Structure/Function Analysis of Peptoid/Lipitoid:DNA Complexes

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ABSTRACT: Previous transfection studies of cationic peptoid polymers (*N*-substituted polyglycines) and cationic lipitoid polymers (peptoid–phospholipid conjugates) have shown that only the polymers which possessed a repeating (cationic, hydrophobic, hydrophobic) substituent sequence are efficient in gene transfer *in vitro*. To determine if there is a physical attribute of peptoid and lipitoid complexes that correlates with efficient gene transfection, biophysical, and transfection measurements were performed with polymer:DNA complexes containing each of seven structurally diverse peptoid polymers and two lipitoids that possess different hydrophobic substituents. These measurements revealed that the biophysical properties of these complexes (size, ζ-potential, ethidium bromide exclusion) varied with polymer structure and complex (+/−) charge ratio but were not directly predictive of transfection efficiency. Unique alterations in the circular dichroism spectra of DNA were observed in complexes containing several of the peptoids and both lipitoids, although FTIR spectroscopy demonstrated that the DNA remained in the B-form. The lack of correlations between the physical properties and the transfection activities of these polyplexes suggests that a further subpopulation examination of these complexes by these methods may reveal hidden structure–activity relationships.
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INTRODUCTION

A number of basic, synthetic polymers efficiently facilitate the delivery of high molecular weight DNA into cells. These include among others the polyethylenimines (PEI), polyamidoamine (PAMAM) dendrimers, and peptoids (*N*-substituted polyglycines) and lipitoids (peptoid–phospholipid conjugates).[1–4] Their present utility, however, is limited by lower levels of gene expression compared to viral vectors and the inability to understand the influence of polymer structure on the efficiency of gene delivery. The establishment of structure–activity relationships with nonviral gene delivery vectors would assist in the identification of structural or chemical properties that permit their complexes to overcome the various cellular barriers to gene delivery and should aid in the development of more efficient vectors.

Several studies have suggested empirical relationships between polymer structure and/or physical properties and the level of transfection efficiency of polyelectrolyte complexes. The level of gene expression among a series of cationic peptides containing hydrophobic residues was correlated with the hydrophobic content of the polymer, which enabled the polymers to form α-helical structures.[5] Several reports have attributed the mechanism of activity of the cationic polymers PEI (polyethylenimine)

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and dendrimers to the presence of protonatable amines.[4,6,7] These amine groups are believed to buffer the pH of the endosome ("proton-sponge" hypothesis) and cause osmotic swelling and/or physical rupture of the endosome, which results in the endosomal release of DNA and enhanced transfection. These hypotheses, however, remain uncertain because contrary evidence has been put forth concerning the actual degree of buffering by PEI in the lysosome[8] and at moderate pH values.[9]

In this study, several physical properties of peptoid:DNA and lipitoid:DNA complexes (which may relate in an indirect manner to transfection activity) were investigated using a variety of biophysical methods to determine if they correlate with biological potency. The hydrodynamic size and surface charge of these complexes were probed using dynamic and phase analysis light scattering, respectively. These colloidal properties may affect the amount of complexes that are in contact with and taken up into cells. The relative affinity of each cationic polymer for DNA was measured using an ethidium bromide exclusion assay. This parameter may reflect the relative abilities of these polymers to protect the DNA in the extracellular medium and to release the polynucleotide for transcription in the nucleus. Furthermore, it is possible that differences in transfection activity could result from any alterations in the secondary structure of DNA in these complexes (if they exist). To test this hypothesis, the structural characteristics of DNA when complexed to several peptoids and lipitoids were probed using circular dichroism (CD) and Fourier-transform infrared (FTIR) spectroscopies. This biophysical analysis of peptoid/lipitoid:DNA assemblies should also provide new analytical approaches to better define the structure and stability of nonviral gene delivery complexes in a pharmaceutical context.

Peptoids and lipitoids possess physical, chemical, and synthetic characteristics that make them unique candidates for gene delivery. Peptoids and lipitoids are efficiently synthesized using a robotic solid-phase submonomer process that allows the generation of a diverse series of polymers with novel structures and defined molecular weights.[10] The structural uniqueness of peptoids results from the transfer of an aliphatic, aromatic, or charged side chain that would normally be located on the α-carbon of an amino acid to the amide nitrogen to form the corresponding peptoid. The *N*-substituted structure of peptoids renders them resistant to protease degradation.[11]

The ability of peptoids and lipitoids to complex with DNA and mediate efficient gene delivery *in vitro* is highly dependent upon peptoid structure.[2,3] Of a series of peptoids that varied in polymer length, charge density and hydrophobicity, only a 36-mer peptoid with 12 cationic side chains was efficient in transfection. The activity of this peptoid was not reduced in the presence of serum, and was comparable to or exceeded that of commercial cationic lipid formulations. Furthermore, the efficiency of this peptoid was not enhanced upon addition of endosomal buffering agents such as chloroquine, which suggests that the endosomal release of the peptoid:DNA complex was not a limiting factor in delivery. The unique ability of this particular peptoid to mediate transfection is intriguing because other peptoids that were inactive in transfection were able to bind to DNA and protect it from nuclease degradation. The transfection competence of this peptoid was further enhanced when conjugated to the phospholipid dimyristoylphosphatidyl ethanolamine (DMPE) to form a lipitoid.[3] This lipitoid:DNA complex was also active in the presence of serum, and was able to protect the polynucleotide from nuclease degradation. The lipitoid: DNA and peptoid:DNA complexes that were active in transfection were observed to be homogenous particles of approximately 60–100 nm in diameter by light scattering and electron microscopy.[2,3]

The structures of the seven peptoid and two lipitoid polymers employed in this study are presented in Figure 1. Each peptoid contains 36 monomer units, which may contain a cationic (*N*-aminoethyl; Nae) aromatic (*N*-phenylethyl; Npe) or aliphatic (*N*-isoamyl; Nia) substituent on the amide nitrogen. Each lipitoid contains a nonamer of peptoid units (cationic, hydrophobic, hydrophobic) in the form of a headgroup. The hydrophobic substituent in these lipitoids is either an *N*-(2-(*p*-methoxyphenylethyl)) (Nmpe) or a *N*-(2-isoamyl) (Nia) moiety. The abbreviations of the chemical names of these peptoid and lipitoid polymers are indicated in Figure 1.

