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Using Ethidium To Probe Nonequilibrium States of DNA Condensed for Gene Delivery[†]

Kristina Fant,* Bengt Nordén, and Per Lincoln

Department of Chemical and Biological Engineering, Chalmers University of Technology, SE-412 96 Göteborg, Sweden

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ABSTRACT: Here we explore the use of ethidium to determine relative affinities of different gene delivery vectors for DNA and describe an improved method for studying the interaction. Specifically, we investigate the binding of poly(amidoamine) dendrimers and show that the DNA−dendrimer−ethidium system is far from thermodynamic equilibrium. Moreover, dendrimer surface modification through PEGylation appears to make the interaction with DNA more reversible, which is favorable from the perspective of vector unpacking. Probing the nonequilibrium state of DNA during condensation processes is thus important for developing novel vectors, and further, it could also be useful in the study of chromatin folding.

Methods of investigating the condensation state of nucleic acids are of great interest for the improvement of vectors for gene therapy. An ideal vector facilitates or actively stimulates the cellular uptake of the vector−nucleic acid complex, while at the same time, it also provides protection of the nucleic acid from extra- and intracellular nucleases. Eventually, it should still be able to release the nucleic acid at the appropriate location inside the cell. The degree of protection is closely linked to the exposure of nuclease binding motifs of the nucleic acid to the surroundings. This can be indirectly investigated using dyes such as ethidium that upon intercalation between the base pairs exhibit a large increase in fluorescence. The most common procedure is to first load the nucleic acid with ethidium at a ratio of approximately one ethidium molecule to every 3−4 bp and then perform a titration with the vector to be investigated. Upon complexation of the nucleic acid with the vector, sterical or electrostatical exclusion of ethidium from the intercalation sites is observed as a decrease in fluorescence intensity (*1*−*6*). Another procedure is to reverse the mixing order, so that the nucleic acid is first condensed with the vector and then the complex is probed by titration with ethidium, for which apparent binding constants and remaining binding site density can be calculated (*7*, *8*). This method was first described by Chen et al. (*7*) and has since been further developed in our laboratory.

Poly(amidoamine) dendrimers are interesting vectors that have been used extensively for the delivery of plasmid DNA as well as antisense oligonucleotides and siRNA in vitro (*9*−*15*), and a few reports are available that indicate their usefulness also in vivo (*16*, *17*). They are synthesized in a stepwise manner into a fractal structure with well-controlled sizes that depend on the number of generations (*18*). The interaction between DNA and dendrimers, as well as the biophysical properties of the resulting dendrimer−DNA complexes (termed "dendriplexes"), has been studied extensively by us and other research groups (*2*, *7*, *8*, *19*−*25*). Dendrimer binding causes condensation of the DNA into a more or less compact structure, the degree of compactness depending on the choice of dendrimer generation and the dendrimer:DNA charge ratio *r* ([N]/[P]). Reports of the relative cytotoxicity of cationic PAMAM dendrimers have led to the development of various types of surface-modified dendrimers with a more suitable biocompatibility profile (*26*−*29*). We recently investigated the biophysical characteristics and the transfection efficiencies of a library of surface-modified dendrimers and the parent generation 4 and 5 PAMAM dendrimers (*30*). In that study, we found that there is a delicate balance between the biocompatibility profile of a cationic dendrimer, promoted by low surface charge, and the DNA condensation capacity that is a prerequisite for high transfection efficiency and is promoted by high surface charge. In this study, we employ the ethidium cation to investigate the extent of condensation of DNA by PAMAM dendrimers in further detail to thoroughly assess how the PEG chains on the surface-modified dendrimers affect the interaction with DNA. We show that the use of ethidium extends beyond mere intensity measurements; by utilizing all information from the titration spectra in our model for ethidium binding, we can isolate the effects on binding affinity and on the number of available binding sites arising upon condensation of the nucleic acid. Further, by varying the order of mixing for solutions containing DNA, ethidium, and dendrimers, we can obtain information regarding the thermodynamic state of the system.

