Charge-Shifting Cationic Polymers That Promote Self-Assembly and Self-Disassembly with DNA

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ABSTRACT: Synthetic materials that assemble reversibly with polyanions under physiological conditions are of interest for a broad range of biotechnical applications. Cationic polymers are used widely as agents for the condensation of DNA, but polycations also introduce practical limitations in applications for which subsequent dissociation or disassembly of polycation/DNA complexes is desired. The design of cationic polymers that promote the release of associated DNA presents a challenge because it requires the introduction of functionality that is inherently opposed to that required for efficient DNA condensation. Here, we report the synthesis and biophysical characterization of linear poly(ethylenimine) (LPEI) functionalized with methyl ester side chains. The gradual hydrolysis of the ester functionality in these materials under physiologically relevant conditions results in a controlled reduction in cationic charge density and a change in the nature of the electrostatic interactions between the polymers and plasmid DNA, as determined by agarose gel electrophoresis. Using this approach, it is possible to mediate the dissociation of DNA from polymer over a period of hours to days by varying the mole percentage of methyl esters incorporated into the polymer. Polymers having a high degree of substitution (e.g., 80 or 100 mol %) release DNA more rapidly than less-substituted polymers (e.g., 40 and 60 mol % functionalized). Polymers having 20 mol % substitution did not release DNA in these experiments. These charge-shifting materials could provide a basis for the design and fabrication of polyelectrolyte complexes and assemblies that sustain the release of DNA in solution and at interfaces.

Introduction

Cationic polymers self-assemble with anionic polymers through electrostatic interactions and are therefore used widely to condense DNA in gene delivery applications[1] and to fabricate polyelectrolyte assemblies on surfaces.[2,3] The use of cationic polymers in these applications creates subsequent problems, however, in cases where the dissociation, disassembly, or release of the associated polyanion is later desired. The influence of pH, temperature, and ionic strength on the disruption of polycation/polyanion interactions is well understood,[4] and abrupt changes in these environmental parameters can be used to promote the dissociation of polyelectrolyte assemblies on "demand". For therapeutic applications, however, changes in pH, temperature, and ionic strength are restricted and, therefore, not generally available as tools to promote disassembly. The work described here was motivated by our interest in designing macromolecular assemblies that provide temporal control over the nature of the electrostatic interactions between polycations and DNA without concomitant stimulatory changes in environmental conditions.[5−8] We report the design, synthesis, and biophysical characterization of a family of "charge-shifting" cationic polymers that form self-assembled complexes with DNA and undergo subsequent structural changes in physiologically relevant media that lead to substantial changes the nature of the electrostatic interactions in these assemblies.

Cationic polymers assemble spontaneously with DNA through electrostatic interactions to yield condensed interpolyelectrolyte complexes—a process that is, in part, driven entropically by the formation of small counterion-derived salts (e.g., NaCl).[9,10] The ability of polycations to condense DNA into nanometer-scale complexes and stimulate the cellular internalization of DNA has placed these materials at the core of many polymer-based approaches to DNA delivery.[1,11−20] Despite the benefits conferred by self-assembly, however, most cationic polymers used to deliver DNA are unable to surmount subsequent barriers in the delivery process.[1] In particular, the self-*disassembly* of DNA from a cationic polymer with which it is associated presents a significant challenge. In the absence of changes in ionic strength, pH, or temperature, the dissociation of DNA from a cationic polymer requires the introduction of functionality (e.g., a low density of positive charge) that is inherently opposed to that required for efficient DNA condensation (typically, a high density of positive charge).[18,21] Thus, the driving forces that contribute to the initial process of self-assembly also place constraints on the extent to which, and the conditions under which, polycation/DNA complexes will dissociate under physiological conditions. Several recent reports suggest that conventional polycation/DNA assemblies are stable toward intracellular dissociation.[15,22,23] Because DNA must ultimately dissociate from polymer before it can be processed by a cell, the inefficiency of this disassembly step is cited increasingly as a factor limiting the effectiveness of polycationic gene delivery agents.[15,22−27] Cationic polymers that facilitate the reversible assembly of polyelectrolytes at surfaces and interfaces are also of interest with respect to the design of thin films that permit the controlled release of therapeutics.[5−8]

The design of materials that provide temporal and spatial control over DNA self-assembly and disassembly under physiological conditions is an active area of research. Several groups have described hydrolytically or reductively degradable polycations that function to release DNA upon chemical hydrolysis[11,12,28−38] or ex-

