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Intrinsic Dynamics of DNA-Polymer Complexes: a Mechanism for DNA Release

Lisa E. Prevette, **Evgenia N. Nikolova**, **Hashim M. Al-Hashimi**, and **Mark M. Banaszak Holl**
University of Michigan, Department of Chemistry and Division of Biophysics, 911 N. University Ave., Ann Arbor, MI 48109

Abstract

The transfer of genetic material into cells using non-viral vectors offers unique potential for therapeutics; however, the efficacy of delivery depends upon a poorly understood, multistep pathway, limiting the prospects for successful gene delivery. Mechanistic insight into DNA association and release has been hampered by a lack of atomic resolution structural and dynamic information for DNA-polymer complexes (polyplexes). Here, we report a dendrimer-based polyplex system containing poly(ethyleneglycol) (PEG) arms that is suitable for atomic-level characterization by solution NMR spectroscopy. NMR chemical shift, linewidth, and proton transverse relaxation rate measurements reveal that free and dendrimer-bound polyplex DNA exchange rapidly relative to the NMR timescale (< millisecond). The dendrimers retain a high degree of mobility in the polyplex, whereas the DNA shows restrained mobility, suggesting that the polyplex is a highly dynamic complex with a rapidly exchanging dendrimer atmosphere around a more rigid DNA framework.

Keywords

Polyplexes; polyplex NMR spectroscopy; DNA dynamics; PAMAM dendrimer dynamics; polyplex DNA release

INTRODUCTION

The transfer of genetic material into cells using non-viral vectors offers unique potential for therapeutics;[1-4] however, the efficacy of delivery depends upon a poorly understood, multistep pathway, limiting the prospects for successful gene delivery.[5] Mechanistic insight into DNA association and release has been hampered by a lack of atomic resolution structural and dynamic information for DNA-polymer complexes (polyplexes). Poly(amidoamine) (PAMAM) dendrimers represent a well-studied class of transfection agents[6-8] and are well-suited for mechanistic studies due to their homogeneity in terms of size and number of cationic amines per polymer particle.[9] Structure and dynamics of PAMAM dendrimer-DNA complexes have been investigated at large length scales. Braun et al. studied entropy-driven electrostatic association between plasmid DNA (pDNA) and many generations of PAMAM dendrimer that provided evidence of direct interaction at both the guanine base and phosphate backbone of the nucleic acid.[7] The Tomalia group studied the binding of high molecular weight linear DNA and PAMAM dendrimer generations 2, 4

SUPPORTING INFORMATION

Gel electrophoresis shift DNA binding assay for G5-NH_2. Fraction of bound DNA as a function of dendrimer concentration as determined by global fit of the relaxation data. Observed R_2 values and amplitudes from biexponential fitting of the decay of integrated NMR signals versus delay time for increasing +/- ratios. This information is available free of charge via the Internet at http://pubs.acs.org.

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and 7 (G2, G4, and G7, respectively) and proposed a dendrimer-DNA binding model based on regions of both tightly bound and "linker" DNA.[10] Despite these reports, essential features of the PAMAM dendrimer-DNA polyplex structure, particularly the dynamics of the DNA and dendrimer components, remain poorly understood. Examples of recent progress include the application of fluorescence methods to highlight the importance of release in the nucleus for expression.[11] Using ethidium bromide intercalation into DNA to probe DNA-dendrimer interactions, Fant et al. showed that polymer and linearized pDNA are exchanging and that the PEGylated PAMAMs bind pDNA less tightly than the parent polymer.[12] Atomic Force Microscopy (AFM) methods have been employed to examine polymer-DNA dynamics and DNA release from polyplexes on the time scale of tens of minutes.[13,14] Still lacking, however, is information on the atomic scale, due to experimental difficulties.

NMR spectroscopy has the potential to be a powerful tool for polyplex characterization due to its atomic-level resolution and ability to elucidate both structure and dynamics; however, NMR has not been extensively used for polyplex characterization because at the concentrations required for spectroscopy, aggregation and precipitation generally result. Indeed, we observed immediate precipitation in our own experiments upon titrating a 20-mer DNA duplex (5′ CCACAGTGTTTGTGCAGCGG 3′) with G5 PAMAM dendrimer. In order to overcome this limitation, we employed G5 PAMAM dendrimers modified with poly(ethyleneglycol) (PEG) arms for this study. High molecular weight, well-hydrated PEG chains restrict aggregation through steric hindrance and by creating a solvation layer around the particle. Such PEGylated materials have clinical relevance and both PAMAM dendrimers[15,16] and other cationic polymer nucleic acid delivery agents[17-19] have been employed to eliminate or reduce flocculation of polyplexes into large aggregates in the presence of physiological salt and serum conditions. This flocculation is a significant hurdle in rendering these delivery methods clinically viable, since large, micrometer-sized particles are quickly eliminated from *in vivo* circulation by the reticuloendothelial system (RES) before reaching their target cells.[20-22] PEGylation has also been shown to limit cytotoxicity by reducing the production of reactive oxidative species and mitochondrial membrane potential collapse induced by the PAMAM dendrimers.[23]

With these PEG-modified dendrimers, we investigated polyplex dynamics by NMR and applied relaxation data to a new interpretation of the spectra obtained at various formulation ratios. These results reveal that fast chemical exchange (< millisecond) exists between free and dendrimer-bound DNA. We conclude that in the polyplex, dendrimer is flexible and mobile while the DNA experiences an increasingly restricted solid-state environment at higher charge ratios. This new information provides mechanistic insight into the association and release of nucleic acids from their delivery vectors.