In light of the potential synthetic and structural advantages of peptoid and lipitoid polymers and their unique structural requirements for transfection activity, these polymers are ideal candidates to investigate the structure/activity relationships in nonviral gene delivery systems as described below.

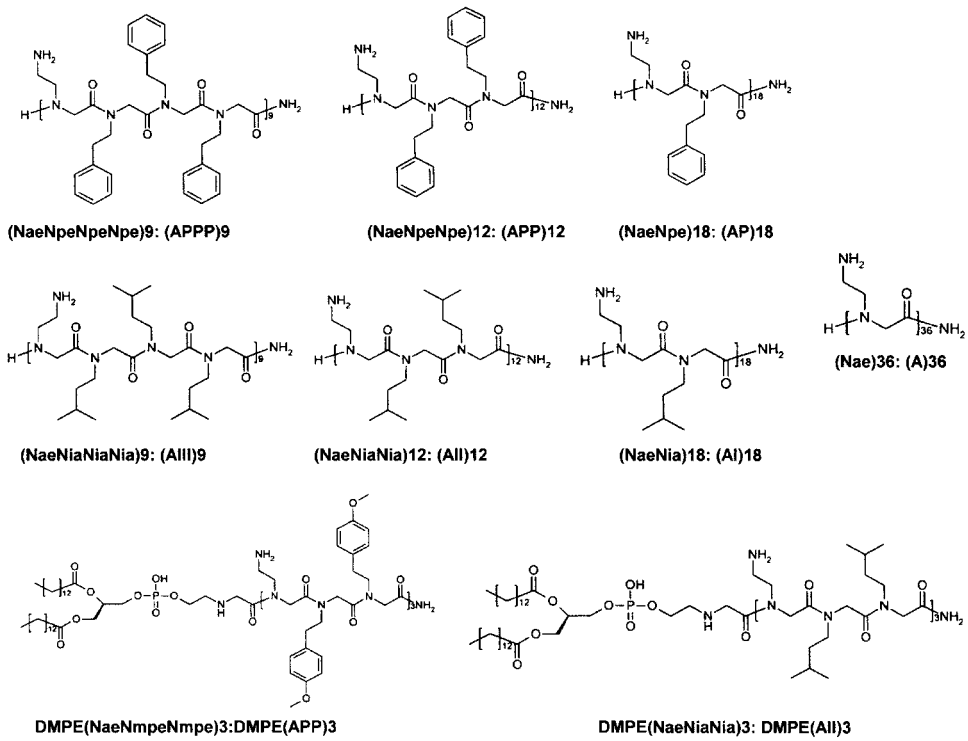


Figure 1. Structures and abbreviated nomenclature of peptoid and lipitoid polymers.

MATERIALS AND METHODS

DNA/Peptoids/Lipitoids

Peptoids and lipitoids were obtained from Chiron Corporation and were prepared as described previously.[3,10] The chloride salt of each peptoid and lipitoid was used as received for the biophysical and transfection experiments. Lipitoid samples that were used for FTIR analysis, however, were received as the trifluoroacetate salt and required conversion to the chloride salt before complex preparation. Salt conversion was achieved by dialysis of lipitoid solutions through a 500 molecular weight cutoff (MWCO) Spectra/Por cellulose acetate membrane into a 0.1 M NaCl solution followed by further dialysis into a 10 mM NaCl solution and final lyophilization to a dry powder. Full salt conversion was confirmed by FTIR spectroscopy and the mass of the chloride salt was used for the calculation of the molecular weight of each lipitoid.

Plasmid DNA (pCMVKm2.GagMod.SF2) in water was supplied by Chiron Corporation.[12] This plasmid was used for all samples and contained less than 5% of nicked and open-circular forms. Cacodylate buffer was procured from Fisher Scientific and Milli-Q purified water was used for all solutions.

Complex Preparation

Complexes were prepared by rapid addition of equal volumes of plasmid DNA to peptoid or lipitoid solutions with stirring. The composition of complexes is defined by the complex charge ratio, which is the ratio of peptoid/lipitoid positive charge to DNA negative charge. To improve the colloidal stability of the complexes, samples were prepared in unbuffered purified water at a final DNA concentration below 100 μg/mL, except complexes formed for FTIR analysis. These complexes were prepared in the presence of 5 mM cacodylate buffer, pH 7.0, at a final DNA concentration of 75 μg/mL. This buffer was selected because the FTIR samples were additionally used for DSC analysis (results not shown), and this buffer possesses a minimal temperature-dependence to its protonic dissociation. Plasmids were dialyzed into two sequential 4.0-liter volumes of cacodylate buffer at 5°C for 12 h using a Pierce 10-K MWCO dialysis cassette. Due to the lesser sensitivity of FTIR, complexes that

were prepared for FTIR measurement were concentrated to 1 mg/mL DNA using a Millipore Ultra-free 100K MWCO centrifugal concentrator and a Sorvall RC 5C Plus centrifuge, for 20 min at 4,000 rpm at room temperature. These concentrated polymer:DNA solutions showed no visible evidence of aggregation. DNA concentration was determined after dialysis using a Hewlett-Packard Model 8453 spectrophotometer at 260 nm and an extinction coefficient of 0.02 A.U. mL/μg cm.

Transfection Efficiencies of Peptoid and Lipitoid:DNA Complexes

The transfection efficiencies of the various peptoid and lipitoid polymer/DNA complexes were assessed using green fluorescent protein (GFP) expression in two cell lines: COS-7 and CHO-K1. All cells were maintained in 75 cm^2 flasks at 37°C and 5% CO_2 with COS-7 cells grown in DMEM with L-glutamine and 4.5 g/L glucose and CHO-K1 cells in Ham's F-12 media with L-glutamine, each supplemented with 10% FBS. Cells were subcultured every 4 days using standard procedures with trypsin/EDTA for cell lifting. Prior to seeding, the cells were trypsinized, counted, and diluted to a concentration of about 80,000 cells/mL. Then 0.1 mL of this dilution was added to each well of a 96-well plate and the cells were incubated in a humid 5% CO_2 incubator at 37°C for 18–20 h.

