentration where the DNA accessibility for EB does not change further with the addition of dendrimers (Table 2). This saturation point is at a 1:1 DNA: dendrimer concentration ratio for G2 and G4 whereas it is at a 1:2 concentration ratio for G7. However, even at this point, there is still a small portion of DNA which is accessible for EB binding.

A Proposed Binding Model. Comparing the effect of dendrimer generation on the binding properties of EB to DNA−dendrimer complexes from the changes in K_{EB}' and r_{max}, G2 and G4 have a larger effect at low DNA:dendrimer concentration ratio but G7 dendrimer has a larger saturation effect. At the saturation point, the accessibility of DNA for EB (Table 2, which is calculated by r_{max}-(saturation dendrimer concentration)/r_{max}(DNA alone)) decreases with dendrimer generation, from 61% for G2 to 17% for G7. In order to build a binding model for DNA−dendrimer complexes, we calculated the length of the "tightly bound DNA" region per dendrimer molecule at the saturation point. Our calculation was based on the assumptions that DNA consists of "tightly bound DNA" regions and "linker DNA" regions, and the percentage of the "linker DNA" regions equals the accessibility of DNA for EB. The calculated length of "tightly bound DNA" region per dendrimer increases from 3.2 base pairs (bp) for G2 to 106 bp for G7.

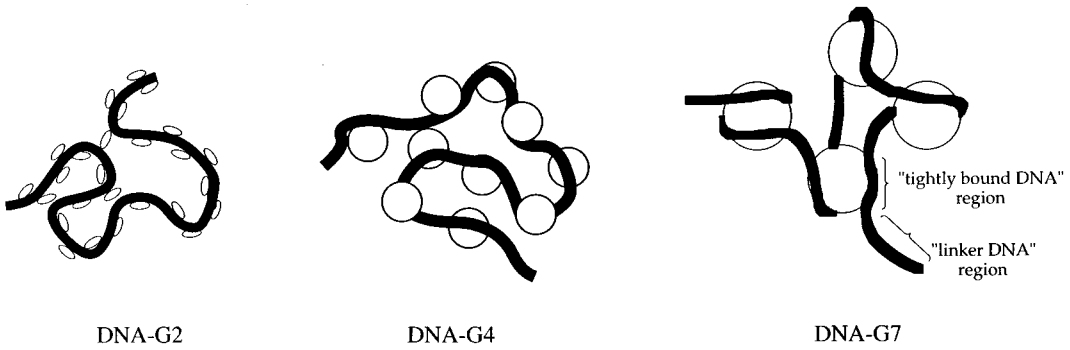

Figure 3. Proposed binding model for DNA−dendrimer complexes.

The binding model for DNA−dendrimer complexes proposed in Figure 3 is consistent with all of the experimental data for DNA−dendrimer binding. In this model one can envision several binding scenarios: one dendrimer molecule may bind only to one successive piece of DNA helix, or bind to two or more separate DNA pieces. The latter binding mode will compact DNA to a higher degree, and it requires less bending of the DNA double strand.

In the case of the first binding mode, one successive piece of DNA helix needs to bind tightly to one dendrimer molecule and, on the basis of our calculations, it will be 11 Å of DNA on G2, 44 Å of DNA on G4, and 360 Å of DNA on G7. Considering the size of dendrimer molecules, the successive DNA pieces would bind with dendrimer molecules covering about 12% of the maximal circumference of G2 dendrimers and 31% of the maximal circumference of G4 dendrimers. For DNA−G7 complexes, in order to make tight contact for complete stretch of 360 Å with the DNA helix which is 1.4 times the maximal circumference of the G7 dendrimer, the DNA double helix has to bend in order to wrap around the G7 dendrimer for about 1.4 turn. In this sense, a DNA−G7 complex would be an analogue to a DNA−histones complex, where the DNA wraps around the histones for about 1.75 turn.[22]

However, there is no direct evidence which shows that DNA−dendrimer complexes would follow only the first binding mode to form an organized three-dimensional assembly like the DNA−histones complex. Most probably the different binding modes discussed above coexist for DNA−dendrimer complexes.