MATERIALS AND METHODS

Materials

Sodium chloride (NaCl), sodium dihydrogen phosphate (NaH_2PO_4), ethylenediamine tetraacetic acid (EDTA), 0.1 N hydrochloric acid (HCl), tris(hydroxymethyl)aminomethane, methoxypoly(ethylene glycol) tresylate (all from Sigma; St. Louis, MO) and 99.96 % deuterium oxide (D_2O; Cambridge Isotope Laboratories, Inc.; Andover, MA) were used as received. Both strands of a DNA 20-mer (5'- CCACAGTGTTTGTGCAGCGG -3' and 5'- CCGCTGCACAAACACTGTGG -3') were ordered from Integrated DNA Technologies, Inc. (IDT; Coralville, IA) and dissolved independently into DNase-/RNase-free water (Gibco; Carlsbad, CA) to final concentrations of 2 mM based on the molar quantity specifications provided by IDT. Then, accurate DNA concentrations were quantified by absorbance at 260 nm, using a molar extinction coefficient of 315,956 cm^{-1} M^{-1} for these

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sequences determined using OligoAnalyzer software from IDT and the two strands mixed at stoichiometric ratio. Phosphate buffer was made by dissolving NaH_2PO_4, NaCl and EDTA in DNase-/RNase-free water to a final concentration of 10 mM, 25 mM and 0.20 mM, respectively. Then, the pH was adjusted to 7.4 with 0.1 N H_3PO_4.

PAMAM Dendrimer Synthesis and Characterization

Generation 5 (G5-NH_2) PAMAM dendrimer was purchased from Dendritech Inc. (Midland, MI). Lower molecular weight impurities and trailing generations were removed from G5-NH_2 by dialysis with a 10,000 MWCO membrane against deionized water for four days, exchanging washes 7 times. The number average molecular weight and polydispersity index or PDI of the dendrimers were determined by gel permeation chromatography (GPC) using a double detection system (refractive index and static light scattering) to be 27,336 g/mol and 1.018. Potentiometric titration was conducted to determine the average number of primary amines per dendrimer (112). Using this number, methoxypoly(ethylene glycol) tresylate or TMPEG was conjugated to the G5-NH_2 dendrimer in a 1:10 PEG:primary amine molar ratio by mixing both in 10 mM phosphate buffer, pH 7.4 and stirring at 25 °C for four days. This product was then dialyzed against deionized water using a 10,000 MWCO membrane for three days, exchanging washes 8 times. The purified and lyophilized product was analyzed by GPC to have a number average molecular weight of 84,730 g/mol. The difference between this molecular weight and that of the non-PEGylated G5-NH_2 (27,336 g/mol) was divided by the average molecular weight of the TMPEG (5,330 g/mol; also determined by GPC) to determine the average number of PEG chains conjugated to each dendrimer of 11. All of the remaining dendrimer primary amines are expected to be protonated at pH 7.4;[24] therefore, the charge of the PEGylated G5-NH_2 (G5-NH_2-PEG) is equal to 101 positive charges/mol (112-11).

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Gel Electrophoresis

Polyplexes were formed by adding increasing volumes of 3.4 μg/μL G5-NH_2 or 11.5 μg/μL G5-NH_2-PEG to 85 μL of 40 μg/mL 20-mer DNA solution, all in 10 mM phosphate buffer at pH 7.4, to result in +/- ratios of 0-20. The final volume was corrected with buffer to result in a final DNA concentration of 34 μg/mL. The two solutions were mixed well for each ratio and incubated at room temperature for 20 min. To each well of a 3% agarose gel made with 1X tris acetate EDTA (TAE) buffer, 18.5 μL of polyplex sample mixed with loading dye was added. The gel was run at 60 mV for approximately 45 min in 1X TAE buffer supplemented with 2 μL of 10 mg/mL ethidium bromide solution.

Dynamic Light Scattering

Polyplexes were formed by adding 300 mg/mL G5-NH_2-PEG to 270 μL of a 350 μM solution of DNA 20-mer both in 10 mM phosphate buffer pH 7.4 to result in +/- charge ratios of 0, 0.25, 0.50, 0.75 and 1.0 at a final DNA concentration of 300 μM. The solutions were mixed well and then incubated at room temperature for 20 min. Hydrodynamic diameter of the complexes was then measured at 37 °C on a Malvern Zetasizer Nano ZS (Worchestershire, UK) with a 4 mW He-Ne laser operating at 633 nm with a 173° scattering angle. Correlation functions were analyzed by global fit to the data of three measurements of three runs each, with sizes reported as the z-average, since the populations were monomodal for all polyplexes with +/- ratio > 0.25.