A stock DNA solution was diluted to 50 μg/mL in 10 mM Tris buffer (pH 7.0) and sterile filtered through a 0.2-μm filter. Aliquots of polymers prepared at 2× concentrations required for each charge ratio in water were sterile filtered through a 0.2-μm filter. Equivalent volumes of polymer and DNA were mixed to prepare complexes at 25 μg/mL DNA concentration. These solutions were diluted with Opti-MEM to a DNA concentration of 2 μg/mL for transfection. Immediately prior to transfection, the cells were washed once with PBS and 100 μL of the diluted complex solution (200 ng of DNA) was added to each well. Cells were incubated with the complexes for 5 hours. The transfection agent was then removed and 100 μL of culture medium was added followed by a further incubation of 48 h. The flourescence intensities of GFP were measured with a Fluostar™ Galaxy microtiter plate reader (BMG, Germany) with excitation and emmision wavelengths set to 485 and 520 nm, respectively. The fluorescence intensities of peptoid and lipitoid complexes and DNA alone are reported as the mean ± standard error for three samples per data point. Detergent disruption of the cells produced similar results.

Dynamic Light Scattering (DLS):

Complexes prepared for CD experiments (see below) were also employed for the DLS studies. Measurements were obtained using a Brookhaven zeta-PALS instrument from the scattered light collected at 90° to the incident laser beam. Each DLS measurement represents the average of four measurements of 1 min duration. Autocorrelation functions were fit to a cumulant analysis (Gaussian distribution of particle size) although a fit to an intensity-weighted two-population model provided similar trends between samples (not shown). The plasmid in 50 mM NaCl possessed a mean diameter of 138.1 ± 3.9 nm.

Phase Analysis Light Scattering (PALS)

Complexes prepared for CD experiments (see below) were used directly for the PALS studies. PALS measurements were obtained using a Brookhaven zeta-PALS instrument and each measurement represents the sum of three runs of 10 cycles of the applied electric field. The ζ-potential of the sample was calculated from the measured electrophoretic mobility using the Smulchowski equation.[13] DNA alone at these concentrations did not contribute significantly to the measured signal. Therefore, the ζ-potential of each sample was primarily representative of the complexes in solution alone.

Ethidium Bromide Exclusion Assay

Complexes were prepared at a DNA concentration of 10 μg/mL (0.3 mL total volume) at charge ratios between 0.25 to 1.0 and 2.0 to 3.0 (+/−) in purified water. After complexation, ethidium bromide was added to the complex at a 5:1 ratio (DNA base pairs to ethidium) and samples were equilibrated for 4 h at room temperature before measurement. Samples at each charge ratio were prepared in duplicate for all polymers. Data points are plotted as the average of replicate measurements with the error bars representing each duplicate measurement value. Samples were measured on a BioTek SL600 fluorescence plate reader using excitation and emission wavelengths of 530 nm and 590 nm, respectively. Fluorescence intensities were subtracted from blank samples (ethidium in water)

and normalized to the fluorescence intensity of ethidium with DNA alone. The fluorescence intensity of ethidium was not enhanced relative to blank samples when added to peptoids or lipitoids alone (data not shown).

Circular Dichroism (CD)

Complexes were formed at a DNA concentration of 50 μg/mL (1.5 mL total volume) at charge ratios between 0.25 to 1.0 and 2.0 to 3.0 (+/−) in purified water and were equilibrated for 10 min before measurement. Samples were prepared in duplicate except for $(APPP)_9$ and $(AP)_{18}$ and both lipitoids, which were prepared once for each charge ratio due to the limited amounts of these samples available. Spectra were obtained from 200 to 350 nm with a Jasco J-720 spectropolarimeter at 25°C using a scan rate of 50 nm/min with a 0.1 cm pathlength cell. Each spectrum represents the average of three collections and was baseline subtracted, noise reduced and converted to molar ellipticity using the molar concentration of DNA in the sample. All spectra were normalized to zero molar ellipticity at 320 nm.

Fourier-Transform Infrared (FTIR) Spectroscopy

FTIR spectra were obtained using a Nicolet 560 ESP spectrometer employing a zinc selenide attenuated total reflectance (ATR) cell. Spectra were determined from 256 scans at 4 cm^{-1} resolution.[14] Buffer spectra (5 mM cacodylate pH 7.0) were subtracted from all sample spectra for solvent correction. Peak positions were obtained from inverted second derivative spectra with the data spacing decreased to 0.25 cm^{-1}.

RESULTS

Transfection Activity and Toxicity of Peptoid/Lipitoid:DNA Complexes

The transfection efficiencies of peptoid and lipitoid polymers were evaluated in CHO-K1 and COS-7 cells. Results are shown in Figures 2a and b, respectively. Due to limitations in polymer availability, the $(APPP)_9$ and $(AIII)_9$ polymers were not tested, but the efficiencies of peptoids with this repeating motif were previously determined to be much lower than the $(APP)_{12}$ peptoid.[3] All polymers evaluated were ineffective in transfection at charge ratios below unity. Furthermore, at charge ratios above this value, only $(APP)_{12}$ and $(AII)_{12}$ and both lipitoids were efficient in inducing transfection. With the exception of DMPE $(APP)_3$ at a 2:1 charge ratio, the relative efficiencies of these polymers were similar in the two cell lines. Significant changes in cellular morphology were observed with the $(APP)_{12}$, $(AII)_{12}$ peptoids and both lipitoids at charge ratios greater than 1, which indicated the onset of cellular toxicity (not shown). Therefore, these polymers were not evaluated above a 3:1 charge ratio. These results are quite similar to previous transfection measurements of these polymers.[2,3]

The various peptoid and lipitoid polymers chosen for analysis in this study are particularly appropriate for a structure/function study because they are all able to interact with plasmid DNA, but only those polymers with a trimer substituent pattern are effective in transfection. Several physical properties of these peptoid/lipitoid:DNA complexes were therefore investigated to determine if there are physical differences between the transfection active and inactive complexes. These physical attributes can be grouped into three areas: the colloidal properties of the complex (particle size and ζ-potential), the relative interaction of these polymers with DNA (EtBr exclusion) and DNA structure (CD and FTIR).