First, the method of extracting binding isotherms for ethidium from the titration data was validated (detailed experimental procedures can be found in the Supporting Information). Figure 1 shows measured reference spectra for bound and free ethidium (Figure 1A), as well as one example of a measured spectrum on a sample spectrum from the titration together with the theoretically reconstructed spectrum obtained using a linear combination of the two extreme reference spectra (Figure 1B). As one can see in Figure 1A, ethidium undergoes not only a large increase in quantum yield as frequently reported (*31*) but also a small yet significant spectral shift toward shorter wavelengths upon intercalation into DNA. We noted during the course of the titration with ethidium that the wavelength of the fluorescence intensity maximum at first corresponded to completely bound ethidium but was subsequently shifted to the red, approaching gradually the value for free ethidium. The linear combination in Figure 1B is obtained using the "pinv" command of Matlab as solution vector **x** of the equation system below:

$$\mathbf{t} = \mathbf{R}\cdot\mathbf{x} + \mathbf{r}, \qquad \mathbf{R}^{\mathrm{T}}\cdot\mathbf{r} = 0$$

where the superscript T denotes the transpose of a matrix and the columns of matrix **R** are the respective reference spectra. **t** is a measured spectrum from the titration and **r** the residual, which is

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*To whom correspondence should be addressed. Phone: +46 31 7721000. Fax: +46 31 772 3858. E-mail: kristina.fant@chalmers.se.

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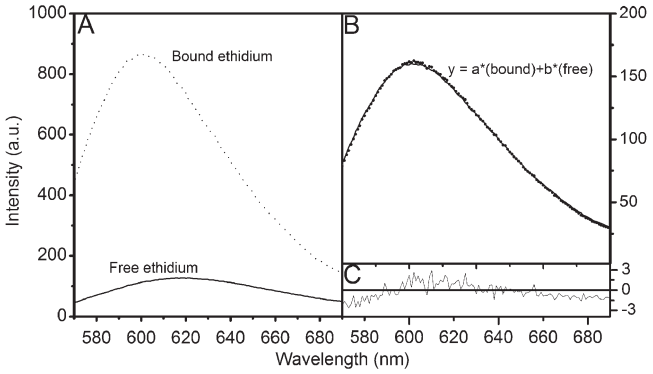

FIGURE 1: Extracting binding isotherms for ethidium from the titration data. (A) Reference spectra for free ethidium and ethidium bound to DNA. (B) Example of one measured spectrum (gray circles) together with the reconstructed linear combination of the reference spectra (—) and (C) the residual *r*.

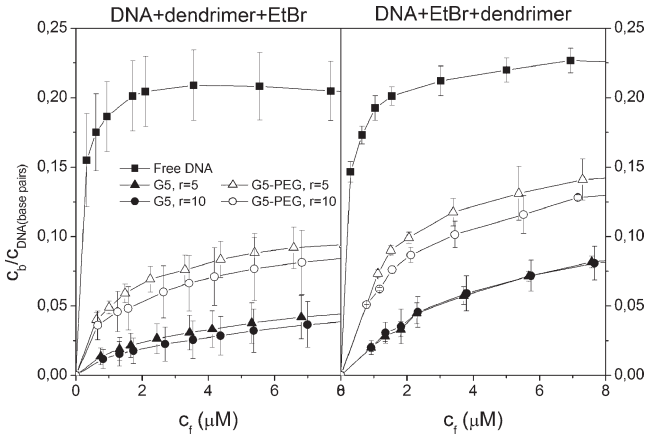

FIGURE 2: Ethidium binding isotherms for titrations of ethidium to dendrimer-condensed DNA. The titrations were performed in the DNA−dendrimer−ethidium order (left) or in the DNA−ethidium−dendrimer order (right). The data represent means ± the standard deviation of at least three independent experiments.