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posure to reductive cytosolic environments.[24−26] In addition, other groups have reported that a reduction of the charge densities of polycations such as linear or branched poly(ethylenimine) (PEI) through permanent chemical conversion of the amines in the polymer backbone can yield significant increases in gene expression,[27,39,40] at least in part through modulation of charge-dependent polymer/DNA interactions. More recently, Funhoff et al. reported a polymer that loses cationic side chains by hydrolysis to release associated DNA.[41] We hypothesized that the gradual introduction of anionic functionality to a cationic polymer could be used to reduce the net charge on the polymer, introduce changes in the nature of the electrostatic interactions between the polymer and DNA, and ultimately, provide temporal control over the dissociation of DNA from the polymer.[42] We report here the synthesis and biophysical characterization of a family of ester-functionalized linear poly(ethylenimine) derivatives that are positively charged at physiological pH, but which undergo dynamic reductions in net cationic charge density via the gradual chemical hydrolysis of ester side-chain functionality. These materials self-assemble with plasmid DNA and make possible the subsequent dissociation of DNA over periods ranging from several hours to weeks by varying the extent to which these polymers are functionalized with masked anionic functionality.

Materials and Methods

General Considerations. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on Bruker AC+ 250 (250.133 MHz) and Bruker AC+ 300 (300.135 MHz) spectrometers. Chemical shift values are given in ppm and are referenced with respect to residual protons from solvent. Gel permeation chromatography (GPC) was performed using a Waters 515 HPLC pump (Waters Corporation, Milford. MA), a Rheodyne Model 7725 injector with a 20-μL injection loop, and two Waters Styragel HT 6E columns in series. THF containing 0.1 M triethylamine was used as the eluent at a flow rate of 1.0 mL/min. Data were collected using a Waters 2410 Refractive Index Detector and processed using the Waters Empower software package. Molecular weights are reported relative to monodisperse polystyrene standards. Attenuated total reflectance infrared spectroscopy data were collected on a Bruker TENSOR 27 FTIR instrument (Billerica, MA) outfitted with an ATR transmission cell from PIKE Technologies (Madison, WI).

Materials. Linear poly(ethylenimine) was synthesized by hydrolysis of the side chains of poly(2-ethyloxazoline) [MW = 50 000; obtained from Polysciences, Inc., Warrington, PA] and purified prior to use in analogy to procedures previously described.[40,43] Plasmid DNA [pEGFP-N1 (4.7 kb), >95% supercoiled] was obtained from a commercial supplier (Elim Biopharmaceuticals, Inc., San Francisco, CA). All other materials were used as received without further purification.

Synthesis of Ester Substituted Linear Poly(ethylenimine) (Polymers 1a−e). The conjugate addition of methyl acrylate to linear poly(ethylenimine) was performed in the following general manner. Methyl acrylate was added to a solution of linear PEI (5 wt % in methanol) in a screw-capped vial equipped with a magnetic stir bar. The amount of methyl acrylate added was varied (e.g., from 0.20 to 1.2 equiv relative to amine functionality in PEI) to achieve desired mole percent substitutions. The reaction mixture was stirred for 2 h at room temperature. Longer reaction times and higher temperatures resulted in the formation of amide cross-links, as determined by attenuated total reflectance infrared spectroscopy (see text). The resulting reaction product was concentrated by rotary evaporation, dissolved in dichloromethane, and precipitated into hexanes. The isolated material was dried under vacuum to yield the desired product as a viscous oil in near quantitative yield. Representative ^{1}H NMR data for a polymer with 100% substitution: ($CDCl_3$) δ (ppm) = 2.45 (t, $CH_2CH_2N(CH_2CH_2CO_2CH_3)$), 2.5 (s, $CH_2CH_2N(CH_2CH_2CO_2CH_3)$), 2.8 (t, $CH_2CH_2N(CH_2CH_2CO_2CH_3)$), 3.71 (s, $CH_2CH_2N(CH_2CH_2CO_2CH_3)$).

Synthesis of Carboxylic Acid Substituted Poly(ethylenimine) (Polymers 2a−e). The synthesis of carboxylic acid-functionalized polymers used in DNA-binding control experiments was conducted in the following general manner. Ester-functionalized polymer (100 mg) was dissolved in NaOH (2 mL, 1.0 N) in a screw-capped vial equipped with a magnetic stir bar and the solution was allowed to stir overnight (10 h) at room temperature. The pH of the resulting solution was adjusted to 7.0 with aqueous HCl (1.0 N) and dialyzed against 18 MΩ water (4.0 L) for 24 h using a dialysis membrane with a 100 molecular weight cutoff (Spectra/Por, Rancho Dominguez, CA). Water was removed from the dialyzed product in vacuo and the resulting product was used in subsequent assays without further purification.