Particle Sizes and ζ-Potentials of Peptoid/Lipitoid/DNA Complexes

To investigate the colloidal properties of the complexes formed from peptoids and lipitoids with DNA, the mean cumulant diameters of the complexes were measured using DLS (Figure 3) and their ζ-potentials were obtained using PALS (Table 1). Overall, the $(AII)_{12}$ and $(APP)_{12}$ complexes possessed the smallest particle size while the $(AI)_{18}$ and $(A)_{36}$ peptoids formed the largest DNA complexes. This finding suggests that the ability of these polymers to condense DNA into small particles is mediated by factors other than the charge density of the peptoid. Despite the diversity of particle sizes among these complexes, there is no apparent correlation between particle size alone and transfection efficiency. The smallest particle sizes (<100 nm) were produced with the $(APP)_{12}$ peptoid and DNA at 0.25:1, 2:1, and 3:1 charge ratios, while only the 2:1 and 3:1 complexes are transfection efficient. As shown in Table 1, the ζ-potentials of complexes are negative when the DNA is in molar excess, and are positive when the cationic polymer is in molar excess. At a

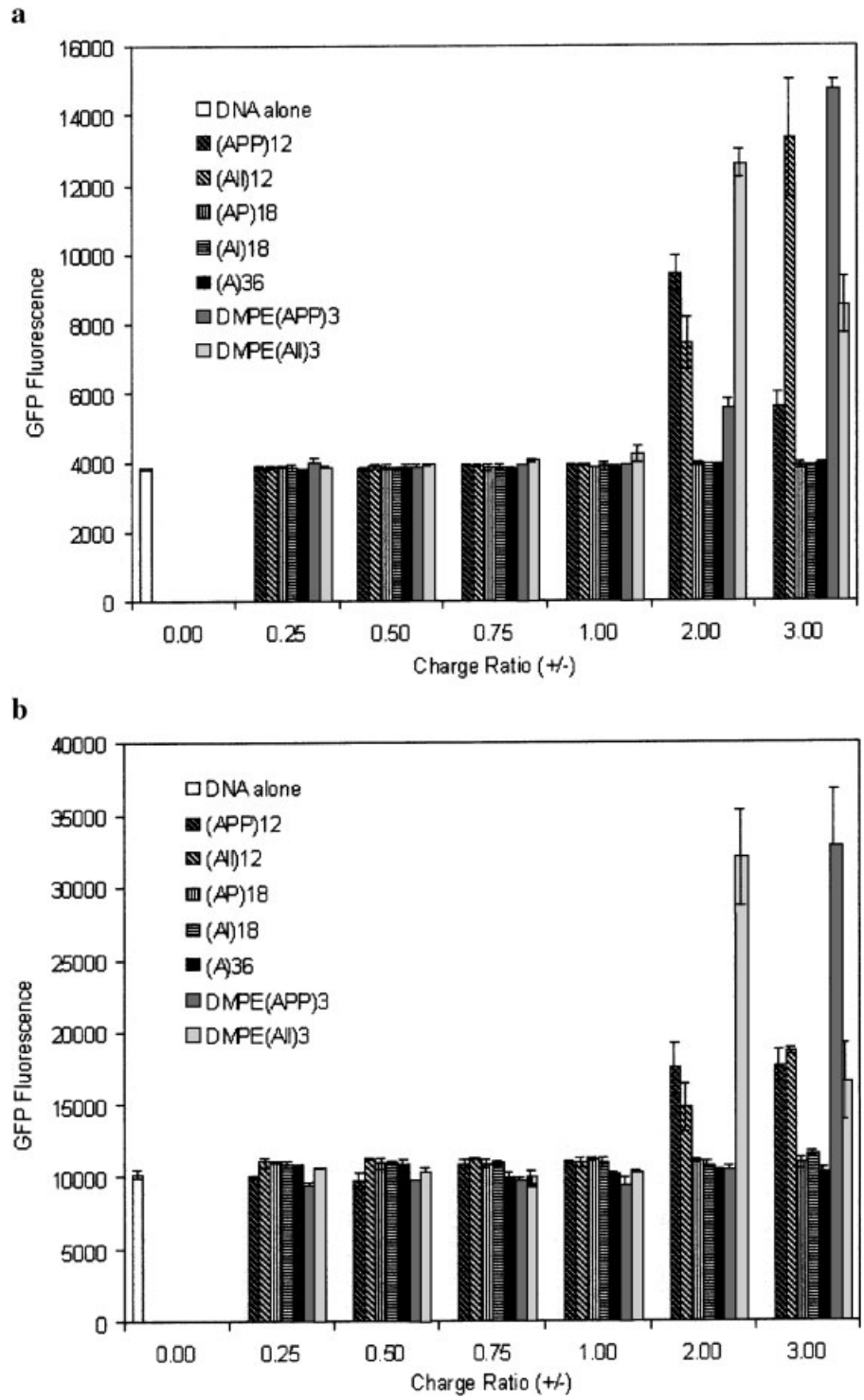


Figure 2. Transfection efficiencies of peptoid:DNA, lipitoid:DNA complexes and DNA alone in CHO-K1 (a) and COS-7 cells (b). The amount of GFP protein expressed was measured by fluorescence 24 h after application of 200 ng of DNA to cells per well in triplicate. Patterns within each column that represent each polymer are indicated in the figures.

