A Fluorescence Study of the Structure and Accessibility of Plasmid DNA Condensed with Cationic Gene Delivery Vehicles

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ABSTRACT: The cationic lipids 1,2-dioleoyl-3-trimethylammonium-propane and dimethyldioctadecylammonium bromide, with or without the helper lipids 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine or cholesterol, and the cationic polymer polyethyleneimine, were compared for their ability to displace fluorescent dyes from DNA. Differences in displacement of the intercalating dyes ethidium bromide and ethidium homodimer correlate with their relative affinities with DNA, with the extent of ethidium homodimer displacement significantly less. Differences in ethidium homodimer and ethidium bromide displacement as a function of the ratio of polycation to DNA and the charge density of the polycation suggest a greater sensitivity of the former to topological changes in condensed DNA. Marked differences in the ability of these cationic delivery systems to displace the minor groove binding dyes 4′,6-diamidino-2-phenylindole and Hoechst 33258 upon interaction with DNA are also apparent, with the majority of Hoechst 33258 remaining bound to DNA. Changes in the spectral properties of Hoechst 33258 were further used to characterize polycation-induced changes in solvent accessibility of the DNA minor groove. Taken together, these studies demonstrate differences in the interaction of various cationic lipids and polyethyleneimine in terms of regional displacement of dyes, polycation-induced structural changes in DNA, as well as polycation-mediated changes in solvent accessibility of the minor groove. The relevance of these studies to current models of the structure and assembly of polycation/DNA complexes are discussed. © 2003 Wiley-Liss, Inc. and the American Pharmaceutical Association
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INTRODUCTION

In the last decade, a significant emphasis has been placed on the development of various synthetic cationic lipids and polymers for the purpose of nonviral gene delivery. Although many clinical trials have been initiated using cationic lipids, little information is available concerning the physical basis for differences in efficiency of transgene expression. Of central importance to this understanding is a comprehensive description of the nature of the interaction of various polycationic delivery systems with DNA. To this

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end, numerous biophysical approaches have been used.[1–12]

The interaction of cationic delivery vehicles with DNA is a spontaneous reaction driven by the electrostatic attraction between the positive charges of the cationic agent and the negatively charged phosphate backbone of the DNA. Neutralization of the negatively charged backbone results in the condensation of DNA. In the case of cationic lipids, these compacted phases take the form of stacked layers of alternating lipid bilayers and DNA strands.[13] Additional honeycomb-like structures have also been observed upon incorporation of significant amounts of nonbilayer forming lipids.[14] Non-lipid cationic polymers condense DNA into tightly packed toroidal or rod-like structures depending on the solution conditions and the chemical structure of the polymer.[15,16] Because the DNA used for gene delivery is usually highly supercoiled, significant topological changes in the writhe and twist of the plasmid double helix must occur for the formation of the tightly packed structures such as those possessing DNA helices sandwiched between lipid bilayers or hexatically packed in toroidal condensates.

Attempts to describe structural transitions in DNA upon binding by cationic delivery systems have involved a variety of spectroscopic and calorimetric techniques. Major changes in the circular dichroism spectra of DNA upon binding various cationic lipids and polymers have been interpreted to indicate changes in the secondary structure of DNA.[17–19] In contrast, X-ray diffraction studies of condensed DNA have shown that the DNA retains its native B-form in the condensed phase.[20] Furthermore, infrared (IR) spectroscopy also suggests that plasmid DNA retains its B-conformation when condensed with several cationic lipids and polymers.[3] Thus, the observed changes in the circular dichroism (CD) spectra of condensed DNA may actually reflect other differences in DNA topology as a result of changes in the winding angle of the double helix.[21] This model for the origin of cation-induced structural changes in DNA is further supported by calorimetric studies of the thermal stability of DNA when bound by various cationic lipids and polymers.[22]

Coupled to the limited structural rearrangements in DNA observed upon binding to various cationic lipids and polymers is the release of bound water molecules from the interface of the two interacting species. This dehydration has been characterized most directly by IR methods[3] but has also been confirmed by studies using differential scanning calorimetry and changes in the generalized polarization of the fluorescence of the lipid probe, laurdan.[23] In the case of cationic lipids, it seems that the type of lipids used can significantly affect the regions of the DNA that are dehydrated upon binding.[3]

One of the first and most common approaches used to characterize the interaction of cationic delivery systems with DNA uses a competitive displacement assay using the fluorescent dye, ethidium bromide (ETBR).[24] Currently, assays monitoring the displacement of ETBR[4,11,17] or other intercalating dyes[25,26] from DNA by various cationic delivery systems are routinely used to verify the condensed state of DNA in these complexes. The mechanism of displacement has been proposed to involve increased rigidity of the DNA double helix upon condensation.[27–29] In an effort to further explore the interaction of cationic delivery systems with DNA, a series of fluorescent dyes that bind to DNA with differing affinities, stoichiometries, and modes of binding are used here to monitor the nature of the interaction of cationic lipids and polymers with DNA. Differences in the ability of these cationic species to displace dyes from DNA provide further insight into the structure of the complexes themselves. Furthermore, the finding that some dyes remain bound to DNA upon formation of complexes with cationic lipids and polymers permits their use to monitor DNA environmental changes within these complexes.

MATERIALS AND METHODS

Materials

Supercoiled plasmid DNA (pMB290, >95% supercoiled) was obtained from Valentis Inc. (Burlingame, CA). The lipids 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), dimethyldioctadecylammonium bromide (DDAB), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and cholesterol (CHOL) were obtained from Avanti Polar Lipids (Alabaster, AL). High-molecular-weight polyethyleneimine (PEI) was obtained from Aldrich (no. 18,197-8; Milwaukee, WI). The fluorescent dyes ETBR, ethidium homodimer (ETHD-1), 4′,6-diamidino-2-phenylindole (DAPI), and Hoechst 33258 (HOC) were obtained from Molecular Probes (Eugene, OR). All other chemicals were from Fisher Scientific (St. Louis, MO).

Determination of Association Constants and Stoichiometries for Dye/DNA Interactions

Binding affinities and stoichiometries of several dyes for DNA were determined spectrofluorometrically based on the substantial increase in quantum yield of the fluorophores upon binding DNA. Dyes were titrated into solutions of DNA (25 μM in base pairs, 10 mM Tris pH 7.4). Binding parameters were determined for ETBR (excitation 518 nm, emission 620 nm), DAPI (excitation 360 nm, emission 455 nm), and HOC (excitation 358 nm, emission 462 nm). Measurements of the fluorescence intensity were made after allowing the solution to equilibrate for 3 min after each titration. Affinity constants and stoichiometries were determined using the methods described by Ward.[30] Briefly, the fractional saturation of binding sites, θ, was determined by the difference in fluorescence intensity upon titration of dye into DNA versus buffer alone (ΔF) divided by the maximum change in fluorescence intensity (ΔF_{max}). The affinity constants and stoichiometry were then determined by using linear least squares fitting of experimental data to the following equation:

$$1/(1-\theta) = (K[\text{dye}]/\theta) - (nK/[\text{DNA}])$$

where K is the equilibrium association constant, n is the stoichiometry, [dye] is the total molar concentration of fluorophore, and [DNA] is the molar concentration of DNA base pairs.