Synthesis of Amide Substituted Linear Poly(ethylenimine) (Polymers 3a−e). The conjugate addition of *N*,*N*-dimethylacrylamide to linear poly(ethylenimine) was performed in the following general manner. *N*,*N*-Dimethylacrylamide was added to a solution of linear PEI (5 wt % in methanol) in a screw-capped vial equipped with a magnetic stir bar. The amount of *N*,*N*-dimethylacrylamide added was varied (e.g., from 0.20 to 1.2 equiv relative to amine functionality in PEI) to achieve desired mole percent substitutions. The reaction mixture was stirred for 4 h at room temperature. The resulting reaction product was concentrated by rotary evaporation, dissolved in dichloromethane, and precipitated into hexanes. The isolated material was dried under vacuum to yield the desired product as a viscous oil in near quantitative yield. Representative ^{1}H NMR data for a polymer with 100% substitution: ($CDCl_3$) δ (ppm) = 2.46 (t, $CH_2CH_2N(CH_2CH_2CON(CH_3)_2)$), 2.5 (s, $CH_2CH_2N(CH_2CH_2CON(CH_3)_2)$), 2.8 (t, $CH_2CH_2N(CH_2CH_2CON(CH_3)_2)$), 2.9 (s, $CH_2CH_2N(CH_2CH_2CON(CH_3)_2)$), 3.0 (s, $CH_2CH_2N(CH_2CH_2CON(CH_3)_2)$).

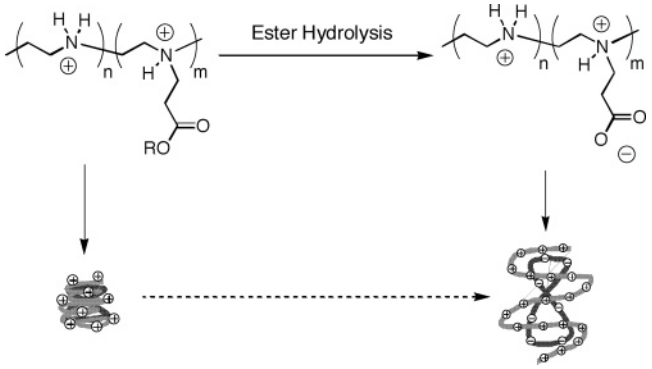
Kinetics of Ester Hydrolysis under Physiological Conditions. ^{1}H NMR experiments used to determine the kinetics of ester hydrolysis for functionalized polymers in solution were conducted in the following general manner. Polymer (8.3 mg) was dissolved in deuterated HEPES buffer (0.6 mL, 100 mM, pH = 7.2), 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (3.6 mg) was added as an internal standard, and the resulting solution was placed in a glass NMR tube. The NMR tube was heated to 37 °C in an oil bath and removed periodically for analysis by ^{1}H NMR spectroscopy. The disappearance of the methyl ester resonance at 3.71 ppm was monitored and integrated versus the trimethylsilyl protons of the internal standard.

Formation of Polymer/DNA Complexes and Agarose Gel Retardation Assays. DNA/polymer complexes were formed by adding 50 μL of a plasmid DNA solution (2 μg/50 μL in water) to a gently vortexing solution of polymer (50 μL in either 20 mM HEPES, pH = 7.2 or 20 mM acetate buffer, pH = 5.0). In every case, the concentration of polymer in this volume of buffer was adjusted to yield a desired DNA/polymer weight ratio (e.g., 1:1, 1:2, 1:3, etc.). These samples were incubated at room temperature for 30 min, after which a 30 μL aliquot of each sample was mixed with a loading buffer and analyzed on a 1% agarose gel (HEPES, 20 mM, pH = 7.2, 108 V, 50 min). DNA bands were visualized by ethidium bromide staining. Samples used to evaluate time courses of DNA/polymer interaction were prepared by doubling the amounts and volumes in the above protocol. These samples were incubated at 37 °C and aliquots were removed at desired time periods (typically 0, 24, 48, and 72 h) for analysis by agarose gel electrophoresis.

Results and Discussion

Design and Synthesis of Ester-Functionalized Polyamines. The central hypothesis of our approach is that the controlled introduction of anionic functionality to a cationic polymer (e.g., via the gradual hydrolysis

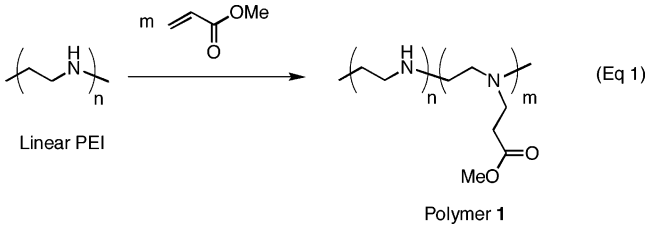
Scheme 1. General Concept Demonstrating the Dynamic Introduction of Carboxylate Groups to a Cationic Polyamine via Side-Chain Ester Hydrolysis[a]



[a] The gradual introduction of negative charge is expected to reduce the net charge of the polymer and promote a change in the nature of the interactions between the polymer and DNA.

of masked anionic functionality) should effectively reduce, neutralize, or even reverse the net charge of the polycation, and that these changes in charge density should translate to a change in the nature of the interactions between these materials and DNA.[42] This approach differs from the design of cationic polymers that degrade completely upon reduction or chemical hydrolysis[11,12,24−26,28−38] and it is complementary to a recently reported strategy that reduces polymer net charge through the hydrolysis and loss of cationic side chains.[41] Our general approach is illustrated in Scheme 1. In this example, the extent to which polymer net charge is reduced by the introduction of carboxylate groups would depend on both environmental pH and the range of pK_a values of the amines and carboxylates in the polymer structure.

