Structure/Function Relationships of Polyamidoamine/DNA Dendrimers as Gene Delivery Vehicles

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ABSTRACT: PAMAM dendrimers are members of a class of polyamine polymers that demonstrate significant gene delivery ability. In this study, a selection of PAMAM dendrimers, spanning a range of sizes (generations 2, 4, 7, and 9) and transfection efficiencies, are characterized by various biophysical methods to search for structural properties that correlate with transfection. Measurements of colloidal properties (size and zeta potential) as a function of charge ratio reveal that highly transfecting dendrimer/DNA complexes have size/zeta potential values between 4 and 8. Circular dichroism (CD) and FTIR spectroscopy of complexes confirm the DNA component remains in B form when associated with all dendrimer generations up to a 5:1 charge ratio (±). Isothermal titration calorimetry and differential scanning calorimetry detect changes that are related to polymer structure and charge ratio but do not directly correlate with transfection efficiency. Despite DNA structural and stability changes detected by CD, FTIR, DSC, and ITC that are similar to those seen with other cationic delivery vehicles [e.g., cationic lipids, peptoids/lipitoids, peptides, polyethyleneimines (PEIs), etc.], clear correlations with transfection activity are not readily apparent. This may be due, at least in part, to the heterogeneity of the complexes. © 2004 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 94:423–436, 2005

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INTRODUCTION

The mechanism by which nonviral gene delivery vehicles transfect cells remains poorly understood.1 This is especially problematic because it makes the rational design of more effective vectors very difficult. One approach to this problem is to systematically vary the structure of such complexes by altering the ratio of delivery polymer to DNA and attempt to correlate the different species formed with transfection ability. In a further extension of this approach, the nature of the polymer itself can be varied and structure/function correlations probed. To this end, we have systematically studied complexes formed between plasmid DNA with various cationic lipids,2–9 different molecular weight polyethyleneimines (PEIs),10 and peptoids with altered side chains11,12 as a function of charge ratios to seek significant correlations with in vitro transfection efficiency. The result of these efforts has so far been somewhat disappointing. This may reflect the wide differences among the various delivery vehicles as well as the intrinsic heterogeneity of the complexes so far studied. To obviate some of these difficulties, we have now extended this
approach to the study of polyamidoamine (PAMAM) dendrimer complexes.

The PAMAM dendrimers are polymers containing both tertiary amines at branch points as well as primary amines at the termini (Fig. 1). PAMAM dendrimers (Fig. 1) are formed by exhaustive Michael addition of ethylene diamine with methyl acrylate followed by addition of the resulting ester core to an excess of ethylene diamine. This synthesis gives a high yield of first-generation PAMAM dendrimers, with higher generations arising from repetition of the previous two steps. Generations up to 10 can be formed before further assembly is limited by the “de Gennes dense packing” phenomenon. The dendrimers so prepared have a consistent size, structure, and charge characteristic of their generation. Transfection efficiency mediated by PAMAM dendrimers appears to be dependent on dendrimer generation, with larger sizes providing higher efficiency, and the charge ratio of the complexes, in which a net positive charge is optimal. In addition to the simple complexation of DNA with intact dendrimers, further transfection enhancement by the use of fractured (hydrolysis degraded) dendrimers or addition of cationic excipients such as DEAE-dextran have also been reported.

The representative PAMAM dendrimers investigated here (G2, 4, 7, and 9) have well-defined chemistry and molecular weights in contrast to the cationic component of nonviral complexes formed from cationic lipids, peptoids/lipitoids, and PEI, providing a system in which transfection efficiency varies, but with less complication from heterogeneity and variable chemistry. Therefore, we have conducted an extensive biophysical characterization of PAMAM dendrimer/DNA complexes (PDDCs) with the goal of establishing a correlation between their physical properties and in vitro transfection efficiency. To this end, we have investigated the hydrodynamic size and zeta potential of complexes by dynamic light scattering and electrophoretic light scattering. The thermodynamics of the complexation process and the stability of complexes were examined by isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC), respectively. The relative affinities of the DNA/dendrimer interactions were determined by ethidium bromide dye displacement. The secondary structure of the DNA component when complexed to dendrimer was investigated using circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopy.