Because G7 dendrimer has a larger fraction of "tightly bound DNA" regions, it can compact DNA to a higher level than G4 and G2. We can speculate that the more compact DNA−dendrimer complexes can more readily penetrate a cell membrane to enter the cell cytoplasm. This would provide a working hypothesis for the observed low gene transfection efficiency of G4 and G2. In addition, the result of higher accessibilities of DNA to EB for DNA−G4 and DNA−G2 complexes after their saturation point might suggest that a greater portion of DNA in the DNA−G4 and DNA−G2 complexes could still be accessible to nuclease, so that exposed DNA could be digested before it reaches the nucleus. This provides a possible rationale for the low gene transfection efficiency of G4 and G2. In summary, the different transfection efficiencies of G2, G4, and G7, where G7 has high transfection efficiency but G4 and G2 have almost no efficiency at all, are consistent with the model shown in Figure 3.

Base Component Selectivity. We have employed $[poly(dA\text{-}dT)]_2$ and $[poly(dG\text{-}dC)]_2$ as a comparison of ctDNA in an effort to investigate the base component selectivity of dendrimer binding. The strength of the DNA−dendrimer interaction was judged by the apparent binding constant K_{EB}' and DNA accessibility for EB. The stronger the DNA−dendrimer interaction, the lower the apparent binding constant K_{EB}' and the lower the DNA accessibility for EB.

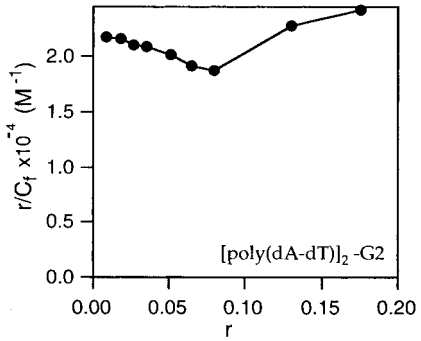

Figure 4. Scatchard plot for EB titration with $[poly(dA\text{-}dT)]_2$−G2 (1:1 DNA:dendrimer concentration ratio).

For DNA−G7 interactions, results for $[poly(dA\text{-}dT)]_2$−G7 and $[poly(dG\text{-}dC)]_2$−G7 were the same as that from ctDNA−G7 within experimental error. In addition, for DNA−G2 interactions, results for $[poly(dG\text{-}dC)]_2$−G2 were also the same as that from ctDNA−G2 within experimental error. This clearly indicates that there is no base component selectivity for DNA−dendrimer interactions. However, a different phenomenon was observed from the $[poly(dA\text{-}dT)]_2$−G2 complex. In this case, EB showed positively cooperative binding at high EB concentration which can be seen from its Scatchard plot (Figure 4). This result indicates that G2 dendrimers began to be displaced from DNA by EB at high EB concentrations (around $r = 0.08$) and interaction between $[poly(dA\text{-}dT)]_2$ and G2 is weaker than that of $[poly(dG\text{-}dC)]_2$−G2 and ctDNA−G2. It has been shown by fluorescence depolarization experiments that alternating self-complementary $[poly(dA\text{-}dT)]_2$ has the ability to locally denature on its double helix below the melting transition.[23] It is also known that alternating self-complementary $[poly(dA\text{-}dT)]_2$ can form hairpin helix branches.[24] These structural properties of $[poly(dA\text{-}dT)]_2$ could account for the weaker interaction of $[poly(dA\text{-}dT)]_2$−G2 complexes. The reason that these properties do not affect $[poly(dA\text{-}dT)]_2$−G7 interactions might be that the local denaturing and hairpin helix branches are small and well-distributed so they do not affect the wrapping of DNA over dendrimer G7.

pH Dependence. PBS buffer solutions with the same ionic strength but different pH values were used to study

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Table 3. Binding of EB to CtDNA in the Presence of Various Amount of Dendrimers at pH 6.5

	G2		G7	
[DNA]:[G*n*]	K_{EB}' (M^{-1})	r_{max}	K_{EB}' (M^{-1})	r_{max}
1:0	30×10^4	0.17	30×10^4	0.17
1:0.5	3.3×10^4	0.11	6.4×10^4	0.12
1:1	1.5×10^4	0.10	0.78×10^4	0.07

Table 4. Binding of CtDNA−Dendrimers at Different Ionic Strengths at the Saturation Dendrimer Concentration at pH 7.5[a]

	100 mM NaCl		500 mM NaCl	
	percentage decrease of K_{EB}' (%)	accessibility of DNA (%)	percentage decrease of K_{EB}' (%)	accessibility of DNA (%)
DNA−G2	91	61	4	73
DNA−G7	97	17	48	47