We selected linear poly(ethylenimine) (LPEI) as a scaffold from which to synthesize model ester-functionalized polyamines for three reasons: (1) LPEI is readily synthesized by the hydrolysis of commercially available poly(2-ethyloxazoline),[40,43] (2) it has been used in many past studies as a DNA-condensing and delivery agent,[18,44] and (3) the dense array of secondary amines in this material facilitates the introduction of new functionality through well-defined chemistry.[45] We selected the conjugate addition of secondary amines to methyl acrylate as a general strategy for the introduction of ester side chain functionality (eq 1) because this reaction proceeds



(Eq 1)

quantitatively under mild conditions[11,12,29,30] and was used previously to functionalize LPEI in the context of dendrimer synthesis.[46] Although the strategies reported below should also be applicable to the derivatization of hyperbranched PEI[14,18] and other polycations functionalized with primary or secondary amines, we selected LPEI for these initial studies because it is easier to characterize derivatives of this linear polymer.

LPEI was synthesized by hydrolysis of commercially available poly(2-ethyloxazoline) (M_w = 50 000).[40,43] Treatment of LPEI with an excess of methyl acrylate at room temperature for 2 h yielded polymer **1** with substitution of > 95% of the secondary amines (eq 1, n = 0), as determined by integration of the ^{1}H NMR spectrum of the resulting material. To investigate the influence of polymer backbone substitution and changes in charge density on DNA/polymer interactions (described below), we synthesized five derivatives of polymer **1** having approximately 100%, 80%, 60%, 40%, and 20% mol % substitution (referred to hereafter as polymers **1a**−**e**, respectively) by varying the stoichiometric mole percentage of methyl acrylate used. For polymers **1b**−**e**, reactions conducted at temperatures higher than ambient or for times longer than 2 h resulted in the formation of amide cross-links due to the reaction of unreacted secondary backbone amines with methyl ester side chains (as identified by attenuated total reflectance FTIR spectroscopy in comparison to independently prepared amide-functionalized model polymers; see below). This side reaction was completely eliminated by conducting the synthesis of these polymers at ambient room temperature for 2 h. Analogues of polymers **1a**−**e** having dimethylamide side chains (polymers **3a**−**e**) were synthesized by conjugate addition of LPEI to *N*,*N*-dimethylacrylamide. Derivatives of polymers **1a**−**e** functionalized with carboxylic acid side chains (polymers **2a**−**e**) were synthesized via saponification of the esters in these materials as described in the Experimental Section.

Polymers **1a**−**e** were obtained as viscous, sticky materials in near quantitative yield after precipitation into hexanes, and each polymer was soluble in water and HEPES buffer (pH = 7.2) at the concentrations required for subsequent assays and experiments. Analysis of polymer **1a** by gel permeation chromatography yielded a molecular weight (M_w) of 33 000 relative to monodisperse polystyrene standards (M_n = 10 200; PDI = 3.23). A direct comparison between the molecular weight of this polymer and the LPEI starting material was not possible, as LPEI (as well as less-substituted polymers **1b**−**e**) was insoluble in the tetrahydrofuran GPC eluent used in this study. A molecular weight of 33 000 for polymer **1a** is reasonable in view of the molecular weight of the poly(2-ethyloxazoline) used to synthesize the LPEI (Mw = 50 000). We caution, however, that a direct or quantitative comparison of these two different materials is not possible because our GPC results, calibrated to polystyrene standards, reflect relative molecular weights based on the hydrodynamic volumes of these two different materials and not their absolute molecular weights.