Figure 1. Chemical structure of a generation 1 PAMAM dendrimer.
These same methods were utilized in the characterization of a number of other nonviral gene delivery preparations studied previously in our laboratory, as indicated above. The current analysis of the dendrimer/DNA delivery system was compared to these previous studies.

**MATERIALS AND METHODS**

PAMAM dendrimers of generations 2, 4, 7, and 9 were a generous gift from Dendritic Nanotechnologies (Mt. Pleasant, MI). Plasmid DNA pMB 290 (4.9 kbp) and pMB 401 (5.9 kbp, encoding firefly luciferase), both >95% supercoiled, were provided by Valentis, Inc. (Burlingame, CA). The former (pMB 290) was used for all the characterization studies while the latter (pMB 401) was used for the in vitro transfection studies. The luciferase marker gene was under the control of the CMV promoter. Ethidium bromide was purchased from Molecular Probes (Eugene, OR). Dulbecco's modified Eagle media was acquired from Cambrex (Rutherford, NJ). Fetal bovine serum was purchased from Atlanta Biologicals (Nocross, GA). Accutase was obtained from Innovative Cell Technologies (San Diego, CA). Opti-Mem was acquired from Invitrogen (Carlsband, CA). 1,2-Dioleoyl-3-trimethylammonium propane (DOTAP) was obtained from Avanti Polar Lipids (Alabaster, AL). Tris and phosphate buffer salts were purchased from Sigma (St. Louis, MO). Nano-purified water was used for all buffer preparation.

**Preparation of Complexes**

The dendritic polymers were received at high concentration (>10% w/w) in methyl alcohol, and were diluted directly into buffer for analysis unless otherwise stated. The plasmids were diluted from a stock solution. The concentration of DNA solutions was determined using their UV absorbance at 260 nm (A260) and a molar extinction coefficient of 0.02 (mg⁻¹ cm⁻¹ mL⁻¹). Individual dilutions were performed for DNA and dendrimer at each charge ratio, calculated as the mol of dendrimer primary amines per DNA phosphates. PAMAM dendrimer/DNA complexes (PDDCs) were formed by addition of equal volumes of DNA and dendrimer while stirring. The order of addition was selected to avoid passing through charge neutrality. These complexes were allowed to equilibrate for 20 min prior to analysis. Complexes were prepared in 10 mM Tris buffer, pH 7.4 unless otherwise noted.

**In Vitro Transfection Efficiency**

The transfection efficiency of dendrimer/DNA complexes was determined by luciferase expression in CHO-K1 cells. The CHO-K1 cells were obtained from the American Type Culture Collection (Rockville, MD). Cultures were maintained in 75 cm² flasks at 37°C and 5% CO₂ and grown in Ham’s F-12 media containing L-glutamine and 10% FBS. Cells were split every 4 days using standard procedures, and accutase was employed for cell lifting. Plates were prepared by adding ~8000 cells (e.g., ~16% confluence) to each well of a 96-well plate and incubating for 18–20 h at 37°C and 5% CO₂ before transfection.

Approximately 1 mg of PAMAM dendrimer of each generation was dried under vacuum with silica gel desiccant to remove methyl alcohol until a stable weight was achieved. The pMB 401 and 10 mM Tris buffer (pH 7.4) used for reconstitution of the dendrimers was filtered through a 0.22-μm filter. Appropriate volumes of polymer and DNA were mixed to prepare complexes at a 25 μg mL⁻¹ DNA concentration. Complexes at various charge ratios were diluted to a final transfection DNA concentration of 2 μg mL⁻¹ with Opti-MEM. Immediately before transfection, the cells were washed once with PBS followed by addition of 10 μL of solution containing complexes (200 ng of pMB 401) to each well. Cells were incubated with complexes for 5 h. The 100 μL of transfection solution was then removed and replaced by 100 μL of culture media. The cells were incubated for a further 48 h at 37°C and 5% CO₂.