Preparation of Polycation/DNA Complexes

For cationic lipid-containing complexes, liposomes were first prepared by placing a specified amount of a chloroform solution containing lipid in a glass vial and evaporating the solvent under a stream of nitrogen gas. The resulting lipid film was then placed under vacuum for a minimum of 2 h before hydrating in the appropriate Tris buffer with vortexing. After equilibrating at room temperature for 30 min, the liposomes were extruded 11 times through a 100-nm pore polycarbonate membrane. Liposomes were stored at 4°C and used within 3 days. Before complex formation, DNA was first equilibrated with a given fluorescent dye for 1 h. Complexes of dye-labeled DNA with various polycations were then formed by mixing the DNA solution with an equal volume of a polycation solution with stirring for 20 s. The complexes were allowed to equilibrate for 20 min before performing fluorescence measurements.

Competitive Dye Displacement by Polycations

Fluorescent measurements were performed using either a QuantaMaster™ spectrofluorometer (PTI, Monmouth, NJ) to obtain both fluorescence intensities and the wavelength of the emission maximum or a Fluostar™ fluorescence microplate reader with appropriate filters (BMG, Offenburg, Germany) when only intensity measurements were desired. When using the PTI instrument, the excitation and emission slits were set for a bandpass of 3 nm and the spectra were obtained by scanning every 1 nm using a 1-s integration time. The percentage of dye displaced upon polycation binding was calculated using the following equation:

$$\text{Relative Displacement} = (F_{obs} - F_0)/(F_{DNA} - F_0)$$

where F_{obs}, F_0, and F_{DNA} are the fluorescence intensities of a given sample, the dye in buffer alone, or the dye complexed to DNA alone, respectively.

Circular Dichroism as an Indicator of Dye Binding Mode in Cationic Lipid/DNA Complexes

The mode of binding of HOC to cationic lipid/DNA complexes was verified by near UV CD. Samples were prepared at a DNA concentration of 50 μg/mL and a HOC concentration of 3 μM (1:25 dye/DNA base pair). CD spectra were collected from 230–400 nm using a Jasco 710 spectrapolarimeter. The sample was placed in a 1-cm path length microcuvette at 25°C and spectra were collected at 0.1-nm increments using a scan rate of 20 nm/min. Three scans were collected for each sample and averaged.

Assessment of HOC Environment in Cationic Lipid/DNA Complexes

To evaluate environmental changes in the minor groove of DNA upon complex formation with cationic lipids, fluorescence measurements of DNA bound HOC were made at a lower dye/DNA base pair ratio (1:150). Cationic lipid/DNA complexes were prepared as previously described and emission spectra obtained from 400–600 nm upon excitation at 358 nm. To explore the role of water solvation on these spectral changes, similar studies were conducted by replacing H_2O with D_2O.

RESULTS

Determination of Binding Parameters for DNA Binding Dyes

Representative linearized binding isotherms for ETBR, DAPI, and HOC are shown in Figure 1. Affinity constants and stoichiometries are presented in Table 1. Binding parameters of ETHD-1 to pMB290 were not determined because previous attempts to determine these quantities directly from fluorescent-based measurements did not yield reliable results below an ionic strength of 1.[31] Affinity constants extrapolated from equilibrium values determined as a function of ionic strength originally reported by Gaugain et al.[31] are therefore included in Table 1 for comparison.

Both intercalating dyes possess stoichiometries of ~0.25 dye molecules per DNA base pair. The association constant determined for ETBR to pMB290 is in good agreement with previously reported literature values for binding to calf thymus DNA. The minor groove binding dyes HOC and DAPI, which have strong preferences for AT sites, possess similar stoichiometries (0.04–0.08 dye molecules binding per DNA base pairs) and have similar affinities for DNA ($\sim 10^8\ M^{-1}$) as previously reported.[32,33]

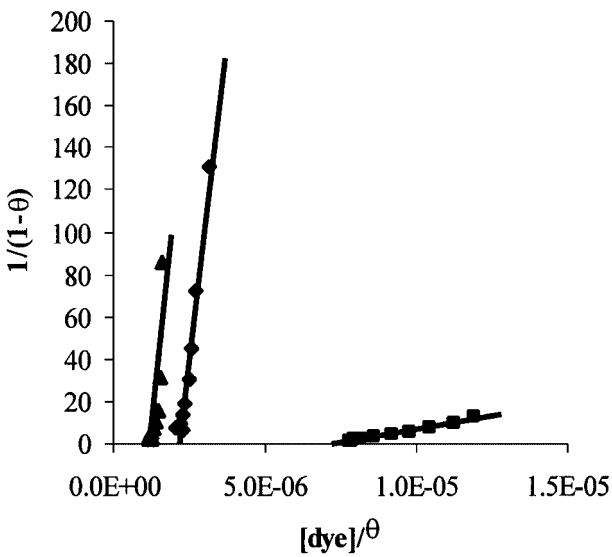

Figure 1. Representative binding isotherms of ETBR, DAPI, and HOC for plasmid DNA in 10 mM Tris pH 7.4 at 25°C. The concentration of DNA was 28 μM in base pairs. ETBR (squares), DAPI (diamonds), HOC (triangles).

Table 1. Binding Parameters of Various Dyes to DNA

Dye	Ka (M^{-1})[a]	n (dye/bp)
ETBR	$2.5\ (0.1) \times 10^6$	0.24 (0.02)
ETHD-1	1.6×10^{12}[b]	0.25[b]
HOC	$1.3\ (0.1) \times 10^8$	0.04 (0.004)
DAPI	$1.1\ (0.1) \times 10^8$	0.08 (0.004)

[a]Average and (SEM).
[b]Data taken from reference 31.