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^{1}H NMR Titrations of DNA with Dendrimer

Separate samples of 270 μL of 350 μM 20-mer DNA were aliquoted in 10 mM phosphate buffer supplemented with 10 % D_2O. Increasing concentrations of 300 mg/mL G5-NH_2-PEG were added to obtain polyplexes of +/- ratios of 0-5 and the final volume of buffer was

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adjusted to obtain a 300 μM solution of DNA. Jump-return 1D ^{1}H spectra were obtained on a 600 MHz Bruker Avance NMR spectrometer equipped with a triple-resonance cryogenic probe at 37 °C with 64 scans. Spectra were processed and analyzed with NMRPipe software.[25]

^{1}H NMR Titrations of Dendrimer with DNA

Separate samples of 18 μL of 300 mg/mL G5-NH_2-PEG were aliquoted in 10 mM phosphate buffer supplemented with 10 % D_2O. Increasing concentrations of 20-mer DNA solution were added to obtain polyplexes of decreasing +/- ratio from 5.0 to 0.25 and the final volume was adjusted to 50 μL. Spectra were obtained on a 600 MHz Varian spectrometer equipped with a Nanoprobe at 37 °C with 2.5 kHz magic angle spinning and 1024 scans including a water saturation pulse. Spectra were processed and analyzed with NMRPipe software.[25]

R_2Relaxation Measurements and Global Fitting of $R_{2,polyplex}$ and p_{free}

Relaxation measurements of polyplexes at +/- ratios of 0, 0.25, 0.50 and 1.0 were conducted at 37 °C using a 1D Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence. To prepare the samples in D_2O buffer, polyplexes formed as above for the ^{1}H titration experiments were lyophilized for 48 h and then the dry solid was resuspended in the same volume of D_2O. Spectra were obtained on a 600 MHz Bruker NMR at 37 °C with 32 (+/- ratio 0 to 0.5) or 64 (+/- ratio 1.0) scans, a constant ν_{cp} = 1250 Hz (τ_{cp} = 0.8 ms) or ν_{cp} = 250 Hz (τ_{cp} = 4.0 ms, data not shown) and variable relaxation delay times t of 0.8 – 160 ms. Spectra were processed and analyzed with NMRPipe software[25] and Origin 7 (OriginLab Corp.). Relaxation rates ($R_{2,cpmg} = R_{2,obs}$) were determined from best fits to mono- and bi-exponential decays of the normalized intensity of the “aromatic” (6.8 – 8.8 ppm) or “ribose” (5.2 – 6.5 ppm) region at increasing delay times. $R_{2,obs}$ obtained from mono-exponential fits of aromatic and ribose regions at different N/P ratios were fit globally to Eq 1. by a non-linear regression algorithm using Mathematica 8.0 (Wolfram Research, Inc.). $R_{2,polyplex}$ for the aromatic and ribose regions and p_{free} (p_{bound} = 1- p_{free}) at different N/P ratios were extracted from 1000 different fits using random initial values for each parameter with suitable constraints (0 p_{free} 1; 0 $R_{2,polyplex}$ 500), and the mean values and standard deviations are reported in Table 2.

Calculation of Observed Polyplex Hydrodynamic Diameter

The rotational correlation time (τ_m) for the DNA-dendrimer polyplex was computed from the fitted $R_{2,polyplex}$ value assuming that dipole-dipole interactions dominate the proton relaxation rate[26]:

$$R_{2,polyplex}=\sum \frac{1}{8}d^2 \quad (4J(0)+3J(\omega_H)+6J(\omega_X)+J(\omega_H-\omega_X)+6J(\omega_H+\omega_X));d=\frac{\mu_0\gamma_H\gamma_X h}{8\pi^2\left\langle r_{HX}^3\right\rangle}$$

where X = H or C, μ_0 is the permeability of free space, h is Plank's constant, γ_H and γ_C are the gyromagnetic ratios of ^{1}H and ^{13}C, r_{HH} and r_{HC} is length of the H-H or C-H bond vector where angular brackets indicate time average. $R_{2,polyplex}$ was calculated as the sum of all proton-proton and proton-carbon contributions weighted by the fraction of H6, H8 and H2 or H1’ and H5 protons in each spectral region (assuming 1% ^{13}C). $J(\omega)$ is the spectral density function as defined by the extended model-free formalism for isotropic tumbling and two timescales of internal motions[27]:

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$$J(\omega)=\frac{2}{5}\left(\frac{S^2\tau_m}{1+(\omega\tau_m)^2}+\frac{S_f^2\tau_e}{1+(\omega\tau_e)^2}\right);\tau_e^{-1}=\tau_m^{-1}+\tau_s^{-1}$$

where S^2 is the generalized order parameter ($S^2 = S_f^2 S_s^2$), S_f^2 is the order parameter for the fast internal motion (< 100 ps), S_s^2 and τ_s is the order parameter and the correlation time respectively for the slow internal motion (> 500 ps). The following parameters were used for estimation of τ_m from $R_{2,polyplex}$: S^2 (H2/H6/H8) = 0.75 - 0.85 and S^2 (H1'/H5) = 0.65 - 0.85; $S_f^2 = 0.95$; τ_s = 1 - 5 ns; r_{CH} is the distance to the directly bonded carbon and r_{HH} reflect distances to neighboring protons within 4 Å from an idealized B-DNA model built with 3DNA[28]. The hydrodynamic diameter ($D_H = 2R_H$) for the polyplex (modeled as a spherical particle) was computed using Stoke's equation below with the range of estimated τ_m values

$$R_H=\left(\frac{3k_B T\tau_m}{4\pi\eta}\right)^{1/3}$$

where k_B is Boltzmann's constant, T is temperature in K, and $\eta(T)$ is the solvent viscosity.

RESULTS AND DISCUSSION

Using 10% PEGylation of the dendrimer primary amine end groups with 5 kDa PEG chains, we fully prevented precipitation of polyplexes (+/- ratios 5) up to a concentration of 0.7 mM and, thereby, successfully prepared polyplex samples suitable for characterization by high-resolution solution state NMR. As characterized by dynamic light scattering, the hydrodynamic diameter of the modified polymer (G5-NH_2-PEG, **Fig. 1**) is 5.9 nm, compared to that of the unmodified G5-NH_2 at 5.5 nm (data not shown). This indicates wrapping of poly(ethylene glycol) around the dendrimer, rather than a fully extended conformation of the chains. The ability of G5-NH_2-PEG to still bind DNA was confirmed using gel electrophoresis (**Fig. 2**). DNA migration was completely prevented at a charge ratio of 0.75 by the unmodified dendrimer (**Supplementary Fig. S1**), compared to a charge ratio of 1.0 for the PEGylated analogue, which suggests a change in the stoichiometry of interaction due to the masked charge and steric hindrance of the PEG chains. This gel shift assay indicates the presence of free DNA at +/- ratios 1.0. Quantification of the relative intensity of the gel bands revealed 36%, 16% and 4% free DNA at +/- ratios of 0.25, 0.50 and 1.0, respectively. The results are consistent with the results obtained by Fant et al. probing with ethidium bromide.[12]

To gain further insights into the dynamic behavior of the polyplex, we recorded ^{1}H NMR spectra as a function of increasing +/- ratio (**Fig. 3**). Incremental addition of G5-NH_2-PEG to the 20 base pair DNA duplex resulted in gradual line broadening without any significant changes in the proton chemical shifts. The absence of sharp spectra of free DNA during the course of the titration suggests that the free DNA present, as indicated by the gel shift assay (**Fig. 2**) and ethidium bromide tritration,[12] is in rapid exchange on the NMR timescale (< millisecond) with a dendrimer-bound polyplex state. This is also consistent with the smearing of the band on the gel at +/- ratios less than 1 and the observation of a single population in the dynamic light scattering (DLS) measurements (**Fig. 4**), run under the same conditions as the NMR spectra. As shown in **Fig. 4**, the average particle hydrodynamic diameter estimated by DLS is remarkably constant as +/- ratio increases from 0.50 to 1.0, with little change in distribution. Interestingly, at a +/-charge ratio of 5, we observe an

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increase in particular DNA peak intensities, or narrower linewidths, relative to conditions of charge neutrality (+/- = 1) (**Fig. 3**). This suggests that either DNA is being released from the polyplex (not supported by the gel electrophoresis data; **Fig. 2**), the DNA experiences increased local dynamics on the pico-to-nanosecond timescale due to change of conformation, or that differential levels of nanosecond internal motions are being manifested as a result of their decoupling from overall DNA motions, as the tumbling rate of the polyplex decreases.[29]

To further characterize the nature of the polyplex detected by the NMR experiments, we used the Carr-Purcell-Meiboom-Gill (CPMG) experiment to measure transverse relaxation rate constants (R_2) for base and sugar protons of the DNA at various points of the titration at 37 °C. Experiments were performed in deuterated buffer to remove dendrimer amine signals overlapping with the base and ribose DNA regions. The “aromatic” (base H2/H6/H8; 6.8 – 8.8 ppm) and “ribose” (ribose H1’/base H5; 5.2 – 6.5 ppm) regions of the DNA spectra were integrated separately for determination of the R_2 values. Measurements of R_2 as a function of two τ_{cp} delays (delay between 180° pulses in CPMG experiments) revealed no evidence for slow chemical exchange. We observed little variation in R_2 when changing τ_{cp} from 4 ms to 0.8 ms at +/- ratios of 0 to 0.5, indicating little R_{ex} contribution and negligible chemical exchange around these timescales. This, together with the lack of significant changes in DNA proton chemical shifts, strongly suggests that the DNA line broadening observed with increasing +/- ratio is dominated by an effective increase in the size of the DNA (and hence intrinsic relaxation rate) due to dendrimer binding and not due to chemical exchange contributions, which are expected to be very small when the exchanging states have similar chemical shifts.