 Luciferase expression was assayed using the Luciferase Assay System™ from Promega (Madison, WI) following the supplied protocol. The cells were lysed with Reporter Lysis Buffer for 30 min. Expression was analyzed in 96-well plates with a Fluostar™ Galaxy microtiter plate reader (BMG, Offenburg, Germany). Automated addition of substrate buffer was employed and the luminescence was then measured. The amount of protein in each well was determined by Coomassie Blue assay (Promega). The data were normalized to the total protein content and reported as relative light units per milligram of protein (n = 3).

**Dynamic and Electrophoretic Light Scattering**

All samples intended for light scattering analyses were prepared using 10 mM Tris buffer, pH 7.4, which was filtered with a 0.22-μm filter to remove any trace particulates. Complexes were prepared.
at a 100 µg mL⁻¹ DNA concentration and at various charge ratios between 0.25 and 5. Particle sizes were measured by dynamic light scattering (DLS) using a Brookhaven (Holtsville, NY) instrument equipped with a 9000AT autocorrelator, a 50-mW HeNe laser operating at 532 nm (JDS Uniphase), an EMI 9863 photomultiplier tube, and a BI-200M goniometer. The light scattered at 90° from the incident light was fit to an autocorrelation function using the method of cumulants. The resultant diffusion coefficient was then converted to a mean hydrodynamic diameter by the Stokes-Einstein equation.

Zeta potential measurements were obtained by phase analysis light scattering (PALS) using a Brookhaven Zeta PALS instrument. The electrophoretic mobility of the DLS samples was determined from the average of 10 cycles of an applied electric field. The zeta potential of complexes was determined from the electrophoretic mobility by means of the Smoluchowski approximation.

**Ethidium Bromide Displacement Assay**

Ethidium bromide was bound to DNA at a 4:1 ratio (DNA bp to ethidium molecule). The labeled DNA was complexed to dendrimers at various charge ratios from 0.25 to 5, while the DNA concentration was maintained at 10 µg mL⁻¹. All samples were prepared in triplicate and the relative fluorescence was quantitated by subtracting the fluorescence intensity of ethidium bromide in water and normalizing to DNA alone. Samples were placed in a 96-well plate and fluorescence was measured using a Galaxy Fluorstar plate reader at an excitation wavelength of 530 nm and emission wavelength of 590 nm. The fluorescence intensity of the dendrimer alone was similar to that of ethidium bromide in water.

**Circular Dichroism (CD) Spectroscopy**

CD spectra of complexes (at a DNA concentration of 100 µg mL⁻¹) at various charge ratios were acquired with a Jasco J-720 spectropolarimeter (Easton, MD). Reported spectra are an average of three accumulations over a spectral range of 190–350 nm obtained at a scan rate of 20 nm min⁻¹ with samples maintained at 20°C in a 1-mm pathlength quartz cuvette. Spectra were buffer subtracted and converted to molar ellipticity based on the molar concentration of the DNA using the instrument software. Further processing including smoothing with a Savitsky-Golay function (7 pts) and a fast Fourier transform method. Peak position determination (+0.3 nm) and plotting were performed using Origin 6.0 software (Microcal Software, North Hamptom, MA).

**Fourier-Transform Infrared (FTIR) Spectroscopy**

FTIR spectra were obtained with a Nicolet Magna-IR 560 spectrometer (Madison, WI) configured with a mercury–cadmium–telluride (MCT) detector. Samples were examined at a DNA concentration of 1 mg mL⁻¹ in a zinc selenide (ZnSe) ATR trough cell (Thermo Spectra-Tech, Madison, WI) with an effective pathlength of 12 µm. Spectra were obtained as 256 scan interferograms under dry air purge using Happ-Genzel apodization with no zero filling producing an effective resolution of 2 cm⁻¹ as described previously. Data analyses including buffer subtraction, baseline adjustment, maximum entropy smoothing (Razor Tools, Spectrum Square Associates, Ithaca, NY), and peak position determination were conducted in Grams AI (Thermo Galactic, Salem, NH). Peak positions were determined from the mean of five replicate independent experiments with the standard error plotted as error bars.