ETBR Displacement from DNA by Various Polycations

The displacement of ETBR from DNA upon complex formation with polycations has been used extensively in the development of nonviral gene delivery systems to detect the interaction of various lipids and polymers with DNA. Here, we use this assay as a benchmark with which to compare a variety of DNA binding dyes and their interaction with DNA when complexed with various polycations. When DNA, pre-equilibrated with ETBR at a ratio of 1:4 dye/DNA base pairs, is complexed with DOTAP or DDAB, approximately 85–90% of the ETBR is displaced (Fig. 2A, squares and crosses, respectively). Incorporation of equimolar amounts of the helper lipids DOPE or CHOL into DOTAP complexes results in a significant reduction in the amount of ETBR displaced (Fig. 2A, diamonds and triangles, respectively). The interaction of cationic lipids with DNA, with or without helper lipids, results in an IC_{50} value for the interaction between 1 and 2 (±) in agreement with stoichiometries determined for these complexes by isothermal titration calorimetry.[34]

The relative ability of the cationic polymer PEI to displace ETBR from DNA is shown in Figure 2B. Complex formation of DNA with increasing amounts of PEI results in an almost complete displacement of the dye from DNA when added above a ratio of 3:1 nitrogen/DNA phosphate. It has been demonstrated previously that the negative charge of DNA is completely neutralized by PEI above a 3:1 ratio.[1]

ETHD-1 Displacement from DNA by Polycations

Similar displacement studies were conducted using ETHD-1. DNA, pre-equilibrated with ETHD-1 at a ratio of 1 dye per 4 DNA base pairs, was complexed with increasing amounts of polycation. A striking difference is observed between titrations using ETHD-1 and ETBR (compare Fig. 3 with Fig. 2). Titration with increasing

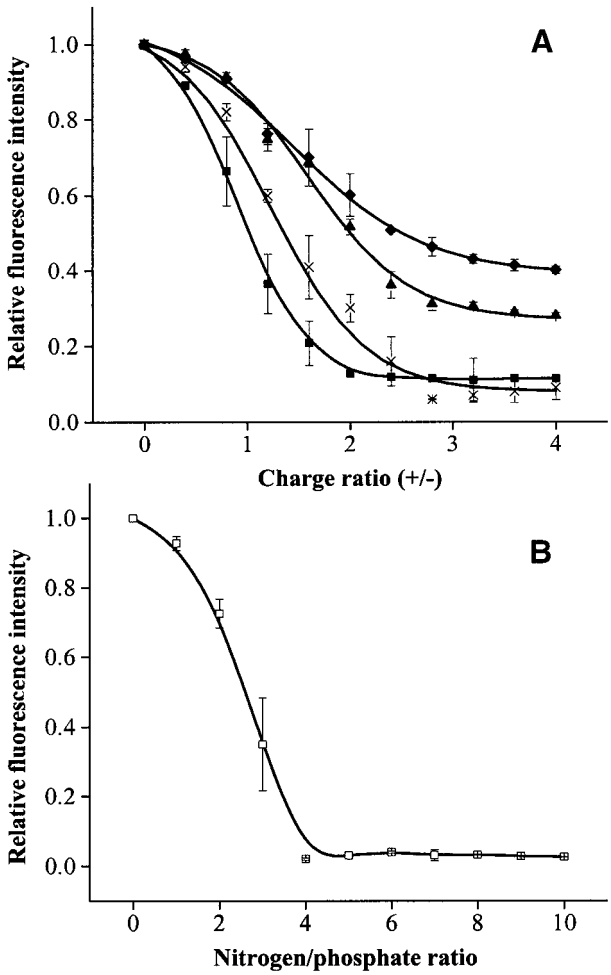

Figure 2. Displacement of ETBR by cationic lipids or PEI. DNA was pre-equilibrated with ETBR at a dye/DNA base pair ratio of 1:4 in 10 mM Tris pH 7.4. (A) displacement by lipids. DOTAP (squares), DOTAP/DOPE (diamonds), DOTAP/CHOL (triangles), and DDAB (crosses). (B) displacement by PEI. Data represent the average and SEM of at least three replicates. Lines represent sigmoidal fits to the data and are meant only as a visual guide.

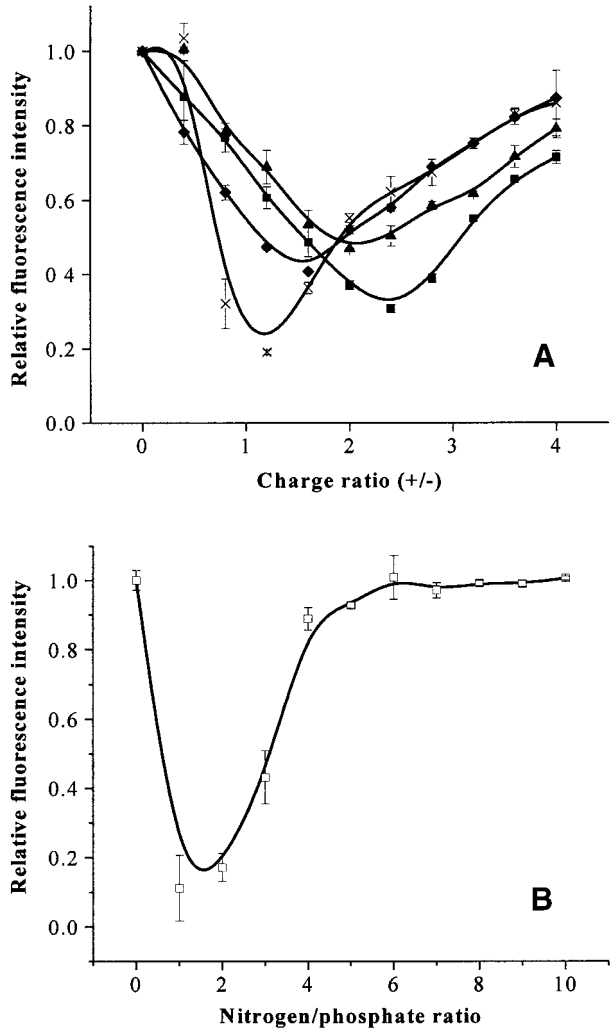

Figure 3. Displacement of ETHD-1 by cationic lipids or PEI. DNA was pre-equilibrated with ETHD-1 at a dye/DNA base pair ratio of 1:4 in 10 mM Tris pH 7.4. (A) Displacement by lipids. DOTAP (squares), DOTAP/DOPE (diamonds), DOTAP/CHOL (triangles), and DDAB (crosses). (B) Displacement by PEI. Data represent the average and SEM of at least three replicates. Lines represent spline fits to the data and are meant only as a visual guide.

amounts of DOTAP results in a decrease in the fluorescence intensity until a charge ratio of 2.5:1, where 30% of the dye is displaced (Fig. 3A, squares). The fluorescence increases above a 3:1 charge ratio. No significant changes in the wavelength of the emission maximum were observed during these titrations. Titrations with DDAB result in a greater displacement of ETHD-1, reaching maximal effect near a 1:1 charge ratio (Fig. 3A, crosses). Above a charge ratio of 1:1, the relative intensity again increases, like that observed for DOTAP.