orthogonal to the columns in **R**, ensuring that the solution $\mathbf{x} = [a,b]$ will give the smallest possible residual in any norm, in particular in the Euclidean "least-squares" sense. The fit of the data was found to be excellent, with residual norms below 2% (Figure 1C). Hence, we are confident that there is only one spectroscopically distinguishable binding mode for ethidium, and that the evaluation method is accurate. By reconstructing the full measured spectra from the whole reference spectra rather than using only the maximal intensity, we exploit all the information in the spectrum. By multiplying the parameters *a* and *b* with the actual ethidium concentration used for the respective reference spectra, we can determine the concentrations of bound and free ethidium, c_b and c_f, respectively, at each point in the titration. However, because of the low quantum yield of free ethidium, there is a relatively large uncertainty in the free ethidium concentration determined this way, and we have preferred to calculate the free concentration by subtracting the concentration of bound ethidium from the total ethidium concentration ($c_f = c_{tot} - c_b$). The parameters c_b and c_f are used to construct Scatchard plots (Figure S1 of the Supporting Information) that are fitted with the modified McGhee−von Hippel equation to yield the binding constant for ethidium and the fraction of available binding sites (see the Supporting Information).

In Figure 2, isotherms for binding of ethidium to dendriplexes prepared using different mixing orders are shown. For uncondensed,

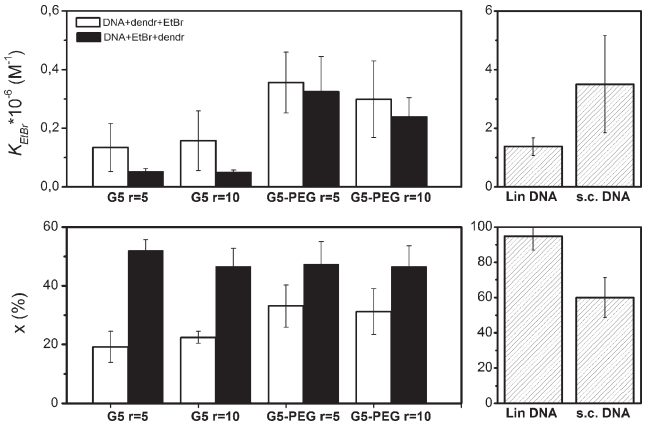

FIGURE 3: Constants for binding of ethidium to DNA (K_{EtBr}) and fraction of available binding sites (*x*), obtained using the modified McGhee−von Hippel equation with binding site size (*s*) of 2.5. The left panels show data for dendrimer-condensed DNA with the DNA−dendrimer−ethidium mixing order (white bars) or the DNA−ethidium−dendrimer mixing order (black bars). The right panels show data for linear and supercoiled DNA. The data represent means ± the standard deviation of at least three independent experiments.

supercoiled DNA, both methods yield as expected the same final amount of bound ethidium, with approximately one ethidium molecule for every 4−5 bp. For dendriplexes, on the other hand, there is a large difference depending on whether the ethidium is first allowed to intercalate into DNA or if it is added after that complexation with dendrimers has occurred. The difference is observed for both PEGylated and unmodified PAMAM dendriplexes. Figure 3 shows the obtained effective binding constants for ethidium, K_{EtBr}, and the fraction of available binding sites, *x*, in free and dendrimer-condensed DNA. For supercoiled DNA, the binding constant increases 2−3-fold while the accessibility decreases by 40% relative to that of linear DNA. This is in agreement with the well-known phenomenon that negative supercoiling increases the affinity for intercalators, and that the closed ring structure can be unwound to a certain extent by only ethidium intercalation, after which threshold the free energy cost becomes too high (*32*). The difference depending on the mixing order for dendriplexes indicates that the DNA is kinetically trapped in a complex with the dendrimers upon condensation, and that the system is not at thermodynamic equilibrium. This hypothesis is supported by previous investigations of DNA−dendrimer systems by cryo-TEM, where different morphologies were observed depending on dendrimer generation and it was suggested that dendrimers of medium to higher generations (fourth and above) bind DNA with an overly high affinity to allow morphological rearrangements after the first contact (*25*). The analysis of the binding isotherms reveals some interesting details. First, the accessibility of the DNA (*x*) is much higher and in fact close to the value for uncondensed DNA if the ethidium is first allowed to intercalate freely before the complexes are formed (i.e., method 2). There is no significant change in the recorded spectra after incubation for up to 30 min of dendrimers with DNA-bound EtBr as compared to the spectra recorded immediately after mixing (data not shown). Further, using method 2, there appears to be no significant difference in the accessibility of the DNA depending on what type of dendrimer is used for the complexation. Using method 1, however, fifth-generation dendriplexes appear to be less available than fifth-generation PEG dendriplexes. Second, the effective ethidium binding constant K_{EtBr} is reduced ≥10-fold upon complexation with dendrimers