**Isothermal Titration Calorimetry (ITC)**

Calorimetric titrations were conducted at 25°C using a Calorimetry Sciences (CSC) model 4200 instrument. The DNA and dried dendrimer (prepared as described above) were buffer matched by dialysis and drying with subsequent reconstitution in the same buffer. Both solutions were then degassed prior to analysis. The titration program consisted of a series of 25 injections of 10 µL of dendrimer into DNA with a 5-min equilibration interval between injections. Binding heats were integrated by Bind Works 3.0 software (Calorimetry Sciences, Provo, UT). Apparent molar binding enthalpies were calculated from the average of the first six consistent injections (typically two to seven), divided by the molar amount on a charge basis of dendrimer per injection as described previously.

**Differential Scanning Calorimetry (DSC)**

DSC thermograms were acquired with a Microcal capillary VP-DSC (Northampton, MA) instrument configured with an autosampler. All complexes were prepared in 5 mM phosphate buffer.
either through dialysis or drying and reconstitution in buffer. A phosphate buffer was used in these experiments because of its low temperature coefficient compared to Tris. Samples were degassed and distributed in a 96-well plate held at 4°C. Thermal scans at a pressure of 40 psi, consisted of a single up scan from 20 to 125°C at a rate of 1°C min⁻¹. Data analysis including subtraction of buffer thermograms, conversion of differential heat to molar heat capacity, and determination of supercoil Tₘ values was conducted in Origin 7.0.

RESULTS

In Vitro Transfection of CHO-K1 Cells

Complexes of DNA with certain polyamidoamine dendrimers substantially enhanced luciferase activity in CHO-K1 cells. The results presented in Figure 2 demonstrate that expression levels are dependent on both the charge ratio of the complex and the dendrimer generation employed. The G2 complexes produced negligible expression compared to higher (G4, 7, and 9) generation PDDCs, with the highest generation considerably less effective than the middle two sizes. In the latter three cases, expression is generally enhanced at higher charge ratios with maximal expression seen at the same charge ratio of 10:1. This general trend of the highest transfection efficiency seen with complexes of moderate generation size (4–7) and in the presence of a significant excess of dendrimer has been previously reported.¹⁴⁻¹⁶ The expression produced by G4 PDDCs at modest charge ratios (1–4) contrasts sharply with that of the other generations. The transfection studies reported here are in contrast to previous dendrimer transfections because the dendrimers were not fractured and additional transfection agents (i.e., dextran sulfate) were not employed. This permits a more rigorous structural analysis of the complexes as described below.

Particle Sizes and Zeta Potentials

The mean hydrodynamic diameter and zeta potential of the complexes were determined by DLS and PALS, respectively (Fig. 3a and b). Regions in which large aggregates (>1 μm) were formed are excluded from this analysis. In general, particle sizes of complexes at high charge ratios (4–5) range over a narrow distribution of ~100 to 175 nm in diameter. In the charge ratio range where complexes have significant biological activity (1–10), the G2 PDDCs have the largest hydrodynamic diameters, while G4 PDDCs possess the smallest dimensions. Particle sizes were only characterized up to a charge ratio of 5; however, all complexes that show significant transfection efficiency have a minimal size at this ratio (average of 122 nm). The zeta potential of the complexes is negative at charge ratios less than unity. In general, dendrimer/DNA complexes display poor colloidal stability near neutrality (0.75–1.75), resulting in aggregation and precipitation, except complexes formed from G4 dendrimers. The zeta potential of complexes prepared with charge ratios in excess of 3 appear to form particles with a stable positive surface charge, the value of which is dependent on generation size. Generation 4 PDDCs show a high positive zeta potential (>15 mV) at all charge ratios greater than and equal to 1:1. The surface charge appears related to dendrimer generation with G2 and 4 showing a similar positive zeta potential near theoretical charge neutrality while G7 and 9 maintain a negative value.

Ethidium Bromide Displacement

Interactions between PAMAM dendrimers and DNA were evaluated from the ability of dendrimers to displace the intercalating dye, ethidium
bromide, from the DNA. The smallest dendrimer, G2, predictably displaces the least ethidium bromide, a modest 57% at the highest charge ratio examined (5) (Fig. 4). The largest dendrimer, G9, appears to have the greatest relative affinity for DNA, displacing 81% of the dye at charge ratios in excess of 2. Thus, the relative binding affinity of PAMAM dendrimers appears to correlate with a generation size in the order G9 > G7 > G4 > G2.