Titrations with DOTAP in the presence of molar equivalents of DOPE and CHOL show differences in the stoichiometries of maximum displacement of ETHD-1 and the maximum extent of displacement. DOPE-containing complexes produce maximal displacement at a 1.5:1 charge ratio with 60% of the dye displaced (Fig. 2A, diamonds). Complexes containing CHOL manifested the greatest displacement at a charge ratio around 2:1 with 50% of the dye displaced (Fig. 2A, triangles). Again, above charge ratios of 2:1, incorporation of CHOL resulted in an increase in the relative

amount of dye remaining bound to DNA. Titrations performed with PEI resulted in the greatest displacement of ETHD-1, with approximately 90% displaced near a nitrogen/phosphate ratio of 2 (Fig. 2B). Above a ratio of 2:1, the fluorescence intensity increased to that of DNA in the absence of ETHD-1.

Displacement of DAPI from DNA by Polycations

Whereas the previous two dyes studied interact with DNA by intercalating between the bases along the double helix, DAPI binds in the minor groove. DOTAP titrations of DNA pre-equilibrated with DAPI at a ratio of 1:12.5 dye/DNA base pairs result in a decrease in fluorescence intensity, with only 5% of the dye still bound to DNA at a charge ratio of 2:1 (Fig. 4A, squares). Titrations performed using DDAB show a similar trend with 10% DAPI still bound to DNA above a charge ratio of 1.5 (Fig. 4A, crosses). When DOPE is included at a 1:1 molar ratio with DOTAP, DAPI is displaced to a lesser degree (20% remaining) when lipid is added above a charge ratio of 1.5:1 (Fig. 4A, diamonds). No significant effect of incorporating CHOL in DOTAP complexes is observed with a similar extent of DAPI displaced compared with DOTAP alone (Fig. 4A, triangles). Whereas PEI is able to displace the majority of DAPI from its binding site, the stoichiometry for saturation of dye displacement occurs at the significantly higher nitrogen/phosphate ratio of 7:1 (Fig. 4B).

Displacement of HOC from DNA by Polycations

The other minor groove binding dye studied, HOC, was used at a dye/DNA base pair ratio of 1:25. Titrations with DOTAP result in a 10% increase in fluorescence up to a charge ratio of 1.5 before showing a net 15% decrease in intensity above a 1:5:1 charge ratio (Fig. 5A, squares). Titrations with DDAB show an opposite trend with a nearly 15% decrease in intensity initially, followed by an increase back to no displacement above a charge ratio of 2 (Fig. 5A, crosses). Incorporation of the helper lipids DOPE and CHOL into DOTAP complexes produces similar effects in intensity changes like those seen with DOTAP itself (Fig. 5A, diamonds and triangles, respectively). Titrations with PEI show that the polymer can displace a significant amount of HOC with 65% displaced above a nitrogen/phosphate ratio of 3 (Fig. 5B).

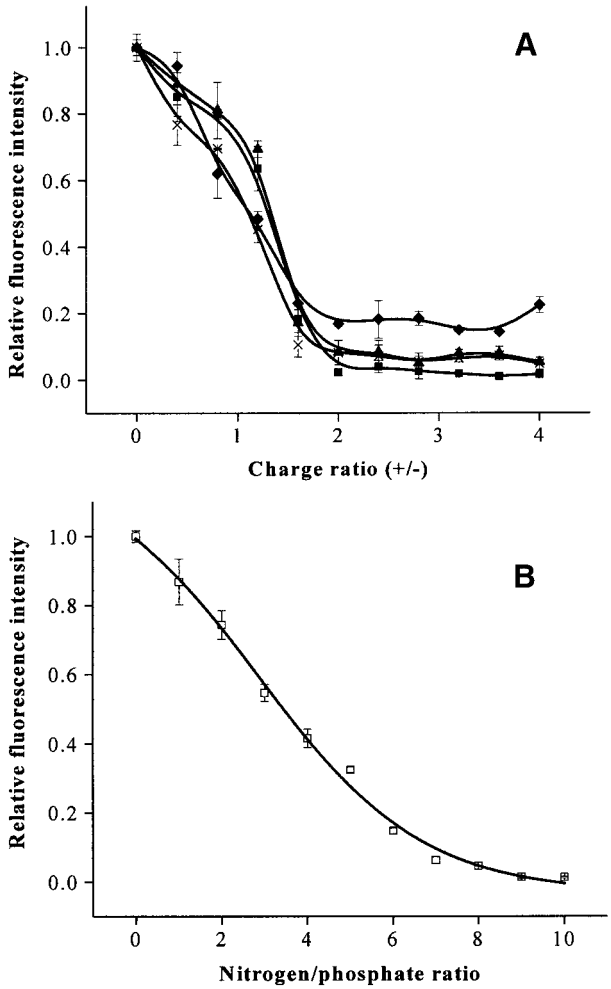

Figure 4. Displacement of DAPI by cationic lipids or PEI. DNA was pre-equilibrated with DAPI at a dye/DNA base pair ratio of 1:10 in 10 mM Tris pH 7.4. (A) Displacement by lipids. DOTAP (squares), DOTAP/DOPE (diamonds), DOTAP/CHOL (triangles), and DDAB (crosses). (B) Displacement by PEI. Data represent the average and SEM of at least three replicates. Lines represent spline fits to the data and are meant only as a visual guide.

Analysis of the Mode of Binding of HOC to Complexes of Cationic Lipids and DNA

Given the intriguing observation that the majority of HOC remains bound to complexes of cationic lipids with DNA, we sought to determine whether or not the dye was still bound to the minor groove of DNA by using CD. Numerous reports of the use of CD to characterize the mode of binding of chromophores to DNA exist in the literature.[35,36] HOC does not possess a strong CD signal itself, but when bound to DNA, a significant CD signal is