with both titration methods. Most remarkably, there is also variation with respect to how the PEGylation of the dendrimers affects the value. For the unmodified fifth-generation dendrimers, the difference in the binding constant is larger between the two titration methods than it is if the dendrimer surface is partly PEGylated. This suggests that the PEG chains render the DNA−dendrimer interaction more reversible, which is most likely achieved by decreasing the affinity of the dendrimer for DNA as we reported in a recent study (*30*). This reversibility is highly desired for gene delivery vectors, because the unpacking event has been suggested as one of the rate-limiting steps in the transfection process (*33*).

Intercalation of ethidium into DNA lengthens and unwinds the double helix and introduces a tilt between the adjacent base pairs (*34*). These changes affect the torsional and bending flexibilities of the DNA, and moreover, binding of the cation leads to a reduced effective charge density of the polymer. All of these parameters are likely to influence the DNA condensation process induced by various gene delivery vectors and will therefore also influence the morphology of the resulting complexes. We have here demonstrated a clear difference in the efficiency of ethidium exclusion depending on whether the molecule is intercalated during the condensation process. This difference is apparent already at low ethidium:DNA ratios, i.e., far from saturating values of ethidium. Therefore, if the ethidium exclusion assay is performed according to method 2, with ethidium present from the start, there is a risk that the condensation efficiency of gene delivery vectors will be underestimated. Furthermore, the differences between different vectors will be less apparent, and it might be more difficult to draw conclusions regarding the effect of structural modifications of the vectors. Another advantage of preforming complexes before the addition of ethidium, which was also shown in a recent study (*30*), is the possibility of studying the effects on complexes of dilution in buffers of varying ionic strengths. This provides invaluable information regarding the stability of gene delivery complexes during the transfection event.

We note that the linearity of the Scatchard plots (Figure S1 of the Supporting Information) shows that there are not, as one could have expected, two sharply distinct classes of binding sites, close or distant from the dendrimer, but rather a smooth distribution from weak to strong to complete inhibition of ethidium binding effected by the dendrimers. On the other hand, it was not possible to satisfactorily fit the data of the competition of ethidium and dendrimers using a cooperative generalized McGhee−von Hippel model, further supporting the lack of thermodynamic equilibrium in the dendrimer distribution.

In conclusion, we show here a simple method of characterizing the thermodynamic state of condensed DNA, with direct implications for the development of new gene delivery vectors. From a wider perspective, probing folded configurations in protein−nucleic acid systems is important in a variety of contexts, including studies of mechanistic pathways for chromatin regulation. Our method might also be extended to investigate catalytic states and mechanisms for avoiding unwanted trapped states in the folding of proteins and for detecting long-lived biologically essential metastable forms of other complex aggregated systems.

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SUPPORTING INFORMATION AVAILABLE

Detailed experimental procedures and an additional figure. This material is available free of charge via the Internet at http://pubs.acs.org.

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