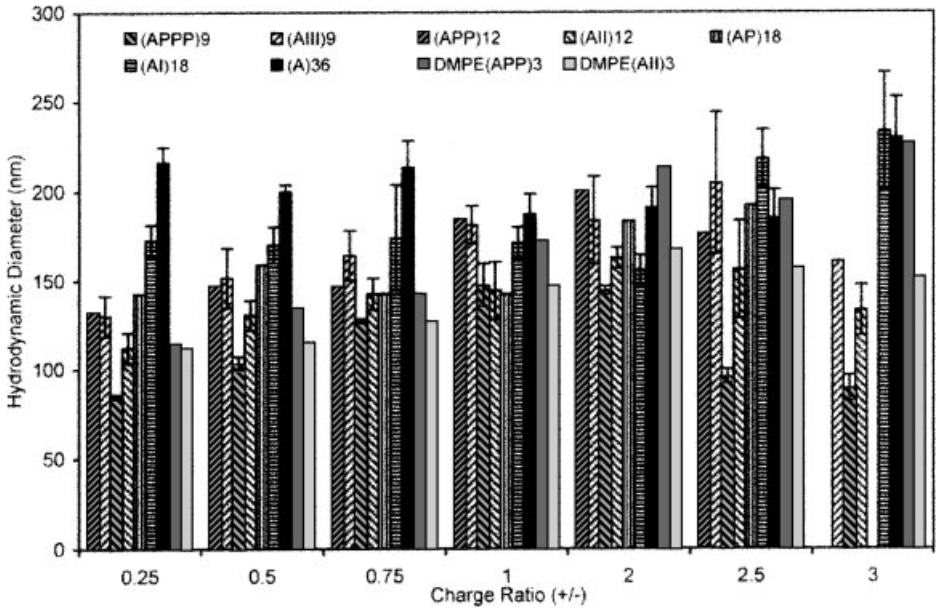


Figure 3. Mean cumulant diameters of peptoid:DNA and lipitoid:DNA complexes as a function of polymer: DNA charge ratio measured by DLS. Complexes were prepared at a DNA concentration of 50 μg/mL in water in duplicate (except for $(APPP)_9$ and both lipitoids). Patterns within each column that correspond to each polymer are indicated in each panel.

charge ratio of 1.5:1(±), the complexes display poor colloidal stability and visually aggregate.

Overall, attempts to correlate transfection activities with the colloidal properties (size and surface charge) of these complexes have been unsuccessful. At a 3:1 charge ratio, positively charged complexes that possess both relatively large [DMPE $(APP)_3$ complexes] and small [$(APP)_{12}$ complexes] sizes were efficient in transfection. Alternatively, the particle sizes and ζ-potentials of DMPE $(APP)_3$ and $(A)_{36}$ complexes were comparable at high charge ratios, but only the lipitoid was transfection competent.

Table 1. Effect of Polymer Composition and Complex (±) Charge Ratio on the ζ-Potentials of Peptoid/Lipitoid:DNA Complexes Measured by Phase Analysis Light Scattering

	ζ-Potential	
Polymer	1:1 ±	2:1 ±
$(APPP)_9$	−46.3	30.3
$(AIII)_9$	−31.0	29.6
$(APP)_{12}$	−36.4	33.5
$(AII)_{12}$	−38.9	29.3
$(AP)_{18}$	−44.2	39.5
$(AI)_{18}$	−31.2	35.4
$(A)_{36}$	−38.1	36.4
DMPE$(APP)_3$	−37.8	44.6
DMPE$(AII)_3$	−45.6	43.6

Values represent the mean of two samples of each polymer:DNA complex, except for the $(APPP)_9$, $(AP)_{18}$ complexes and both lipitoid complexes, which were prepared singly.

Ethidium Bromide Exclusion

The relative binding strength of peptoids and lipitoids to DNA was estimated from the ability of increasing amounts of cationic polymer to exclude the intercalation of the fluorescent probe ethidium bromide (EtBr) from DNA. Complexes containing $(APP)_{12}$, $(AII)_{12}$, $(AP)_{18}$, or $(AI)_{18}$ at charge ratios less than 2:1 were least able to exclude EtBr (Figure 4). These differences were more apparent when the cationic polymer is in molar excess. In this region, $(AII)_{12}$ is able to exclude only 60% of the DNA, compared to the near quantitative exclusion by $(A)_{36}$. The lower dye displacement produced by $(APP)_{12}$ and $(AII)_{12}$ suggests that these peptoids bind to DNA with the weakest affinity or form a complex in which the DNA is still accessible to the dye. The nearly complete exclusion of ethidium from the $(A)_{36}$:DNA complexes indicates that this polymer binds with the tightest affinity to DNA. These results are quite different

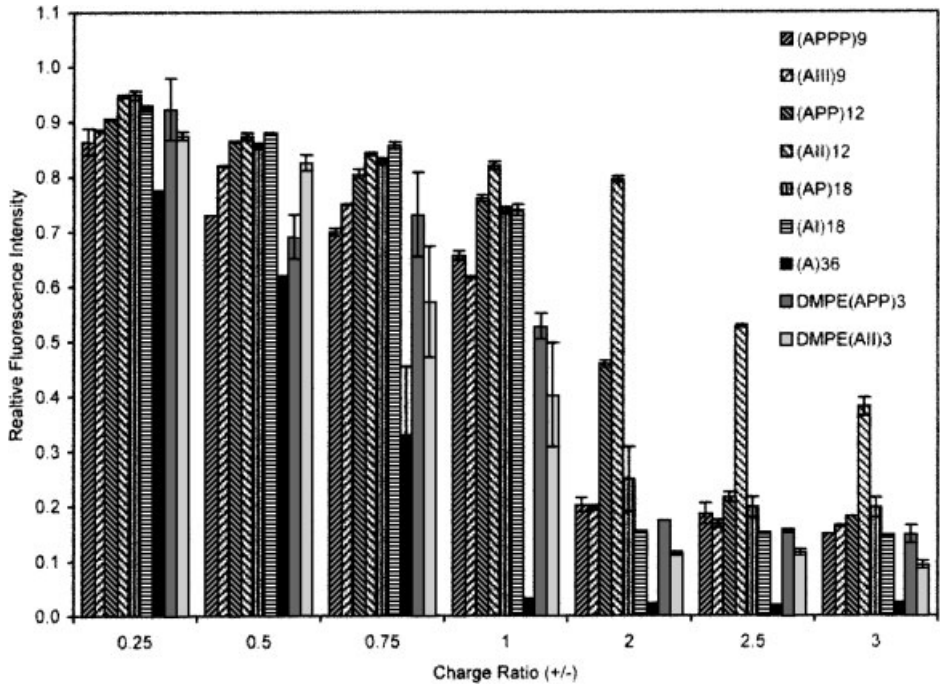
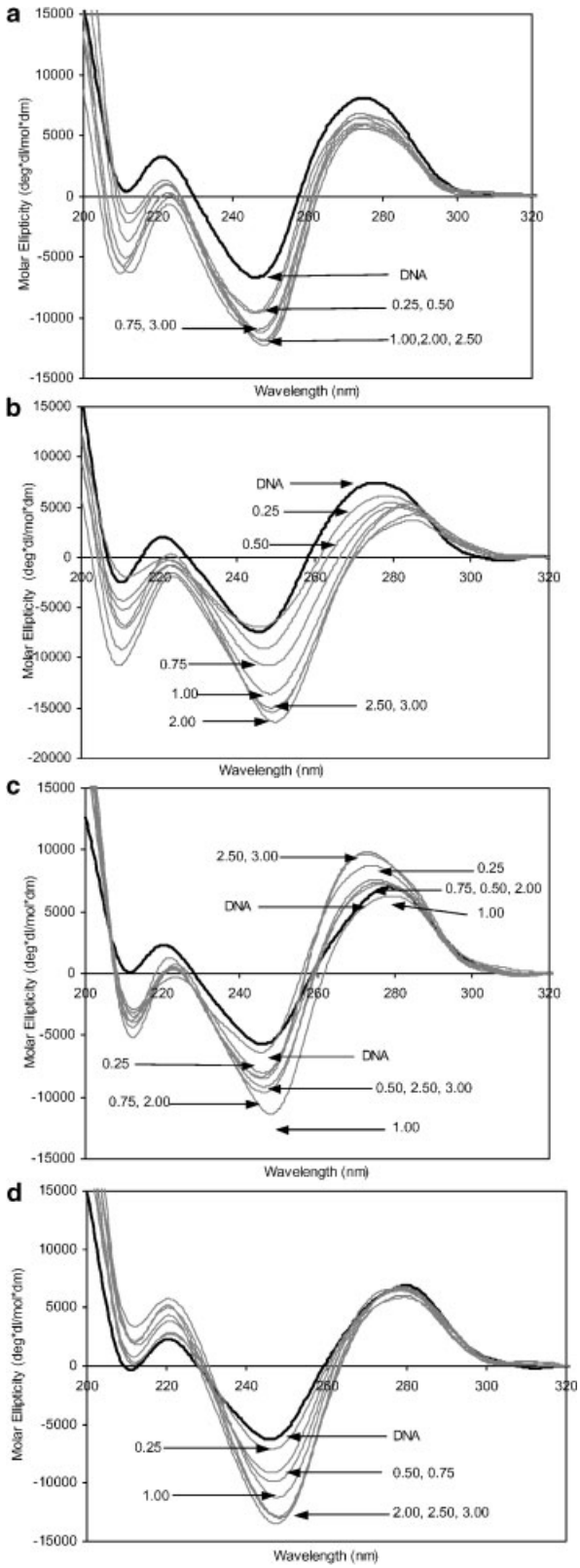