Self-Assembly of Ester-Functionalized Polyamines with Plasmid DNA. The ability of polymers **1a**−**e** to form self-assembled complexes with plasmid DNA at physiological pH was determined using an agarose gel shift assay. Agarose gel electrophoresis separates DNA on the basis of size and charge, and the immobilization or retardation of DNA in the presence of a polycation is frequently used to characterize polyamine/DNA self-assembly processes.[17,20,47] We selected a monodisperse plasmid DNA construct (pEGFP−N1, 4.7 kb, >95% supercoiled) on the basis of the relevance of this construct as a reporter gene in DNA delivery applications.[48] Figure 1 shows the results of electrophoresis assays using DNA/polymer complexes prepared using polymers **1a**−**e** at various DNA/polymer ratios, in which DNA is bound by polymer and pre-

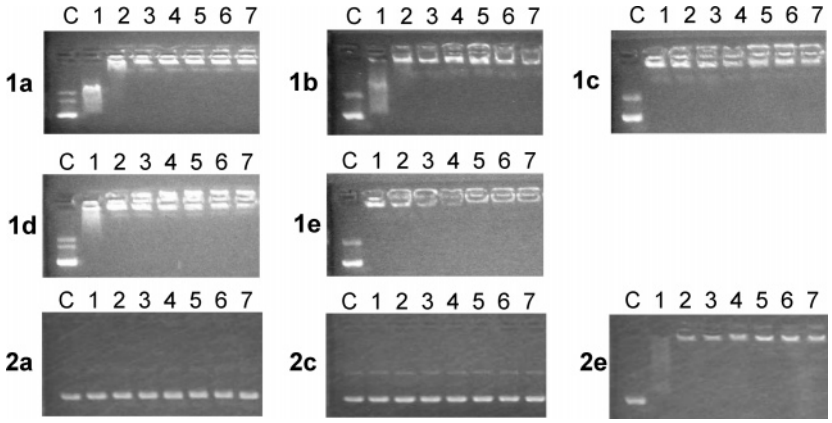


Figure 1. Agarose gel electrophoresis experiments demonstrating the self-assembly of methyl ester-functionalized polymers **1a**−**e** and carboxylate-functionalized polymers **2a**, **2c**, and **2e** with plasmid DNA. The leftmost lanes labeled "C" correspond to a DNA control (no polymer). Additional lanes labeled 1−7 correspond to DNA/polymer weight ratios ranging of 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, and 1:7, respectively.

vented from migration through the gel at weight ratios as low as 1:1 or 1:2. These experiments demonstrate that the structural changes introduced by the functionalization of the LPEI backbone (i.e., the introduction of side chains and the conversion of secondary amines to more sterically hindered and less basic tertiary amines) do not prevent these polymers from forming electrostatic complexes with DNA.

We note that polymers **1c**−**e**, having lower percentages of substitution, completely retard the migration of DNA at DNA/polymer ratios of 1:1, but a greater amount of polymer is required to completely inhibit migration using polymers **1a** and **1b** (e.g., a DNA/polymer ratio of 1:2; Figure 1). These differences could arise from steric constraints or the higher percentages of less basic tertiary amines in polymers **1a** and **1b**. These differences may also result from the use of DNA/polymer weight ratios, rather than N:P ratios, to prepare and describe the complexes used in these experiments. Polymers **1a** and **1b** are more substituted and thus have higher molecular weights than polymers **1c**−**e**. As such, a sample of polymer **1a** or **1b** would contain fewer moles of amine than an equal mass of a less substituted polymer; greater masses of polymer would thus be required to inhibit the migration of DNA as percent substitution increases. We note that alternative descriptions based on ratios of nitrogen to phosphate in these complexes would introduce a similar limitation, as the pK_a values of polymers **1a**−**e** could vary substantially as backbone substitution is varied.

Completely hydrolyzed, acid-functionalized polymers **2a**−**d** (i.e., containing >40 mol % acid groups) did not retard the migration of DNA at DNA/polymer ratios of up to 1:10. Figure 1 shows representative electrophoresis data for experiments using 100%- and 60%-functionalized polymers **2a** and **2c**. These results provided an initial indication that the hydrolysis of the ester side chains in polymers **1a**−**d** could change the nature of the electrostatic interactions of these materials with DNA at levels that might be sufficient to promote the disruption of electrostatic polymer/DNA assemblies in subsequent experiments (discussed below). By contrast, polymer **2e** (containing only 20 mol % acid groups) *did* prevent the migration of DNA into the gel at DNA/polymer ratios as low as 1:2 (Figure 1). The migration of DNA through the gel was only partially inhibited at DNA/polymer weight ratios of 1:1.

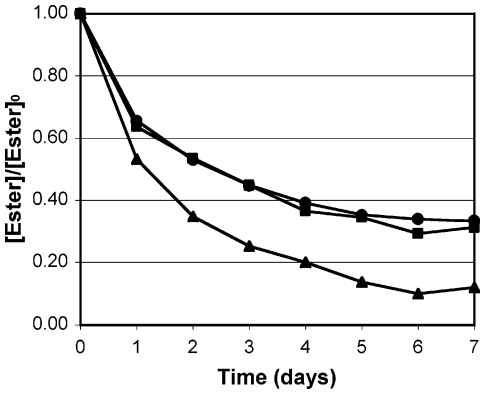


Figure 2. Kinetics of side chain ester hydrolysis for polymers **1a** (●), **1c** (■), and **1e** (▲) in HEPES-d_{18} buffer (100 mM, pH = 7.2) at 37 °C, determined by ^{1}H NMR spectroscopy.