Fig. 5 shows measured decay curves as a function of delay time for increasing +/- ratio. Best fits of this data to mono- and bi-exponential decays are reported in **Table 1** and **Supplementary Table S1**. Decays for the aromatic region fit better to a bi-exponential model, which can be explained by the intrinsically different relaxation properties of proton spins (i.e. H2 vs. H8)[30] contained in the integrated regions, while decays for the ribose region dominated by H1’ protons were described well by a mono-exponential model. As shown in **Table 1**, R_2 values from mono-exponential fits of the two regions increase similarly in a non-linear fashion with increasing dendrimer concentration up to +/- ratio of 1. This is consistent with an increase in the effective DNA molecular weight due to increased association with dendrimer. Again, we observe a decrease in $R_{2,obs}$ from +/- ratio 1 to 5, consistent with the narrower linewidths in the ^{1}H DNA spectra and/or consistent with increased dynamics in the DNA.

The NMR data suggest that the DNA is in rapid exchange with increasing amounts of dendrimer at increasing +/- ratios (up to 1). However, it remains difficult to resolve whether the DNA exchanges with one well-defined polyplex species that increases in population with increasing +/- ratio, or with a growing number of dendrimers condensed on the DNA or if aggregation of multiple polyplexes at high charge ratios results in many exchanging species. Assuming a two-state equilibrium between free DNA and a single polyplex species in the fast NMR timescale, where the chemical exchange constant is much larger than the chemical shift difference between the two states ($k_{ex} >> \Delta\omega$), the observed relaxation rates can easily be expressed as a population-weighted average of values for free and polyplex states[31]

$$R_{2,obs}=p_{free}R_{2,free}+p_{bound}R_{2,polyplex} \quad \text{Eq. 1}$$

in which we ignore chemical exchange contributions. Since $p_{free} + p_{bound} = 1$ and both $R_{2,obs}$ and $R_{2,free}$ are measured experimentally, we used the above equation and a global fit

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of the relaxation data ($R_{2,obs}$ obtained from monoexponential fits) at different titration points to extract $R_{2,polyplex}$ and the fractions of free (p_{free}) and bound (p_{bound}) DNA at each +/- ratio (**Table 2**). This relationship was previously considered for the spin-lattice relaxation rates of free and bound DNA by Bonechi et al.[32,33] A similar method was also recently used to extract the relaxation profiles, chemical exchange, and binding affinities of two weakly interacting proteins.[34] The computed value of $R_{2,polyplex}$ (**Table 2**) using the constraints outlined in **Methods** suggests a species with an estimated overall rotational correlation time, τ_m, of ~ 50–220 ns obtained from a range of motional parameters for aromatic and ribose protons and a corresponding polyplex hydrodynamic diameter of ~8-14 nm. This diameter is in good agreement with the predicted diameter of one DNA-dendrimer complex (~15 nm at theoretical charge neutrality based on 2-3 20mer DNA molecules per PEGylated dendrimer, which was measured to have a hydrodynamic diameter of 5.9 nm by DLS, data not shown). However, calculated p_{bound} values at different +/- ratios, even with large error, do not show the characteristic saturation profile expected from a two-state binding (**Supplementary Fig. S2**). Instead, p_{bound} values increase more rapidly, and point to a more complex behavior that likely involves a greater number of species. The average diameter of the polyplex species observed by NMR is also significantly smaller than the one obtained from DLS bulk measurements of polyplex particle size (~60 nm; **Fig. 4**). Thus, it is likely that small DNA-dendrimer complexes are in equilibrium with NMR-invisible higher aggregates, also supported by the decrease in the overall integrated area of the DNA imino and aromatic/ ribose protons with increasing +/- ratios (**Table 2**). Differences in size, composition, and packing of the polyplex at +/- ratio of 1 versus 5 could lead to conformational changes (i.e. bending, fraying or helical deformations) in the DNA with elevated internal/global flexibility and account for the increased peak intensities and increase in apparent p_{free} at +/- ratio of 5.

As an inverse experiment, we performed the NMR titration in the opposite direction to monitor dendrimer resonances upon increasing the concentration of DNA. Interestingly, while we do observe chemical shift perturbations that are consistent with rapid exchange of dendrimer with DNA, we observe little line broadening, even at very high DNA ratios (**Fig. 6**). We do not observe the increase in dendrimer proton line broadening (maximum estimated increase in $R_{2,obs}$ is ~ 2-fold as compared to ~ 20-fold for DNA) that would be expected upon formation of a large molecular weight polyplex. These data suggest that the dendrimer remains highly mobile in the polyplex, locally and globally, and exhibits solution-like dynamics even at high DNA ratios.