Figure 4. EtBr exclusion of complexes containing DNA (10 μg/mL) and increasing amounts of peptoid and lipitoid polymers. Samples were prepared in duplicate and the error bars represent the individual values of each measurement. Data are shown in terms of the reduction in fluorescence intensity of the intercalated dye (relative to DNA alone) induced by binding of the indicated peptoid/lipitoid. Patterns within each column that correspond to each polymer are indicated in the legend.

than those reported for the ability of these polymers to protect DNA from DNase I digestion, where $(APP)_{12}$ provided protection but $(A)_{36}$ did not.[3] One might be tempted to infer that the higher transfection efficiency of $(APP)_{12}$ and $(AII)_{12}$ results from a lower affinity of the peptoids for DNA. This finding may be coincidental, however, because both lipitoids were able to exclude EtBr to a much greater extent but mediated transfection efficiently. Furthermore, at the highest charge ratios examined, $(APP)_{12}$ excluded EtBr to the same extent as several inactive peptoids.

CD of Peptoid/Lipitoid/DNA Complexes

The CD spectra of DNA complexed to increasing amounts of each peptoid or lipitoid were measured to determine if any alterations in DNA secondary structure were produced upon complexation (Figure 5). The DNA spectral features of primary interest are the positive band near 275 nm and the negative signal at 245 nm (indicative of B-form DNA). In contrast to peptoids containing chiral substituents,[15,16] these polymers do not possess an intrinsic CD signal or ordered secondary structure. The spectral changes induced in the CD of DNA are quite similar when DNA is complexed $(APPP)_9$, $(AIII)_9$, $(APP)_{12}$, $(AII)_{12}$, or $(AP)_{18}$. The CD spectra of $(APP)_{12}$:DNA complexes are shown in Figure 5a, and are representative of the spectral changes produced by these five polymers. A decrease in the ellipticity of the 275 nm band and an increase in the negative ellipticity near 245 nm band are observed as polymer was complexed to DNA. Shifts in peak position of each band to longer wavelengths are also evident with increasing charge ratio. Changes of a greater magnitude are detected in complexes containing the $(AI)_{18}$ peptoid (Figure 5b), which resemble the CD spectra of poly-L-lysine/DNA complexes.[17,18] The samples prepared with $(A)_{36}$ (Figure 5c) are distinct in that the 275 nm band of DNA *increases* in magnitude at high charge ratios, with an accompanying shift in peak position to *shorter* wavelengths. Complexation of DNA with either of the two lipitoids produced comparable CD spectra (Figure 5d). The 275 nm band of DNA is unperturbed while the magnitude of the 245 nm minima progressively increases with addition of the lipitoid. In general, the changes observed in the CD of DNA in



several of these complexes [e.g., $(APPP)_9$, $(AIII)_9$, $(APP)_{12}$, $(AII)_{12}$, and $(AP)_{18}$ in Figure 5a] more closely resemble the changes in the CD spectra of DNA in cationic-lipid/DNA complexes[19] than the intense ϕ-like spectra observed in polyethylenimine/DNA complexes[9] and of DNA condensed with polyethylene glycol or salts.[20,21]

Several published reports have attributed such decreases in the 275 nm band of cationic lipid:DNA complexes to the existence of C-DNA.[22,23] It has been clearly demonstrated by FTIR spectroscopy, however, that DNA remains in the B-form upon complexation with several cationic lipids and with polyethylenimines.[9,14] FTIR measurements in this report also confirm that DNA is maintained in the B-form in peptoid/lipitoid:DNA complexes (see below). A recent and more detailed investigation into the source of the altered CD spectra of DNA in cationic lipid:DNA complexes using a combination of molecular dynamics simulations and FTIR and Raman spectroscopies suggests that these spectral changes more probably result from local perturbations in DNA base geometry than from alterations in the helical structure of DNA.[19]