Kinetics of Ester Hydrolysis and Release of DNA. To evaluate the kinetics of ester side chain hydrolysis under physiologically relevant conditions, we incubated polymers **1a**, **1c**, and **1e** in HEPES-d_{18} buffer (100 mM, pH 7.2) at 37 °C and monitored the disappearance of the methyl ester resonance at 3.71 ppm by ^{1}H NMR spectroscopy. As shown in Figure 2, the half-lives for the disappearance of ester in 100%- and 60%-functionalized polymers **1a** and **1c** were approximately 48 h. The disappearance of the ester groups for 20% substituted polymer **1e** occurred more rapidly under these conditions ($t_{1/2} \approx 24$ h).

The ^{1}H NMR data in Figure 2 reflect the disappearance of the ester functionality in these materials rather than the direct formation of acid functionality. We considered that the disappearance of the ester groups in these experiments could also arise from the amidation of the ester side chains, i.e., the reaction of secondary amines in the backbone of a polymer with a side chain methyl ester (as discussed above for the synthesis of these materials). Amide formation is not possible for 100% substituted polymer **1a**, because this polymer contains only tertiary amines. However, the formation of amides could become more favorable during the incubation of polymers **1b**−**e** because the relative ratio of secondary to tertiary amines increases for these polymers as mol % side chain substitution decreases. We used attenuated total reflectance FTIR spectroscopy to analyze freeze-dried samples of polymers **1a**, **1c**, and **1e** that were incubated in phosphate buffered saline for

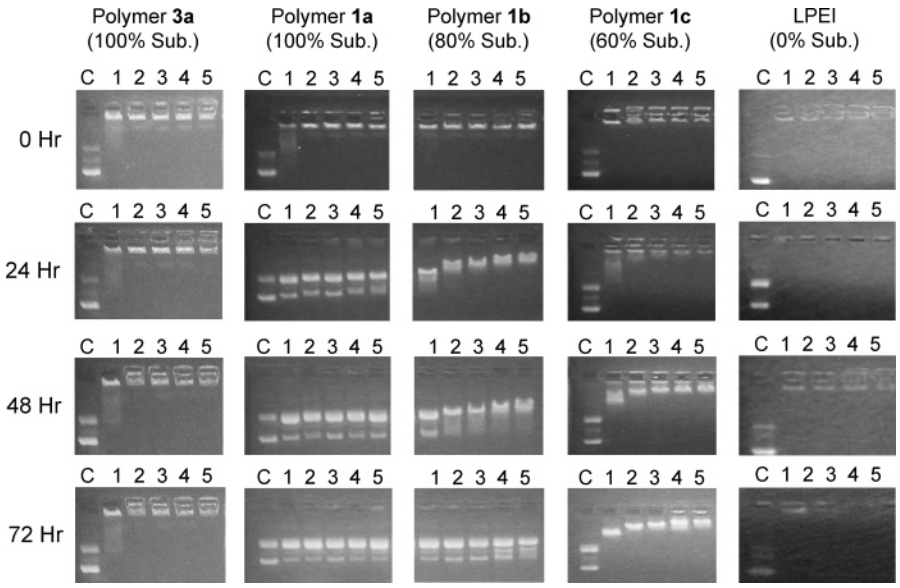


Figure 3. Agarose gel electrophoresis experiments showing time courses for the release of DNA from DNA/polymer complexes formed using dimethylamide-functionalized polymer **3a** and methyl ester-functionalized polymers **1a**, **1b**, and **1c** at 0, 24, 48, and 72 h. The leftmost lane in each gel (labeled "C") corresponds to a DNA control (no polymer added). Lanes labeled 1−5 in each gel correspond to DNA/polymer ratios (w/w) of 1:1, 1:3, 1:5, 1:7, and 1:9, respectively. Note: the DNA control in lane "C" shown in column 2 (for experiments using polymer **1a**) also serves as the internal DNA control for the data located in column 3 (for experiments using polymer **1b**). These experiments were conducted in the same row of lanes but have been separated in the figure to enhance the clarity and readability of the figure.