Polymers used in conducting materials have been shown to exhibit dynamic chain motion, providing multiple coordination sites for counterions, leading to their transport.[35] Polymer electrolytes are often formed using poly(ethylene oxide) or PEO, such as in PEOPMMA-H_3PO_4 (with hydrogen ions as charge carriers) or PEO_3:$LiCF_3SO_3$ (with lithium and triflate ions as charge carriers). Both Przyluskie et al and Gadjourova et al showed that NMR linewidth of the species in these materials can reveal their dynamics.[36,37]

The polyplex structure of mobile dendrimer in a rigid framework of DNA has many implications both in polymeric gene delivery vector design and understanding the mechanisms of transfection and protein expression inside the cell. Fant et al. proposed a model for G5 PAMAM dendrimer-DNA binding that included higher order chiral DNA stacking of helices in a well-defined structure with dendrimers woven throughout, attracting the strands.[38] This type of structure is consistent with our NMR observations of a rigid DNA lattice and might explain the formation of toroids (seen for dendrimers and many other polycationic DNA complexes, such as poly-L-lysine[39], chitosan[40] and spermidine[41]) due to the bundling of DNA helices. This polyplex structure polymorphism suggests DNA release could result from the dendrimer sampling multiple binding sites on the DNA, thereby

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regulating transcription, as previously hypothesized for spermine and histone protein DNA condensation.[42] The delivered DNA must be released from the vector long enough to form the DNA transcriptional complex. Fant recently proposed that the uncondensed fraction of DNA in a G5 PAMAM dendrimer polyplex sample is responsible for transcription, based on separating the fractions with ultracentrifugation.[38] Our NMR results indicate that the two fractions could be one in the same, with DNA release controlled by fast exchange and dendrimer mobility in the complex. It should be noted that polycation structure, molecular weight and PEGylation, as well as DNA length, can affect polyplex physicochemical properties, so care should be taken in extending these results to all nonviral gene delivery systems until experimental limitations of studying all systems are overcome.

CONCLUSION

Our results provide a new view of PAMAM dendrimer-DNA polyplexes, as featuring rigid but rapidly exchanging DNA associated with highly flexible and dynamic dendrimer that remains locally mobile. The faster-than-millisecond exchange rate of the DNA with the polyplex, possibly enhanced by the polymer's dynamics, provides an intrinsic mechanism of nucleic acid release from the polymeric delivery vector. Since release of DNA from the polyplex has been proposed as a rate-limiting step in the expression process, NMR approaches that can measure the dynamics of the exchange, such as those presented here, as well as $^{13}C/^{15}N$ relaxation and relaxation dispersion studies of isotopically labeled DNA or dendrimer, are of particular importance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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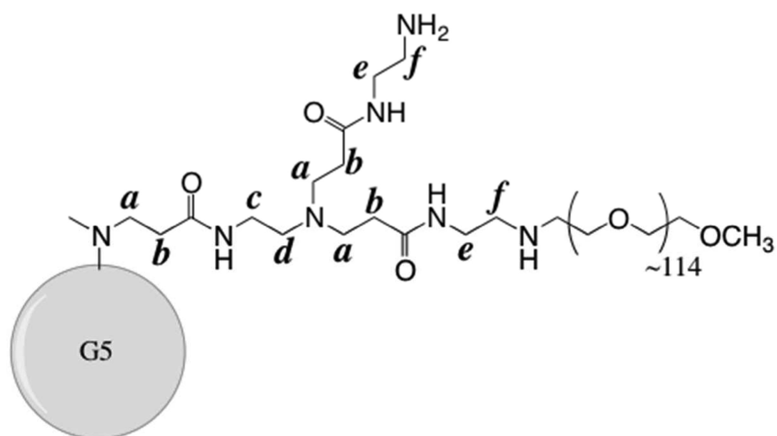

Figure 1.

Structure of G5-NH_2-PEG, showing one arm of the fifth generation. Poly(ethylene glycol), ~5000 MW with ~114 repeat units, is conjugated to 10% (11/112) of the primary amines on the dendrimer.

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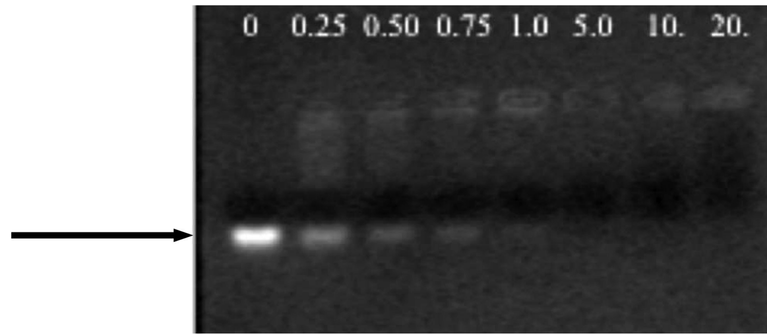

Figure 2.

Agarose gel electrophoresis of 20-mer DNA (5' CCACAGTGTTTGTGCAGCGG 3') at increasing +/- ratios of G5-NH_2-PEG. Free DNA is indicated by the band marked with the arrow. Loss of migration of DNA in the lane indicates complexation with dendrimer.