An interesting comparison can be made of the effects of the degree of hydrophobic substitution and the structure of the substituent (alkyl versus aryl) of the peptoid upon the alteration of the CD spectra of DNA. As shown in Figure 6, an increase in the degree of aromatic substitution of the peptoid polymer (from $(AP)_{18}$ to $(APPP)_9$) correlates with a decrease in magnitude of the molar ellipticity of the 275 nm band. This trend may reflect spectral contributions from the aromatic moiety of the peptoid, if present in a chiral environment in the complex. An increase in alkyl substitution [from $(AI)_{18}$ to $(AIII)_9$], however, produces the opposite effect. Such trends suggest that the spectral properties of the complex are mediated by factors other than simple charge neutralization of the DNA and the charge density of the peptoid. More importantly, the similar CD spectral changes of DNA in both transfection active and inactive complexes suggest no direct correlations between the

Figure 5. Molar ellipticity of DNA obtained by CD spectroscopy in complexes containing $(APP)_{12}$ (a), $(AI)_{18}$ (b), $(A)_{36}$ (c), and DMPE$(APP)_3$ (d). All samples were prepared at a DNA concentration of 50 μg/mL in water. Charge ratios of complexes represented by each trace are indicated. The CD spectrum of DNA alone is shown as the darkest trace.

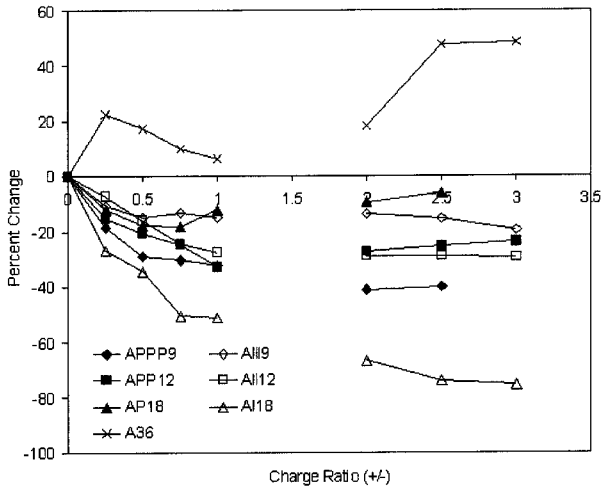


Figure 6. Percent change in molar ellipticity of DNA in peptoid:DNA complexes of increasing charge ratio. Each data point represents the average of two measurements, except for $(APPP)_9$, which was measured once at each charge ratio. Legend: ◆ = $(APPP)_9$, ◇ = $(AIII)_9$, ■ = $(APP)_{12}$, □ = $(AII)_{12}$, ▲ = $(AP)_{18}$, △ = $(AI)_{18}$, × = $(A)_{36}$.

polymer-induced structural changes in DNA and the transfection potencies of these complexes.

FTIR Spectroscopy of Peptoid/Lipitoid: DNA Complexes

FTIR spectroscopy was employed to further probe the secondary structure of DNA in complexes containing several representative peptoids [$(APP)_{12}$, $(AI)_{18}$ and $(A)_{36}$] and each of the two lipitoids. The vibrational spectra of DNA, $(APP)_{12}$: DNA complexes of increasing charge ratio and $(APP)_{12}$ alone are presented from bottom to top, respectively, in Figure 7a. Displayed in Figure 7b are representative FTIR spectra of DNA alone, DMPE $(AII)_3$:DNA complexes at increasing charge ratios and of DMPE $(AII)_3$ alone (from bottom to top, respectively). Vibrational features indicative of B-form DNA include the band near 1718 cm^{-1} produced by the stretching of the C7 guanine carbonyl, the 1225 cm^{-1} peak representing the asymmetric phosphate stretching frequency and the 970 cm^{-1} peak, which results from the combined phosphodiester/deoxyribose stretch of DNA.[24] Due to the limited amount of some samples available for analysis, a quantitative comparison of the vibrational properties of all of the complexes was not performed. The presence, however, of DNA vibrational features in these complexes which are within 4 cm^{-1} of DNA alone suggests that DNA is maintained in the B-form in these complexes and in $(AI)_{18}$ and $(A)_{36}$ complexes.[25]

DISCUSSION

This study has attempted to correlate the biophysical properties of a series of peptoid/lipitoid: DNA complexes with their observed transfection efficiencies. This effort is part of an overall goal to establish structure–activity relationships with nonviral gene delivery vectors through differences in their physical properties.[26–29] The premise underlying this approach is that nonviral vectors that are efficient in transfection also possess unique physical properties or structures that allow them to overcome the physical and cellular barriers to gene delivery. If successful, this strategy should aid in the development of more therapeutically useful nonviral vectors that possess optimized physical/chemical characteristics.

The inability to correlate the physical properties of peptoid/lipitoid:DNA complexes to their transfection capabilities has been observed with other cationic polymer and lipid systems.[30–32] A comparison of linear, grafted, branched, and dendritic polylysines revealed no correlations between transfection activity and complex size, polymer shape, complex ζ-potential or the extent of DNA condensation.[31] The impact of particle size on transfection efficiency is unclear, and studies have shown that both small homogeneous particles[33] and large aggregated complexes[34–36] provide efficient levels of gene expression. Large complexes may settle onto the surface of cells *in vitro*, which can provide a greater amount of material in contact with cells compared to smaller complexes.[37] Several studies have found correlations between the magnitude of the positive ζ-potential of complexes and their *in vitro* activities,[38,39] while other studies have found none.[40,41] Measurements of the relative affinity of the vector for DNA using an ethidium bromide exclusion assay have also been successful[41] and poor[42] predictors of transfection efficiency.