7 days (at 37 °C, and at the same concentrations used in the ^{1}H NMR experiments above). The IR carbonyl stretches observed for these incubated materials were compared to those corresponding to ester (1735 cm^{-1}), carboxylic acid (1570 cm^{-1}), and dimethylamide (1640 cm^{-1}) functional groups in independently prepared model polymers **2** and **3**. For each incubated polymer, we observed carbonyl stretches corresponding to the formation of carboxylic acid groups and did not observe distinct stretches characteristic of amide formation. We cannot discount the possibility that the broad acid carbonyl stretches observed in these experiments (typically ranging from 1650 to 1500 cm^{-1}) could obscure smaller signals corresponding to amide formation. However, these FTIR experiments provide additional evidence suggesting that side chain esters in these materials hydrolyze readily to yield acid-functionalized polymers upon incubation in physiologically relevant media. We note here that in the context of the current study, the formation of amide cross-links during incubation, if it occurs, would have the same qualitative effect as ester hydrolysis in reducing the net charge of these polymers (i.e., amide formation would result in the consumption of one protonatable amine). The specific reasons for the differences in the rates of hydrolysis for polymers **1a,c** and **1e** are not yet clear. In view of the above data, we speculate that more hydrophobic polymers **1a** and **1c** may aggregate in aqueous solution or assume conformations that place the pendant ester groups in a more hydrophobic environment than the esters in less functionalized polymer **1e**.

The experiments above suggest that the hydrolysis of the ester side chains in polymers **1a**−**e** occurs over relatively short time scales at physiological pH and temperature. We conducted an additional series of gel electrophoresis experiments to determine whether ester hydrolysis also resulted in substantial changes in the nature of the electrostatic interactions between these polymers and DNA. For these experiments, DNA/polymer complexes were incubated in HEPES buffer (25 mM, pH = 7.2) at 37 °C and aliquots were removed at 0, 24, 48, and 72 h for analysis by gel electrophoresis. All polymers prevented the migration of DNA upon initial complexation at ratios as low as 1:1 or 1:2, as described above. However, we observed the presence of either uncomplexed DNA or partially complexed DNA at 24-, 48-, and 72-h time points consistent with changes in the nature of the electrostatic interactions in these assemblies.

Figure 3 shows representative results for the release of DNA from complexes formed using 80% substituted polymer **1b** at DNA/polymer weight ratios of 1:1, 1:3, 1:5, 1:7, and 1:9 (third column, read top to bottom). In this example, the migration of DNA is initially inhibited at DNA/polymer ratios as low as 1:1 (lane 1). However, DNA from complexes formed at a DNA/polymer ratio of 1:1 is able to migrate into the gel after 24 h (as indicated by the intermediate migration and streaking of plasmid relative to a no polymer control) and it migrates at nearly the same rate as a DNA control at the 48-h time point. If the rate of ester hydrolysis for the polymer in these assemblies is assumed to be similar to the rate of ester hydrolysis for the polymers in solution, a comparison of these data with the ^{1}H NMR data in Figure 2 suggests that the complete separation of DNA from polymer **1b** (in a complex formed at a ratio of DNA/polymer of 1:1) requires the hydrolysis of fewer than 50% of the side-chain esters.

Further inspection of Figure 3 demonstrates that complexes formed from polymer **1b** and DNA at lower DNA/polymer ratios (e.g., 1:3, 1:5, or 1:7) demonstrate similar behavior, but that DNA is released more slowly than the DNA in complexes formed at a ratio of 1:1. This behavior may result from either the greater amount of polymer incorporated into the DNA/polymer complexes at these ratios or the greater amount of excess polymer that likely remains in solution (i.e., excess polymer that was not incorporated into the complexes at these lower DNA/polymer ratios). The DNA in all samples prepared using polymer **1b** at these lower ratios was able to

migrate at the same rate as a DNA control after 72 h. Inspection of the electrophoresis bands characterizing the DNA present in these samples at the 72 h time point reveals that the plasmid is almost completely converted to an open circular form. This form of degradation may result at least partially from prolonged incubation in HEPES buffer, as the supercoiled DNA used as a control (no polymer) is also significantly converted to an open supercoiled topology over this time period. We note, however, that this change in topology appears to be more significant for the DNA incubated in the presence of polymer, and that the amount of supercoiled DNA remaining in these samples appears to decrease as the amount of polymer used increases (Figure 3). Although open supercoiled DNA remains transcriptionally viable when delivered to cells, it is considered to be less active than supercoiled DNA. As such, the potential for this form of plasmid damage should be borne in mind with respect to the potential future application of these materials as gene delivery agents. The specific reasons for this change in plasmid structure are not completely understood; we speculate that such damage could result from the generation of locally acidic environments within these DNA/polymer complexes as hydrolysis occurs, although additional experiments will be required to examine this hypothesis more thoroughly.