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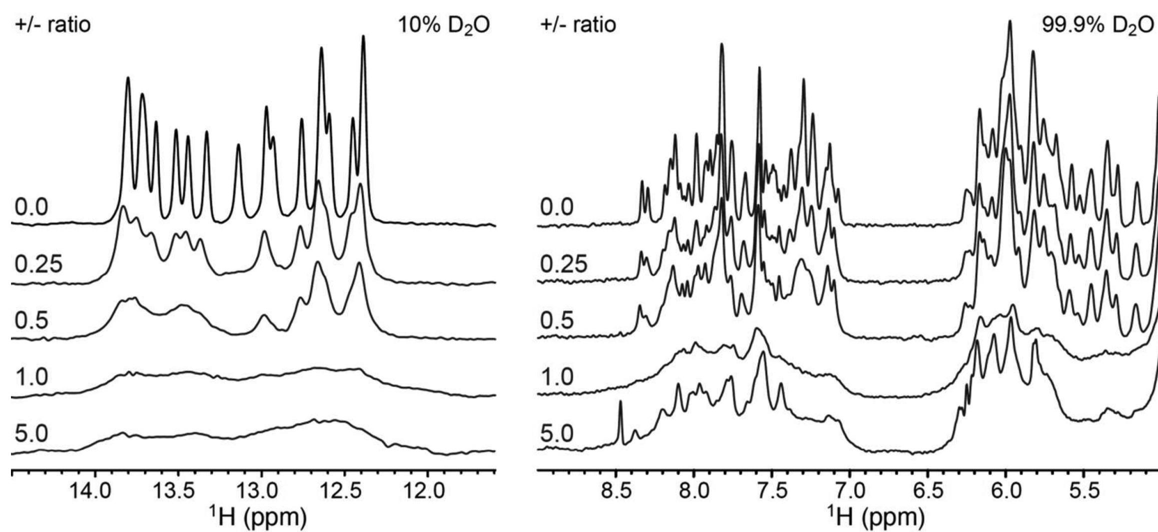

Figure 3.

^{1}H NMR spectra of 300 μM DNA titrated with 300 mg/mL G5-NH_2-PEG to increasing +/- ratio. (Left) DNA imino proton region. (Right) DNA aromatic (7.0 – 8.5 ppm) and ribose (5.0 – 6.5 ppm) regions.

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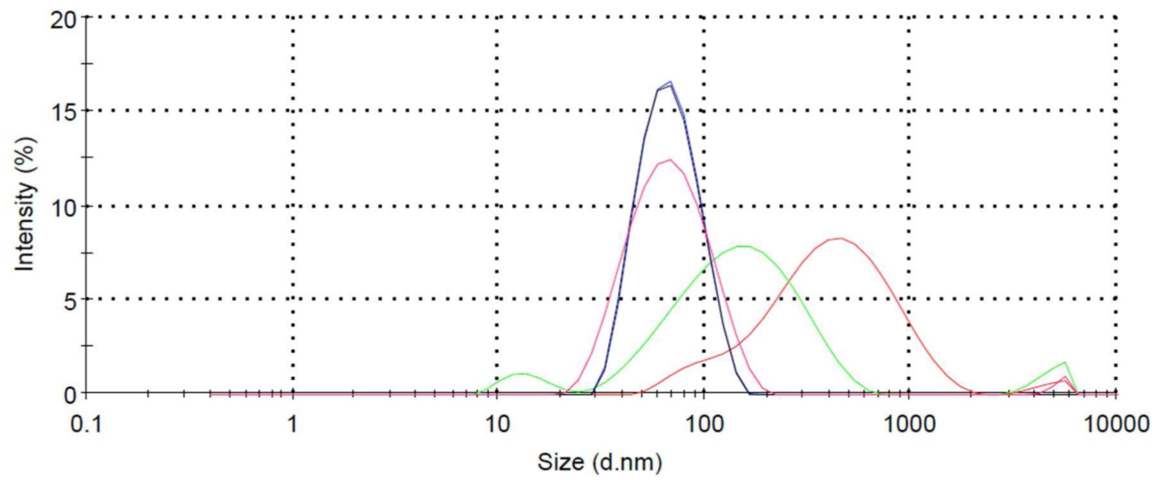

Figure 4.

Distribution of particle hydrodynamic diameter (in nm) of NMR samples of increasing +/- ratio. DNA only (red), +/- = 0.25 (green), 0.50 (blue), 0.75 (black) and 1.0 (pink).