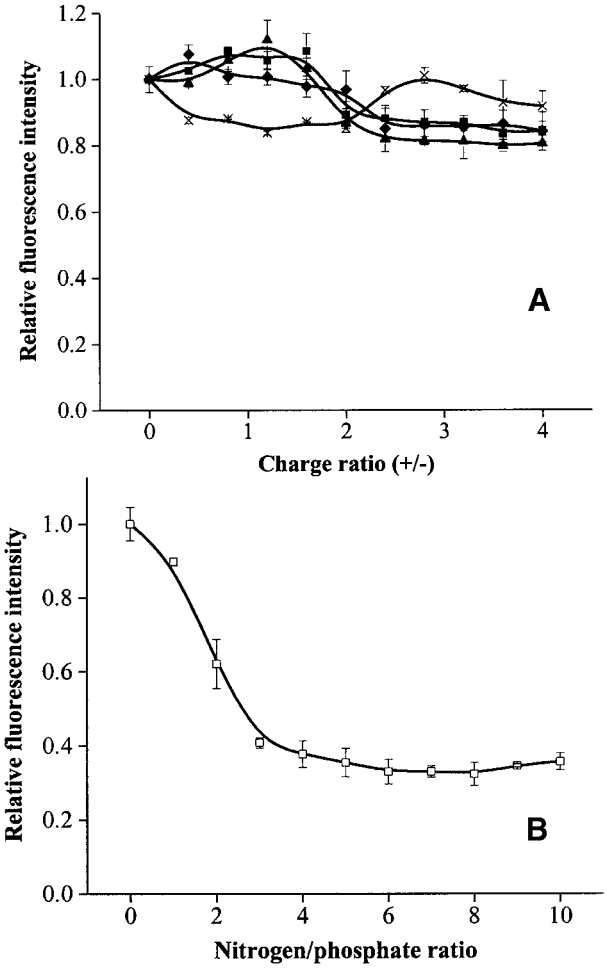

Figure 5. Displacement of HOC by cationic lipids or PEI. DNA was pre-equilibrated with HOC at a dye/DNA base pair ratio of 1:25 in 10 mM Tris pH 7.4. (A) Displacement by lipids. DOTAP (squares), DOTAP/DOPE (diamonds), DOTAP/CHOL (triangles), and DDAB (crosses). (B) Displacement by PEI. Data represent the average and SEM of at least three replicates. Lines represent spline fits to the data and are meant only as a visual guide.

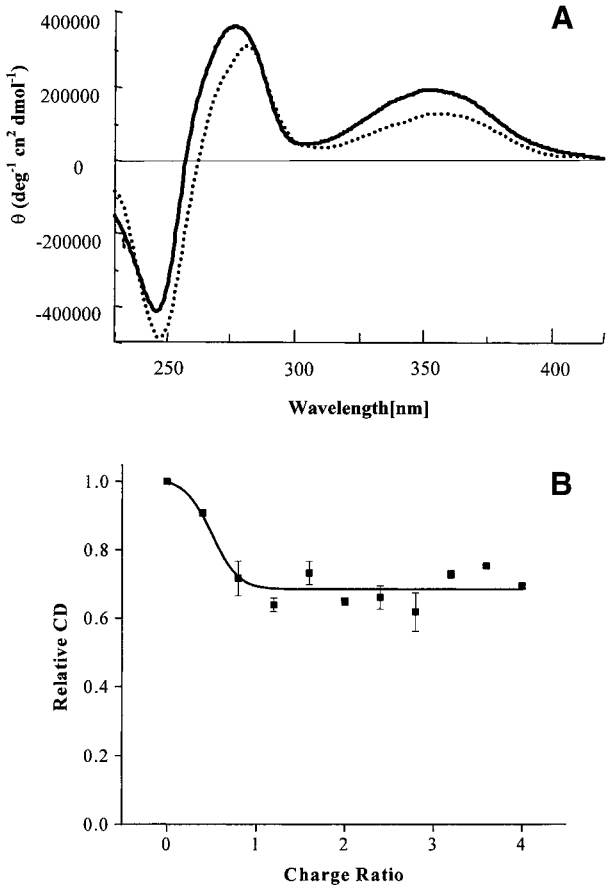

Figure 6. Determination of HOC binding to the DNA minor groove in DOTAP/DNA complexes at various charge ratios. (A) Circular dichroism spectra of HOC/DNA (1:25 dye/DNA base pair, dark line) and a complex of HOC/DNA with DOTAP at a charge ratio of 4:1 ±. (B) Molar ellipticity of HOC in DOTAP/DNA complexes at 360 nm as a function of charge ratio. Data represent the average and SEM of at least three replicates. Lines represent sigmoidal fits to the data and are meant only as a visual guide.

induced because of interactions with the helical structure of the DNA. It has been shown previously that the CD spectrum of HOC bound to DNA possesses a moderate positive ellipticit centered on the absorption maximum of the dye (~360 nm) as a result of binding in the minor groove.[35]

When HOC is bound to DNA at a 1:25 dye/DNA base pair ratio, this positive ellipticity at 360 nm is clearly observed (Fig. 6A, solid line). The positive and negative ellipticities at 275 and 245 nm arise from the DNA itself.[37] The spectra of a DOTAP/DNA complex at a 4:1 charge ratio again shows the positive ellipticity indicative of minor groove binding although to a lesser extent (Fig. 6A, dashed line). No CD signal was observed for free HOC or HOC in the presence of excess DOTAP(data not shown). Overall, the molar ellipticity at 360 nm decreases 25% with increasing charge ratio. This correlates well with the decrease in fluorescence intensity observed with these complexes. The agreement among these results suggests that the fluorescence signal observed for HOC in complexes with cationic lipids arises from dye still bound in the DNA minor groove.

Analysis of Polycation-Induced Environmental Changes in the DNA Minor Groove

The utility of HOC as a probe of the effect of cation binding in the region of the minor groove was explored by labeling DNA with HOC at a lower ratio of dye/DNA base pairs. Because the affinity of HOC for DNA is known to be sequence dependent, the ratio of HOC to DNA was lowered to 1:150 dye/DNA base pairs. This should result in the dye occupying a greater fraction of higher affinity sites with sufficient signal still available for quantitative analysis.

Upon titration of trace-labeled HOC-DNA with DOTAP, a net increase in fluorescence intensity of 45% is observed (Fig. 7A, squares). A 370-nm bandpass filter was used in these studies to minimize light-scattering contributions to the spectra. Light-scattering contributions were found to be negligible as assessed by performing similar titrations in the absence of HOC (data not shown). Concomitant with the observed increase in fluorescence intensity is a significant blue shift in the wavelength of the HOC emission maximum from 462 to 453 nm (Fig. 7B, squares). Titrations with DDAB result in a 25% increase in the fluorescence intensity (Fig. 7A, crosses) and a blue shift in the wavelength of the emission maximum to 454 nm (Fig. 7B, crosses).

Incorporating DOPE at a 1:1 molar ratio with DOTAP results in a 30% increase in the fluorescence intensity upon titration of HOC-DNA (Fig. 7A, diamonds) accompanied by a blue shift in the wavelength of the emission maximum to 454 nm (Fig. 7B, diamonds). CHOL-containing complexes show the greatest increase in fluorescence intensity with a 65% increase observed above a charge ratio of 2:1 (Fig. 7A, triangles). Once again, a blue shift in the wavelength of the HOC emission maximum is observed, with values of 452 nm above a charge ratio of 2:1 (Fig. 7B, triangles). Titrations of trace-labeled HOC-DNA with PEI results in roughly 50% of the dye remaining bound to DNA (Fig. 7C). The apparent plateau in the binding isotherm up to a nitrogen/phosphate ratio of 3:1 results from displacement of bound dye, which re-equilibrates with available binding sites because of the sub-stoichiometric labeling ratio used.[4] This is confirmed when examining changes in the wavelength of the emission maximum which also does not significantly change up to a ratio of 3 (Fig. 7D). Above a ratio of 3, however, a significant blue shift to 445 nm is observed, suggesting that bound PEI induces a much greater change in local polarity in the minor groove of DNA.