This indirect means of determining binding affinity may be misleading, however, because multiple mechanisms for the displacement have been suggested, including conformational changes that modify base stacking and charge repulsion between the dye and polyamine polymers. It appears that in this case, the mechanism at least involves exclusion of the dye from its DNA binding site into bulk solution, because the dye’s fluorescence can be enhanced by the addition of unliganded DNA to high charge ratio complexes (data not shown).

Circular Dichroism Spectroscopy

CD spectroscopy was used to characterize the helical structure of the DNA within PAMAM dendrimer/DNA complexes. The spectrum of unliganded plasmid (Fig. 5A) shows the characteristics of the native B form conformation, a positive band centered near 275 nm, and a negative band near 245 nm. None of the dendrimers manifested any optical activity. The spectra of individual complexes undergo a charge ratio-dependent decrease in intensity of the 275 nm CD bands and a red shifting of the peak position of both the 275 and 245 nm signals. At a charge ratio of 0.5, the 275 nm peak is almost completely lost and the associated 245 nm trough is dramatically intensified. Both bands are significantly red shifted but not to the same degree. As the positive charge...
Figure 5. Representative CD spectra of G4 dendrimer/DNA complexes normalized as molar ellipticity values (A). Plots of the intensity of the 245 nm band (B), the position of 245 nm band (C), the intensity of 275 nm band (D) and the position of 275 nm band (E) versus dendrimer/DNA charge ratio. Samples were prepared at a DNA concentration of 100 μg mL⁻¹ in 10 mM Tris buffer (pH 7.4). Individual points are the mean of at least three measurements with error bars representing the standard error.
increases (charge ratios 3 and 5), spectra continue to manifest altered shapes in comparison to unliganded DNA, but with intensities and positions shifted to a lesser degree than seen at lower charge ratios. Both intensity and peak position are summarized for each dendrimer generation as a function of charge ratio in Figure 5B–E. The gaps in the data near charge neutrality are due to the exclusion of data due to sample precipitation. In general, the maximal spectral perturbation from the unliganded DNA spectra occurs in complexes prepared near charge neutrality. Complexes with a charge excess of dendrimer exhibit spectra with intensities and peak positions still significantly altered from the native plasmid but give nearly identical spectra at charge ratios equal to 3. The spectra of DNA in complexes vary in the intensity of the B form marker bands. The 245-nm trough increases in intensity and the 275-nm peak decreases, with the degree of perturbation from unliganded DNA following the trend G2 > G4 > G9 > G7. Accompanying the change in rotational strength of complexes is a red shift in peak position. The red shifts follow the same trend as the intensity changes with respect to dendrimer generation. The red shifts in the predominant bands vary considerably, with the 245 nm trough shifting as much as 7 nm while the 275 nm peak exhibits shifts of nearly 18 nm (G2). This dramatic peak shifting appears to be related to generation size with smaller dendrimers (2 and 4) near charge neutrality manifesting the largest red shift.

Fourier-Transform Infrared (FTIR) Spectroscopy

FTIR spectroscopy was employed as a complementary technique to further investigate the secondary structure of the DNA component of complexes. The FTIR spectra of DNA, several G4 dendrimer/DNA complexes and G4 dendrimer alone are compared in Figure 6A. The DNA spectrum in the absence of polymer is indicative of B form with conformationally sensitive vibrations arising from guanine/thymine carbonyl stretching (1715 cm\(^{-1}\)), coupled sugar-base thymine vibrations (1328 cm\(^{-1}\) and 1281 cm\(^{-1}\)), antisymmetric phosphate stretching (1224 cm\(^{-1}\)), coupled phosphodiester/deoxyribose backbone stretching (970 cm\(^{-1}\)), and ribose ring vibrations (897 cm\(^{-1}\)).1,10 The dendrimer spectra have three major absorption bands, two broad peaks arising from amine deformation (1635 cm\(^{-1}\)) and amide C–NH stretching (1560 cm\(^{-1}\)) as well as a strong C–OH stretch from the incomplete removal of methyl alcohol used as the dendrimer solvent. In spectra of complexes, the dendrimer bands were poorly resolved, and could not be reliably monitored. The position of the five DNA bands, three conformationally sensitive signals (1715, 1224, and 970 cm\(^{-1}\)) as well as two DNA backbone vibrations considered insensitive to conformation (the symmetric phosphate stretch at 1086 cm\(^{-1}\) and the antisymmetric C–O stretch of furanose at 1053 cm\(^{-1}\)), are plotted as a function of complex charge ratio in Figure 6B–E. The conformationally sensitive DNA backbone band (970 cm\(^{-1}\)) and G/T carbonyl stretch (1715 cm\(^{-1}\)) both increase in frequency upon dendrimer binding up to a 1:1 charge ratio while the asymmetric phosphate stretch (1224 cm\(^{-1}\)) decreases in frequency over the same range. Although these conformationally sensitive peaks shift significantly when DNA is bound to the dendrimers, the positions remain indicative of only the B conformation.