Several factors may affect the ability of biophysical methods to predict the transfection activity of nonviral vectors. Complexes are prepared for biophysical characterization at much higher concentrations (up to 2–3 mg/mL for Raman spectroscopy[19]) and at much lower ionic strengths (~0.01) than those used for

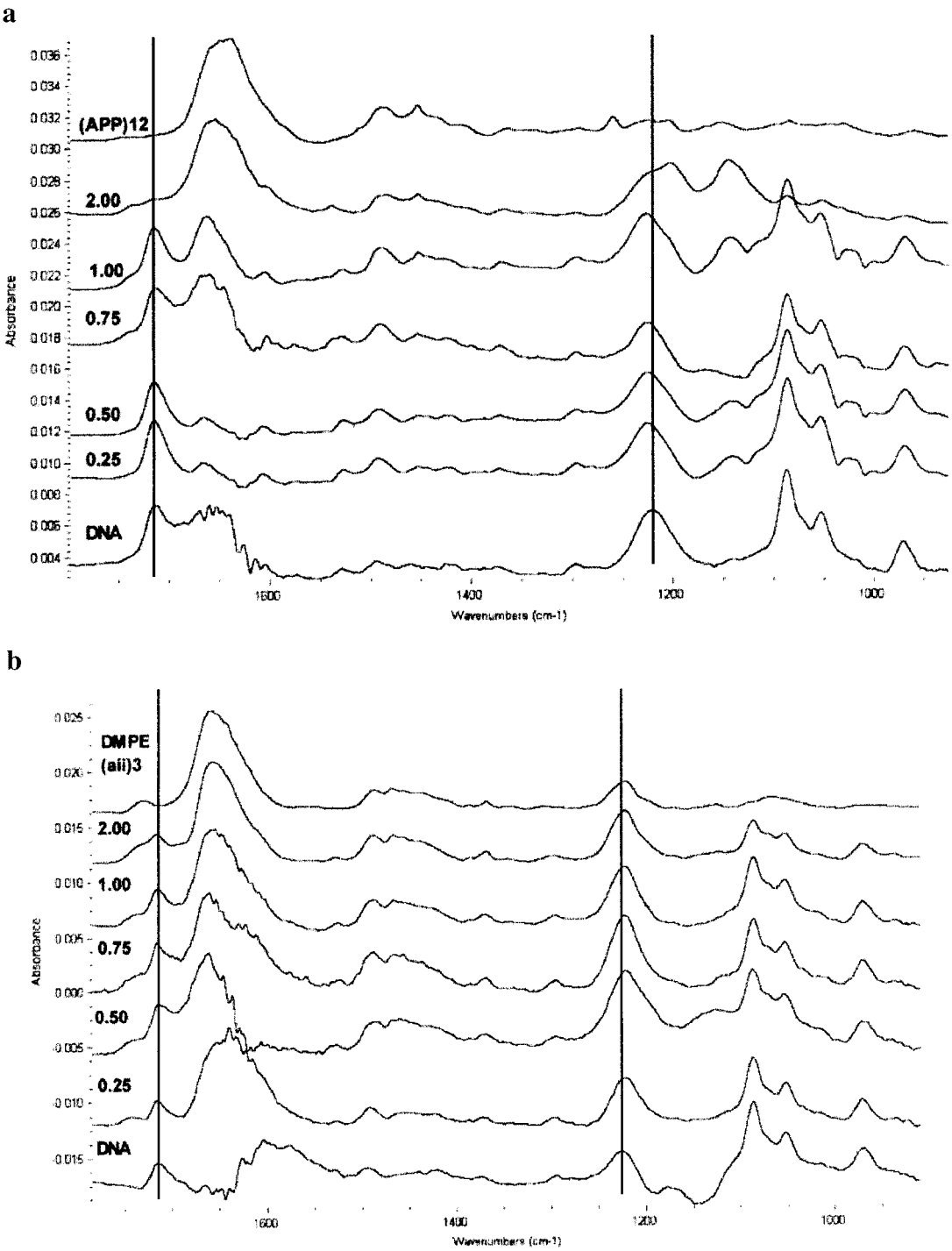


Figure 7. FTIR spectroscopy of peptoid/lipitoid:DNA complexes. (a) Representative FTIR spectra of DNA (bottom), $(APP)_{12}$:DNA complexes of increasing charge ratio and $(APP)_{12}$ alone (top). (b) Representative FTIR spectra of DNA (bottom), of DMPE $(AII)_3$:DNA complexes of increasing charge ratio and of the DMPE $(AII)_3$ lipitoid alone (top). The vertical lines represent the approximate peak positions of the guanine carbonyl and asymmetric phosphate stretching frequencies of DNA alone ($\sim$1715 and 1225 cm^{-1}, respectively) and are meant to guide the eye only. Spectra were obtained using an ATR geometry of solutions containing 1 mg/mL DNA in 5 mM cacodylate buffer pH 7.0.

transfection experiments (~25 μg/mL and 0.15). The lower ionic strength of the experimental conditions is needed to maximize the colloidal stability of the samples at these higher concentrations. The colloidal properties of the complex and the affinity of the vector for the DNA that are measured at lower ionic strengths will not be representative of the complexes that exist under physiological conditions, due to the electrostatic nature of the interaction between the cationic vector and DNA. Finally, the complexes themselves are heterogeneous in composition and structure. Therefore, their physical properties (which reflect the population average) may not accurately reflect the characteristics of any active subpopulation(s).

In summary, these biophysical studies describe features of peptoid/lipitoid:DNA complexes that are qualitatively similar to those obtained with PEI:DNA and poly-L-lysine:DNA complexes, such as the vibrational properties of the DNA in the complex. Other spectroscopic qualities, such as their CD spectra, are novel. Despite this diversity of physical properties among the various peptoid/lipitoid:DNA complexes, there exists no obvious correlation between these parameters and their transfection efficiencies. This lack of any direct relationship may reflect the intrinsic heterogeneity of these complexes as a direct consequence of their relatively nonspecific electrostatic formation. The separation of these complexes based upon their density, size, or surface charge might at least partially resolve their heterogeneity. A compositional and biological evaluation of cationic lipid:DNA complexes that were separated using density-gradient centrifugation suggests the potential value of this approach.[43–45] Alternatively, one may speculate that the physical properties of the complexes measured by these techniques (CD spectra, vibrational properties, etc.), although essential for their pharmaceutical formulation and characterization, may have limited direct relevance to their biological activities. Additional studies employing differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC) also found no correlations between the stability of DNA in peptoid/lipitoid:DNA complexes or the enthalpies of binding between DNA and several peptoid/lipitoids and their transfection activities (not shown).[25] A more mechanistic evaluation of these complexes, which might include the analysis of their extent of interaction with model proteoglycans[46] and their ability to disrupt membranes[47] might elucidate a more direct relationship between their structure and biological activities.

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