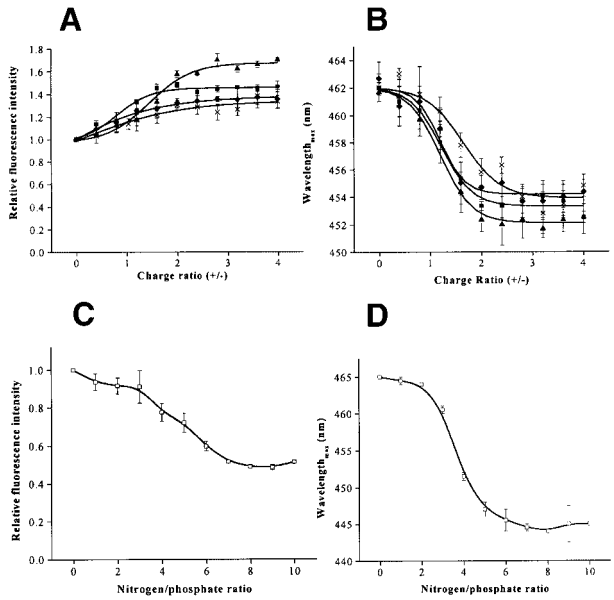

Figure 7. Spectral changes in HOC-DNA fluorescence emission when complexed with various cationic lipids or PEI as a function of charge ratio. DNA was pre-equilibrated with HOC (1:150 dye/DNA base pair) before complex formation with lipids or PEI in 10 mM Tris pH 7.4. (A) Relative fluorescence intensity: DOTAP (squares), DOTAP/DOPE (diamonds), DOTAP/CHOL (triangles), and DDAB (crosses). (B) Wavelength of HOC emission maximum: DOTAP (squares), DOTAP/DOPE (diamonds), DOTAP/CHOL (triangles), and DDAB (cross). (C) Relative fluorescence intensity: PEI. (D) Wavelength of HOC emission maximum: PEI. Data represent the average and SEM of at least three replicates. Lines represent sigmoidal fits to the data and are meant only as a visual guide.

To determine whether dehydration of the minor groove could explain the observed spectral changes in HOC, additional titrations were performed using D_2O as the solvent. Since H_2O and D_2O are known to differentially quench HOC,[38] differences in spectral responses during titrations performed in the two solvents would confirm a role for solvation changes in the observed spectral changes in HOC-DNA upon the binding of cations. When HOC-DNA is dissolved in deuterated buffer, a 30% increase in fluorescence and a substantial red shift in the wavelength of emission maximum are observed. These results are in agreement with previous reports.[38] Titrations of DOTAP into HOC-DNA were performed in both solvents and the wavelength of the emission maximum was monitored as a function of charge ratio (Fig. 8).

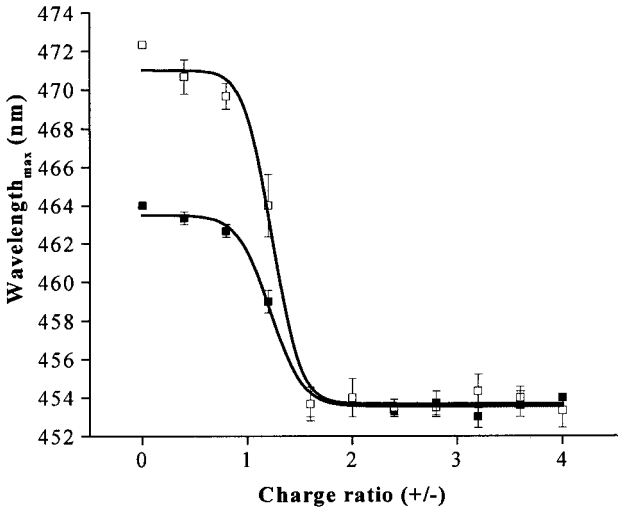

Figure 8. Determination of hydration effects on the observed spectral changes of HOC-DNA complexed with cationic lipids. Identical binding isotherms for DOTAP/DNA were generated in either H_2O (closed squares) or D_2O (open squares) containing 10 mM Tris pH 7.4 (pD 7.8). DNA was pre-equilibrated with HOC (1:150 dye/DNA base pair) before complex formation. Data represent the average and SEM of at least three replicates. Lines represent sigmoidal fits to the data and are meant only as a visual guide.

Substantial differences in the wavelength of HOC in the two solvents are observed for charge ratios below a value of 1.5:1. No difference is observed between titrations done in H_2O and D_2O above this charge ratio, a condition under which DOTAP interaction with DNA is saturated.

The Effect of Ionic Strength on Displacement of ETHD-1 and HOC by Polycations

The effect of physiological ionic strength on this interaction was probed using ETHD-1 and HOC. DOTAP titrations of DNA-ETHD-1 (1:4 dye/DNA base pair) in 10 mM Tris 150 mM NaCl show a substantial decrease in the amount of ETHD-1 displaced at increased ionic strength, with a maximum of 30% of the dye released above a charge ratio of 2:1 (Fig. 9A, squares). The marked increase in fluorescence intensity observed at low ionic strength when complexes were prepared above a charge ratio of 2:1 is not observed at this higher ionic strength. Displacement of ETHD-1 by DDAB at the higher ionic strength shows an overall similar profile to that observed at the lower ionic strength but the maximum extent of ETHD-1 displacement was three-fold less (compare crosses in Fig. 9A with Fig. 3A). DOPE and

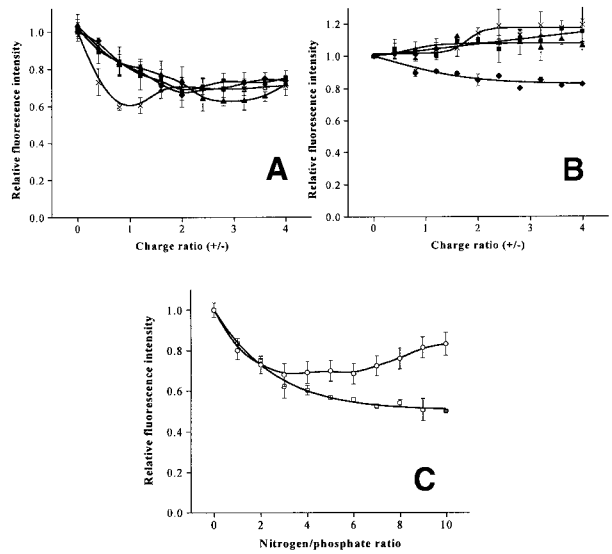

Figure 9. The effect of ionic strength on the displacement of ETHD-1 and HOC by cationic lipids or PEI. Samples were prepared in 10 mM Tris pH 7.4 containing 150 mM NaCl. (A) Displacement of ETHD-1 by cationic lipids. DNA was pre-equilibrated with ETHD-1 (1:4 dye/base pair) before complex formation with cationic lipids. DOTAP (squares), DOTAP/DOPE (diamonds), DOTAP/CHOL (triangles), and DDAB (crosses). (B) Displacement of HOC by cationic lipids. DNA was pre-equilibrated with HOC (1:25 dye/DNA base pair) before complex formation with cationic lipids. DOTAP (squares), DOTAP/DOPE (diamonds), DOTAP/CHOL (triangles), and DDAB (crosses). (C) Displacement of ETHD-1 (circles) or HOC (squares) by PEI. Data represent the average and SEM of at least three replicates. Lines are meant only as a visual guide.