The shifts in position of the vibrations arising from both backbone and bases suggest some direct dendrimer/DNA interaction occurs outside of the expected phosphate–amine electrostatic interactions. The position of a DNA ribose sugar vibration (1053 cm\(^{-1}\)) shifts significantly to lower frequency

![Figure 6. Representative FTIR absorbance spectra of G4 dendrimer, DNA, and complexes (A). Plots of selected peak positions of G2 PDDCs (B), G4 PDDCs (C), G7 PDDCs (D) and G9 PDDCs (E) as a function of charge ratio. All spectra are of complexes prepared at a DNA concentration of 1 mg mL\(^{-1}\). The peak position values and error bars (SEM) reflect an average of five measurements.](Image)
(≈2.3 cm⁻¹) in both G7 and 9 PDDCs. Furthermore, as indicated above, the increases in frequency of the base carbonyl vibrations suggest altered hydrogen bonding occurs within the bases.

The decrease seen in the frequency of the antisymmetric phosphate stretch (1223 cm⁻¹) with polymer addition up to a 1:1 ratio also suggests (as expected) this moiety is directly involved in

Figure 6. (Continued)
the dendrimer/DNA interaction\textsuperscript{10} presumably through a direct electrostatic interaction with dendrimer amino groups.

**Isothermal Titration Calorimetry (ITC)**

To investigate the thermodynamics of binding of dendrimers to DNA, we conducted ITC analysis of each dendrimer generation at 25°C and low ionic strength (<0.01 M). Unfortunately, due to aggregation prior to saturation of binding (at charge neutrality), the number of binding sites or an affinity constant could not be determined. An apparent binding enthalpy ($\Delta H_{\text{app}}$), however, could be estimated by averaging the observed constant binding heats prior to aggregation. These results are summarized in Table 1. Dendrimer generation appears to have little effect on the binding energetics. The average binding enthalpy of $\sim$7.6 kJ mol$^{-1}$ observed for the four complexes is consistent with electrostatic interactions dominating the complexation process.\textsuperscript{8,18–21}

**Differential Scanning Calorimetry (DSC)**

DSC was used to evaluate the thermal stability of DNA complexed to PAMAM dendrimers. Thermograms of DNA display two major melting transitions. The first originates from linear/open circular forms and occurs between 60 and 70°C, while the second arises from supercoiled DNA and appears near 109°C (data not shown).\textsuperscript{9} The thermograms of PAMAM dendrimers alone display no thermal transitions. The melting temperature of the supercoiled plasmid complexed to each generation of dendrimer is plotted versus charge ratio in Figure 7. The supercoiled form is thermally destabilized when complexed to dendrimers below charge neutrality ($\leq$0.5). Complexes prepared with a significant positive charge excess (>2), however, show thermal stabilization of the supercoiled form. Dendrimer/DNA complexes prepared from generations 2, 4, 7, and 9 show similar destabilization at low charge ratio and stabilization at high ratios. Maximal temperature stabilization occurs at a two to three charge ratio regardless of the dendrimer generation employed. The extent to which the SC $T_m$ is altered does not obviously correlate with the transfection efficiency of the various dendrimer generations.