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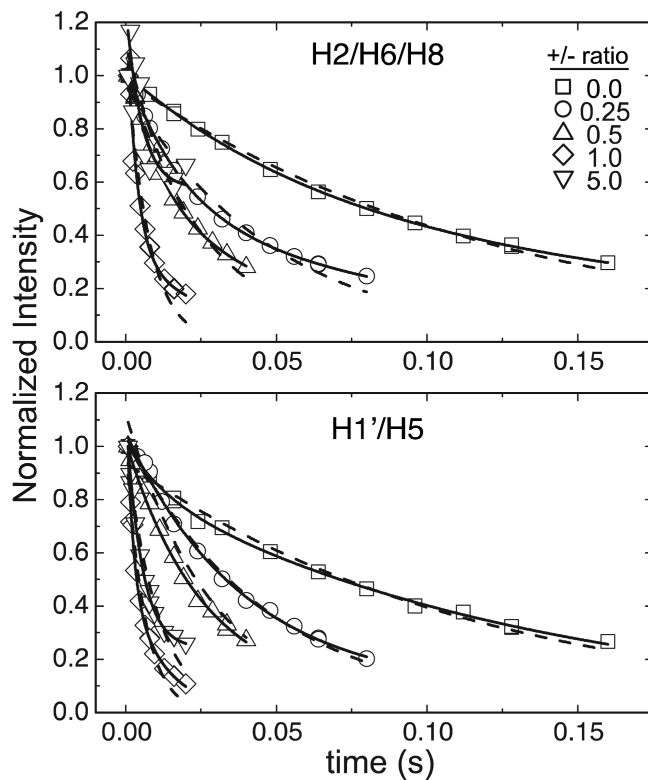

Figure 5.

Intensity of the H2/H6/H8 (top) and H1'/H5 (bottom) region as a function of delay time at 37 °C for 300 μM DNA titrated with 300 mg/mL G5-NH_2-PEG to increasing +/- ratio (inset). Monoexponential (dashed line) and biexponential (solid line) fit to the decay.

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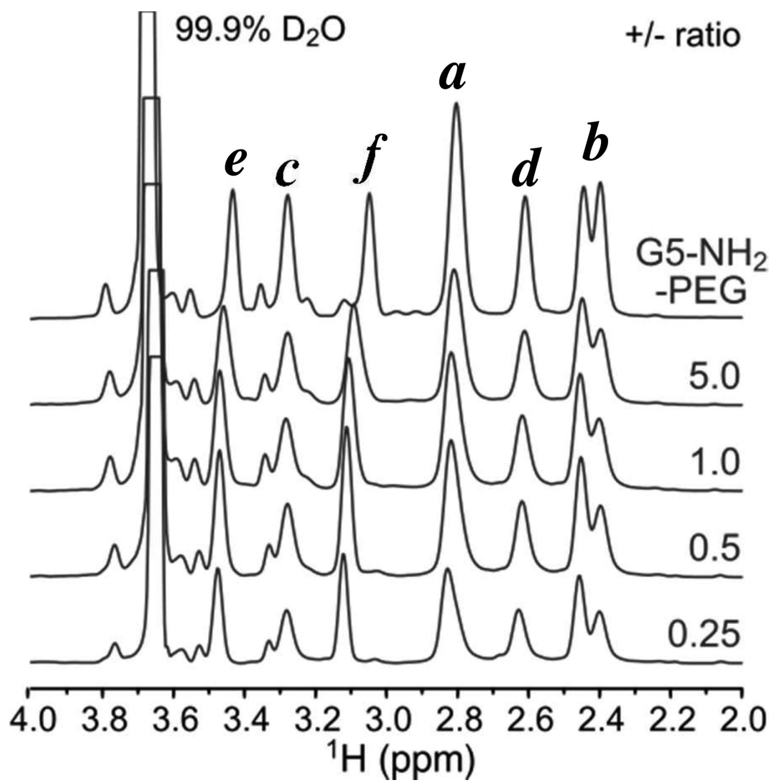

Figure 6.
^{1}H NMR spectra of 300 mg/mL G5-NH$_2$-PEG titrated with 20-mer DNA at 37 °C as a function of +/- ratio. Peak labels refer to those in **Fig. 1**.

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Table 1

Observed R_2 values from fitting the decay of integrated DNA aromatic H2/H6/H8 (6.8-8.8 ppm) and ribose H1'/aromatic H5 (5.2-6.5 ppm) region versus delay time for increasing +/- ratios.

+/- Ratio	$R_{2,obs}$ (Hz)
	Aromatic (H2/H6/H8)
0	8.1 ± 0.2
0.25	20.5 ± 1.0
0.50	36.2 ± 1.4
1.0	135.6 ± 14.5
5.0	42.4 ± 8.5
	Ribose (H1')/Aromatic (H5)
0	8.7 ± 0.4
0.25	21.4 ± 0.6
0.50	34.6 ± 0.6
1.0	162.7 ± 13.5
5.0	94.0 ± 7.2

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Table 2

Best-fit parameters for the fraction of free DNA (p_{free}) and polyplex R_2 ($R_{2,polyplex}$) at increasing +/- ratios obtained from global fitting of Eq. 1 for both the aromatic H2/H6/H8 and ribose H1'/aromatic H5 regions of the DNA relaxation spectra.

+/- Ratio	p_{free}	$R_{2,polyplex}$ (Hz) (*H2/H6/H8*)	$R_{2,polyplex}$ (Hz) (*H1'/H5*)
0	1	256 ± 121	267 ± 116
0.25	0.95 ± 0.02		
0.50	0.90 ± 0.03		
1.0	0.40 ± 0.22		
5.0	0.76 ± 0.08		