CHOL-containing DOTAP complexes produce a similar effect with a decreased amount of ETHD-1 being displaced by these systems compared with complexes at lower ionic strength (Fig. 9A, diamonds and triangles, respectively). Similar titrations with PEI at the higher ionic strength result in significantly different alterations in fluorescence intensity changes compared with lower ionic strength titrations, with the maximal ETHD-1 displacement of 20–30% occurring above a nitrogen/phosphate ratio of 3:1 (Fig. 9C, circles).

Titrations of polycations into HOC-DNA (1:150 dye/DNA base pair) at the higher ionic strength also demonstrate considerable differences compared with similar titrations at low ionic strength. DOTAP titrations show an insignificant increase in fluorescence intensity with increasing charge ratio (Fig. 9B, squares). The wavelength of the HOC emission maximum also showed no significant change upon complex

formation with DOTAP but was somewhat blue shifted for all complexes including DNA alone when compared with the low ionic strength data (data not shown). This observation is in agreement with previous reports of ionic strength dependent changes in hydration of the minor groove of DNA[39] and was observed in all cases. Titrations with DDAB showed an increase in fluorescence intensity of 15% above a charge ratio of 2:1 but were not significantly different from those observed for DOTAP complexes (Fig. 9B, crosses). DOTAP/DOPE binding to DNA resulted in displacement of 20% of HOC (Fig. 9B, diamonds). When CHOL was incorporated at a 1:1 molar ratio with DOTAP, no significant change in fluorescence intensity was observed (Fig. 9B, triangles). Titrations with PEI at higher ionic strength resulted in the gradual displacement of approximately 50% of HOC from DNA (Fig. 9C, squares). This is slightly less than observed for low ionic strength titrations.

DISCUSSION

Changes in the spectral properties of certain dyes bound to DNA upon complex formation with cationic delivery systems provide significant insight into differences in the interaction of several cationic lipids and the cationic polymer, PEI, with DNA. Two classes of dyes were explored: one that intercalates between the bases and another that binds to the minor groove. Although the description of the binding stoichiometry and affinity of these ligands for DNA is not straightforward because of cooperative effects (both negative and positive), the sequence specificity of the interaction, as well as differences in spectral properties of dyes bound to different sequences,[40] the average values of these parameters were determined for each dye studied and are in general agreement with literature values.

Displacement of Intercalating Dyes from DNA

The intercalating dyes ETBR and its homodimer ETHD-1 differ greatly in their relative affinity for DNA ($\sim 6.4 \times 10^5$-fold greater affinity for ETHD-1). The modes of binding of these two dyes to DNA do, however, share some common features. The intercalation of both ETBR and ETHD-1 is not sequence specific and causes the DNA double helix to unwind by 26 degrees.[31] Although synthesized for bis-intercalation, ETHD-1 binding to DNA is the result of intercalation of a single phenathridine ring system between the DNA base pairs whereas the other ring system remains associated with the minor groove.[31]

In the case of ETBR, the charge density of cationic gene delivery vehicles seems to have the most significant effect on dye displacement. Whereas the IC_{50} values for dye displacement all seem to fall within the range of $1-2:1 \pm$ for cationic lipids and 3:1 nitrogen/phosphate for PEI (Fig. 2) near the point at which all of the negative charge from DNA is neutralized in these systems,[1,34] the extent of dye release seems to be most directly related to cationic charge density. This is supported by the fact that both DOTAP and DDAB displace 85–90% of the ETBR whereas incorporation of neutral helper lipids significantly decreases dye displacement. The almost complete displacement of ETBR by PEI, which has the potential for one of every three atoms of the polymer backbone to be positively charged, further supports this idea. Although possible changes in DNA helical geometry and flexibility upon condensation have been suggested as an explanation of the displacement of ETBR upon condensation of DNA with various polycations,[29] a simple competition for binding to the negatively charged DNA seems equally plausible.

Displacement of ETHD-1 by the same cationic agents results in a considerably different result than that obtained with its monomeric counterpart. Most notable is the apparent biphasic nature of ETHD-1 displacement, with both lipids and PEI showing an initial increase in the amount of dye displaced followed by a decrease at higher charge ratios. Several possible explanations can be advanced to rationalize this result. Because it has been proposed that only one of the phenanthridine rings of ETHD-1 inserts between the base pairs, it is possible that the other ring may insert into either the lipid bilayer, an adjacent DNA strand, or the same DNA strand. No binding of ETHD-1 to liposomes alone is observed in separate titrations, excluding the former case (data not shown). Intercalation into an adjacent DNA strand also seems unlikely when complexed to cationic lipids because the interhelical spacing of the DNA at higher charge ratios is greater than at lower ones, with a distance of 4 nm observed for DOTAP above a 3:1 charge ratio and even greater when helper lipids are included.[12] Bis-intercalation of dye remaining bound to DNA cannot, however, be ruled out.

Another potential explanation involves the effect of cationic lipids and PEI on the secondary or tertiary structure of DNA. Previously it has been reported that no significant changes in the secondary structure of DNA have been observed in IR spectroscopic studies of similar complexes with cationic lipid[3] or PEI.[41] Thermal studies of DNA stability when complexed to PEI, however, have described decreases in DNA stability when complexed with PEI between nitrogen/phosphate ratios of 0 to 0.5 whereas increased DNA stability is observed above this charge ratio.[22] This trend correlates nicely with observed fluctuations in ETHD-1 fluorescent intensity upon addition of increasing amounts of polycation to DNA. This has led to the proposal that subtle changes in the winding angle of the double helix as well as possible alterations in the topology of supercoiled plasmid DNA occur upon complex formation with these agents.[22] Thus, displacement of ETHD-1 as a function of charge ratio and polycation structure may therefore prove to be a sensitive tool for an analysis of the condensed DNA structure in these complexes. Further studies are necessary, however, to more comprehensively describe the basis for the observed displacement of ETHD-1 from DNA upon complexation with cationic gene delivery vehicles.