**DISCUSSION**

This study attempts to establish a structure–function relationship between the biophysical properties of dendrimer/DNA complexes and their in vitro transfection efficiencies. A number of biophysical measurements including size and zeta potential measurement, dye (ethidium bromide) displacement, DSC, CD, and FTIR show significant alterations as the ratio of dendrimer to DNA is varied. These changes appear to be greatest up to the point the amount of dendrimer becomes equal to that of DNA on a charge basis. These results suggest structural rearrangements in both DNA and the complex itself as charge neutrality is reached. By comparison, PDDCs that show the greatest biological activity (G4 at a 1–10 charge ratio and G7 at 2–10 charge ratios) exhibit

**Table 1.** Binding Enthalpies of PAMAM Dendrimers to Plasmid DNA at 25°C Using ITC

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<tr>
<th>Ligand</th>
<th>$\Delta H$ (kJ/mol)</th>
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<tr>
<td>Generation 2 Dendrimer</td>
<td>7.6 ± 0.5</td>
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<tr>
<td>Generation 4 Dendrimer</td>
<td>9.3 ± 1.3</td>
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<tr>
<td>Generation 7 Dendrimer</td>
<td>7.4 ± 0.1</td>
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<tr>
<td>Generation 9 Dendrimer</td>
<td>6.1 ± 0.9</td>
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their maximum activity at charge ratios above charge neutrality. All four dendrimer generations examined also show optimal structural stability when the dendrimer is in charge excess ($>2$). A number of studies of various nonviral gene delivery vehicles suggest that complexes formed in significant charge excess reach a structurally stable state that differs depending on the chemistry of the vehicle. Thus, the total of the evidence suggests that structural features of the complexes may be related in some way to their individual transfection efficiencies, but the details of these relationships are currently obscure.

The colloidal properties (hydrodynamic diameter and zeta potential) of PDDCs do not independently appear to correlate with transfection efficiency. Further analysis of the size and zeta potential of complexes at charge ratios of 2–5 where G4 and G7 complexes differ in their transgene expression, do suggest a correlation in which low (4–8) size to charge ratios show high transfection efficiency. Although this correlation exists for G4 and 7 PDDCs, the existence of G2 and 9 PDDCs with size/charge ratios within the postulated range but without significant transfection activity suggests that a narrow range of size/charge ratios may be necessary but insufficient to explain relative transfection abilities. Previous studies of nonviral vectors composed of cationic lipids, peptoids/lipitoids, or PEI show contrasting results with regard to any size/charge correlation. Polyethylenimine complexes formed at N/P ratios with a positive zeta potential ($>4$) manifest no evidence of a size/charge correlation with transfection efficiency. Characterization of cationic lipid and peptoid/lipitoid transfection active complexes find size/charge ratios within the postulated range but the limited number of observations preclude further refinement of any correlation from this data. A similar charge density correlation has recently been proposed for cationic lipid complexes that adopt a lamellar organization.

The two generations of dendrimer that show the greatest biological activity (G4 and 7) both have an intermediate relative affinity for the DNA as determined by ethidium bromide displacement. The lowest affinity dendrimer (G2) has poor transfection efficiency, possibly related to a complex morphology that is different from G4–G9 complexes as suggested by its abnormal gel mobility and greater fluorescent dye accessibility in direct addition experiments. Cationic lipids incorporating the helper lipid DOPE similarly exist in an altered morphology (inverted hexagonal phase) and manifest both low relative affinity for DNA and reduced transfection efficiency when prepared with a cationic charge excess. In contrast, cationic vehicles with a moderate to high relative affinity seem to form complexes with similar overall morphologies at least with respect to the properties discussed above. Peptoids demonstrate a correlation similar to G4 and G7 dendrimers with a high transfection efficiency and moderate relative affinity. A number of cationic vehicles have high relative affinities but differ in their transfection efficiencies. Both PEI (750 kDa) and G9 dendrimer have high relative affinity but lower transfection efficiencies. This observation suggests an upper threshold may exist in which high affinity reduces transfection, perhaps by inhibiting DNA release. It has been previously shown that G9 dendrimer complexes inhibit luciferase expression to a greater extent than lower generations (G5 and 7) in an in vitro gene expression system. An exception to the latter may exist, however, in that many cationic lipids display both high dye binding affinity and high transfection activity. The difference in the chemistry between lipid and polymer vehicles at least leaves open the possibility that a different DNA release mechanism between the two cations may account for this discrepancy. The observation that some fraction of the ethidium is excluded suggests that displacement may be mediated by condensation of the DNA and dendrimer to a more compact state. This mechanism is consistent with the trend in which the largest dendrimers (highest generations) apparently displaced the greatest fraction of dye. It should be remembered, however, that neither ITC nor ethidium bromide exclusion provide an unambiguous measure of the affinity of the cationic vehicle for DNA.