[a] The percentage decrease of K_{EB}' reflects the percentage difference in the apparent binding constant of EB to the DNA−dendrimer complex with respect to that of EB binding to free DNA.

the influence of pH value on the DNA−dendrimer interactions. At lower pH, a greater portion of the dendrimer surface groups is expected to be protonated. The Scatchard plots were the same at pH 8.5 compared with pH 7.5 within experimental error (results not shown). At pH 6.5, the binding affinity of EB and DNA accessibility were slightly less compared with the results at pH 7.5 at the same DNA to dendrimer concentration ratio (Table 3), which indicated slightly stronger interactions at pH 6.5. Overall, the effect of pH on the DNA−dendrimer interactions is small in the pH range 6.5−8.5 and the main change happens between pH 6.5 and 7.5. This pH dependence is in agreement with the gradual change in the protonation status and a transition point around pH 7.[4]

Ionic Strength Dependence. PBS buffer solution with 500 mM NaCl (pH 7.5) was used to examine the effect of ionic strength on the DNA−dendrimer interaction. For the convenience of comparison, we calculated the percentage decrease of the apparent binding constant K_{EB}' relative to K_{EB} (EB binding to free DNA) (Table 4). Comparing with the results obtained at lower ionic strength, the differences between K_{EB}' and K_{EB} are much smaller and the accessibilities of DNA to EB are higher at higher ionic strength. This indicates that the binding between DNA and dendrimer is weaker at higher ionic strength, which is in agreement with electrostatic contributions to the binding.

The results derived with EB are in general agreement with another study,[25] which investigated DNA−dendrimer interactions by an EPR technique using TEMPO-labeled dendrimers. In both cases, "later generation" dendrimers (G6 and G7) show stronger interaction with polynucleotides than "earlier generation" dendrimers (G2); and the interaction of $[poly(dA\text{-}dT)]_2$−dendrimer is weaker than those of $[poly(dG\text{-}dC)]_2$−dendrimer and ctDNA−dendrimer.

$Ru(phen)_2dppz^{2+}$ is a different type of DNA intercalator.[26,27] Preliminary results using $Ru(phen)_2dppz^{2+}$ probe under the same conditions have shown the same trends as those obtained with EB.

Conclusions

Investigations have been carried out to probe the binding interactions between double-stranded DNA (mainly ctDNA) and amine-terminated polyamidoamine dendrimers (G2, G4, and G7). EB was used as a fluorescent probe for this purpose.

Under our experimental conditions, the results show that there is predominantly one binding mode in the binding of EB to DNA−dendrimer complexes. The simplest structural model suggests that DNA binding with dendrimers can be divided into "tightly bound DNA" regions and "linker DNA" regions (Figure 3). The binding of dendrimer to DNA is sufficiently strong that added EB cannot displace dendrimers from DNA. The apparent binding constant of EB and the accessibility of DNA to EB decreases with increasing dendrimer concentration until a saturation dendrimer concentration is reached.

The length of the "tightly bound DNA" region per dendrimer molecule at the saturation dendrimer concentration was calculated and a binding model of DNA−dendrimer complexes was proposed for G2, G4, and G7. From the calculation, one G7 molecule may make "tight" contact with a successive 360 Å of DNA helix so DNA could wrap around G7 for about 1.4 turn, which is analogous to the DNA−histones complex. DNA cannot wrap around the smaller G4 and G2 dendrimer molecules as concluded from both calculations and simple inspection of molecular dimensions. It is also possible that one dendrimer molecule can bind to two or more independent DNA pieces.

The DNA−dendrimer interaction has little preference for the DNA base components. The interaction of $[poly(dA\text{-}dT)]_2$−G2 is weaker than that of $[poly(dG\text{-}dC)]_2$−G2 and ctDNA−G2, which might be caused by the local denaturing and hairpin helix branches of $[poly(dA\text{-}dT)]_2$.

The DNA−dendrimer interaction at pH 6.5 is stronger than that at pH 7.5 as expected from a higher degree of protonation of the amine groups on the dendrimer surface. The binding between DNA and dendrimers became weaker at higher ionic strength, consistent with the importance of the electrostatic interactions in the DNA−dendrimer binding.

Current studies in our laboratory involving other fluorescent probes are being undertaken to further understand the interaction between dendrimers and DNA as well as dendrimers and liposomes.

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