Displacement of Minor Groove Binding Dyes from DNA

In contrast to intercalating dyes, the binding of DAPI and HOC does not significantly distort the helical structure of DNA.[42] Both dyes display a strong preference for binding to the relatively narrow minor groove of AT rich sequences, with DAPI spanning 3–4 and HOC 4–6 base pairs.[33,42] As indicated in Table 1, both dyes possess similar macroscopic affinity constants for DNA but differ somewhat in stoichiometries. Several previous studies have demonstrated that the sequence specific microscopic affinity constants can vary by 200-fold for HOC and 30-fold for DAPI for five possible combinations of tetrameric A/T base pairs.[33,42] Additional differences between the interaction of DAPI and HOC include the observation that HOC is more likely to interact with G/C sequences directly up and downstream from the AT binding sites[42] and apparent differences in sensitivity to the width of the minor groove.[42]

Given the similarities in the relative affinities of DAPI and HOC for plasmid DNA, the significant differences in displacement of these dyes by cationic lipids and PEI is unexpected. The extent of DAPI displacement by cationic lipids is essentially complete with $<10\%$ of the dye remaining bound to DNA for all systems examined. In contrast, the displacement of HOC by cationic lipids is minimal. This apparent difference in dye displacement has also been observed for the condensation of spermatozoal DNA by protamine.[43,44] Differences in the displacement of these two dyes by PEI are also apparent. Because the footprints of DAPI and HOC bound to DNA are typically very similar, the observed differences in displacement of the dyes may reflect dissimilar sensitivities of DAPI and HOC to subtle structural changes in the minor groove that occur upon binding cationic lipids or PEI. Allosteric effects of DAPI binding to model oligonucleotides have demonstrated a significant cooperativity in some cases[36] but the role of this phenomenon in larger polynucleotides, if any, has not yet been described.

HOC can be Used to Probe Environmental Changes in the Minor Groove of DNA in Complexes with Cationic Lipids and Polymers

The intriguing observation that only a small fraction of HOC is displaced upon binding of cationic lipids or PEI to DNA suggests that this dye may be used as an extrinsic probe of environmental changes in the DNA minor groove. Additionally, HOC labeling of DNA provides a relatively inexpensive alternative to commercially available kits for the covalent attachment of fluorescent dyes to DNA. This should permit further studies of the structure and dynamics of cationic lipid/DNA complexes using additional fluorescence techniques such as resonance energy transfer and anisotropy decay. The results of CD studies confirming that the dye remains bound to the minor groove of DNA when complexed with cationic lipids further supports this utility of HOC in these studies.

Changes in the fluorescence spectral properties of DNA labeled with sub-stoichiometric amounts of HOC show considerable sensitivity to the binding of cationic lipids and polymers (Fig. 7). The increase in fluorescence intensity coupled with a blue shift in the wavelength of emission maximum upon complex formation of HOC-DNA with polycations suggests a significant decrease in the polarity of the minor groove. Studies comparing the spectral changes for titrations performed in both H_2O and D_2O argue that this polarity change is attributable at least in part to a significant

change in the hydration of the groove upon binding of cationic lipids and polymers (Fig. 8).

Previous IR spectroscopic studies have demonstrated dehydration of the phosphate backbone and nitrogenous bases (especially guanine) of DNA as well as the interfacial region of lipids upon formation of cationic lipid/DNA complexes.[3] Additionally, studies using differential scanning calorimetry and generalized polarization of laurdan fluorescence have provided evidence for dehydration events upon cationic lipid/DNA complex formation.[23] Results presented above regarding spectral changes in HOC-DNA upon complex formation with various polycations suggest that this method can provide additional information specifically related to dehydration of the DNA minor groove.

In comparing this new evidence for the dehydration of the minor groove to that previously obtained by IR spectroscopy for the same complexes, several similarities are observed. DOTAP appears to facilitate a greater dehydration of the minor groove than DDAB based on the greater changes in HOC fluorescence intensity and the wavelength of the emission maximum. This correlates with the IR spectroscopic results which show that the major groove-oriented C6-carbonyl of guanine is dehydrated to a greater degree by DOTAP than DDAB.[3] The effect of incorporating the helper lipids DOPE and CHOL into DOTAP complexes with DNA has been shown to facilitate a greater degree of dehydration of both the lipid interface[3,23] as well as the guanine carbonyl.[3] Significant dehydration of the DNA minor groove is apparent for CHOL-containing complexes which show a marked increase in the HOC-DNA spectral changes. Spectral changes for DOPE-containing complexes are not very different than those seen for DOTAP alone suggesting little effect of this helper lipid on the dehydration of the minor groove. Whereas complex formation of DNA with PEI results in considerable displacement of HOC from its binding site in the minor groove, a significant shift in the wavelength of emission maximum suggests that the minor groove of DNA is dehydrated to a much greater extent by PEI than any of the cationic lipids tested.

Ionic Strength Effects on the Interaction of Cationic Lipids and Polymers with DNA

Increasing the ionic strength to near physiological conditions (e.g., 150 mM NaCl) has a significant effect on the displacement of the two dyes which show the least amount of displacement upon cationic lipid or PEI binding at low ionic strength. For ETHD-1, the extent of dye displaced by the cationic lipids is greatly reduced in agreement with the Manning's polyelectrolyte theory.[45] Binding of PEI to DNA-ETHD-1 at physiological ionic strength, however, shows drastically different results than the low ionic strength data with maximum displacement of ETHD-1 occurring at the point of stoichiometric neutralization of DNA phosphates and no substantial increase in the amount of dye bound at higher nitrogen/phosphate ratios. The marked differences in ionic strength-dependent trends for displacement of ETHD-1 by cationic lipids and PEI could be explained by structural differences in DNA at the two ionic strengths because the superhelical topology of plasmid DNA is known to be a function of ionic strength.[46,47] This lends further support to the notion that ETHD-1 is sensitive to DNA structural changes upon condensation with cationic gene delivery vehicles.

The utility of HOC as a probe for environmental changes in the minor groove of DNA at increased ionic strength is somewhat diminished. Although it appears that HOC still remains bound to the DNA minor groove in cationic lipid/DNA complexes, dehydration of HOC bound to DNA by increasing the ionic strength does not permit detection of further changes in solvent accessibility.

CONCLUSIONS

These studies provide further insights into the influence of polycation structure on their interaction with plasmid DNA. The use of multiple fluorescent dyes has provided enhanced resolution of differences in these interactions in terms of subtle topological changes in DNA as well as solvent accessibility of DNA within complexes. This information should aid in the further characterization of polycation/DNA complexes and will be useful in exploring the effects of various formulation variables on this interaction.

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