The characterization of PAMAM dendrimer/DNA complexes reveals no other obvious correlations with transfection efficiency. Importantly, however, biophysical methods do reveal a number of common features among a wide variety of nonviral vectors as well as a few measurements that may sensitively reflect differences in vector chemistry. It appears that the DNA conformation within nonviral gene delivery complexes of a wide variety of classes (cationic lipids, peptoids/lipitoids, PEI, and dendrimers) remains in an overall B-form helix, although localized alterations in DNA structure may occur within complexes as reflected by FTIR position shifts and altered CD spectra (see extensive discussion.
within ref. 7). These alterations show a strong dependence on the individual chemistries of the cationic vehicle. The thermal stability of the scDNA melting transition also shows a dependence on the nature of the cationic component. Both dendrimers and cationic lipids stabilize supercoiled DNA when complexes are prepared in positive charge excess, while PEI complexes at charge excess either stabilize the scDNA transition (750 and 25 K branched forms) or destabilize (2 K branched and 25 K linear) depending on the geometry and/or molecular weight of the polymer.10

Biophysical characterization of PAMAM dendrimer/DNA complexes does suggest possible correlations between transfection efficiency and particle size/charge ratio and the relative affinity of the PAMAM dendrimer and DNA components. These correlations are not apparent, however, between chemically dissimilar nonviral vehicles. The lack of any all-inclusive correlation may be due to the different distribution of amine binding moieties among differing vehicles or could be a result of complexes consisting of a number of subpopulations varying in size, density, and charge state due to the nonspecific electrostatic interactions involved in their assembly. Although we attempted to reduce cell-type variability in our studies by using the same cell line (CHO-K1) in a series of studies with different gene delivery systems,5,10,12 we cannot rule out cell-type variability as another factor in our inability to establish a comprehensive correlation.14

Our primary goal was to establish structure/function relationships that are independent of the individual steps of the transfection process. It appears likely, however, that efficient transfection requires balance exist among various structural properties and their role in transgressing the multiple barriers to gene delivery. Thus, we can speculate that an optimal size/charge ratio, consisting of a small size (<150 nm dia.) and positively charged surface, may be important for the cell association and endocytosis processes. In fact, an optimal size/charge ratio for overall transfection efficiency has been reported for at least one cationic lipid/DNA gene delivery system.22 The dye displacement studies reported provide some indication of the extent of compaction of DNA when bound to PAMAM dendrimers. Compaction seems to favor gene delivery by providing small stable particles that protect the DNA from nuclease degradation. Condensation to compact structures, however, has also been shown to inhibit transcriptional activity for both dendrimer/DNA and PEI/DNA complexes.25,26 In fact, studies that monitor the individual events of the gene delivery process (e.g., confocal microscopy and flow cytometry approaches) suggest that endosomal release, nuclear localization, or transcription are the rate limiting processes rather than cellular uptake.27–32 Thus, attempts to establish correlation between the individual stages of the transfection process and the physical properties of nonviral gene delivery complexes would seem to have a higher probability of success than attempts to correlate such properties with gross measures of gene expression.

Fractionating complexes based on relative affinity, size, or density could isolate active sub-species that manifest stronger correlations. Early attempts to separate cationic lipid/DNA complexes by density-gradient centrifugation, however, have met with only moderate success.33–36 It might also be possible to establish correlations by focusing on the individual events of the transfection process (e.g., cellular entry, endosomal release, and nuclear translocation) as discussed above rather than the more complex end point of cellular transfection. Some progress has been reported in this regard. A cholesterol-dependent pathway has been identified for the cellular uptake of dendrimer/DNA complexes,37 and a potential pathway by which dendrimer/DNA complexes may escape endosomes has been detected in an anionic liposome model.38

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