Influence of Backbone Substitution on the Dissociation and Release of DNA. To examine relationships between polymer backbone substitution, dynamic changes in charge density, and resulting changes in DNA/polymer interactions we conducted agarose gel electrophoresis experiments using DNA/polymer complexes formed from the full set of polymers **1a**−**e** and LPEI. The relative rates and extents to which DNA was released from these complexes correlated directly with the extent of polymer backbone substitution. Figure 3 shows the results of experiments using 100%-, 80%-, and 60%-functionalized polymers **1a**−**c** incubated for 0, 24, 48, and 72 h. DNA was substantially released from 100% substituted polymer **1a** in less than 24 h at all DNA/polymer ratios investigated (Figure 3, second column). However, the DNA in samples prepared using 80%-functionalized polymer **1b** remained bound significantly at 24 h and required up to 48 to 72 h for complete release, as discussed above. In further contrast, the DNA in samples prepared using 60%-functionalized polymer **1c** was essentially completely retarded at all DNA/polymer ratios other than 1:1 at 24 h, and the DNA remained substantially retarded even after 72 h. Subsequent characterization of the DNA present in samples prepared from polymers **1c** and **1d** at extended times (e.g., 7 days) suggested that a portion of the DNA in these samples was able to migrate through the gel unhindered (data not shown). However, high levels of plasmid damage, also present in DNA controls incubated in HEPES buffer over this 7 day period, prevent any substantive conclusions regarding the release of DNA from these polymers at these extended times.

Complexes prepared from polymer **1e** did not release DNA under any conditions evaluated within the quantitative limits of our ethidium bromide detection assay (see Supporting Information). This result is consistent with the control experiments described above (Figure 1), in which 20% carboxylic acid-functionalized polymer **2e** was shown to form stable complexes that prevent the migration of DNA through a gel. Complexes formed using LPEI as a control did not release DNA in these experiments within the quantitative limits of this electrophoresis assay (Figure 3, fifth column). In general, efforts to characterize the behavior of complexes formed from polymers **1d**, **1e**, and LPEI were frustrated by an inability to visualize completely the DNA in complexes formed from these less substituted polymers using ethidium bromide staining procedures. As has been documented in past studies, certain polycations,[41,49] including PEI at sufficiently low DNA/polymer ratios,[49] form complexes that are often difficult to visualize using ethidium bromide. This behavior has been attributed to the formation of aggregates in which DNA/polymer binding is sufficiently strong to prevent the intercalation of ethidium bromide into the structure of DNA. In the present study, we observed the absence of ethidium bromide fluorescence in complexes formed using polymers **1d**, **1e**, and LPEI at certain DNA/polymer ratios (see Figure 3 and Supporting Information). This behavior was observed consistently in repeated experiments using these materials. In these cases, we did not observe the reappearance of ethidium bromide fluorescence that would otherwise indicate the migration of released DNA into the gel upon incubation. Given the limits imposed by the irregular fluorescence using these less-substituted materials, however, we interpret these results with caution. We do conclude on the basis of these collective experiments, however, that the hydrolysis of the esters in polymers **1d** or **1e** leads to changes in the nature of the electrostatic interactions with DNA that are less substantial than those resulting from the hydrolysis of more substituted polymers **1a**−**c**.

To further correlate the release of DNA using polymers **1a**−**c** with the hydrolysis of the esters in these materials, we conducted a series of control experiments using DNA/polymer assemblies prepared using structural analogues of these materials functionalized with dimethylamide side chains (polymers **3a**−**c**). Complexes formulated using these amide-containing materials did not release DNA under incubation conditions and electrophoretic conditions identical to those used to evaluate the release of DNA from complexes formed using polymers **1a**−**c**. Figure 3 shows representative data for experiments using 100%-functionalized polymer **3a**, in which DNA is completely prevented from migration through the gel at ratios as low as 1:1 for up to 72 h (Figure 3, first column). These experiments further support our hypothesis that the observed changes in the nature of the interactions between polymers **1a**−**c** and DNA are a result of side chain ester hydrolysis and not other factors resulting from the incubation, handling, and analysis of the DNA/polymer complexes themselves.

The results above are consistent with the gradual hydrolysis of the ester functionality in polymers **1a**−**d** and a subsequent change in the nature of the electrostatic interactions between the polymers and DNA. Moreover, these results suggest that increasing the percent substitution of the polymer backbone yields a more extensive reduction in net polymer charge upon hydrolysis and promotes larger changes in polymer/DNA interactions. Taken together, these experiments demonstrate that it is possible to design cationic polymers that self-assemble with DNA under physiological conditions and subsequently disrupt the resulting electrostatic complexes over a wide range of time scales simply by varying the mole percentage of ester-functionalized side groups incorporated into a cationic polymer backbone.

Summary and Conclusions

The design of cationic materials that balance forces governing the self-assembly and disassembly with DNA or other polyanions under physiological conditions is of interest in both fundamental and practical contexts. We have demonstrated that linear poly(ethylenimine) derivatives functionalized with methyl ester side chains are capable of forming self-assembled complexes with plasmid DNA, and that the principles introduced here can be used to achieve temporal control over the electrostatic interactions between these cationic materials and DNA. We used agarose gel electrophoresis to demonstrate that it is possible to control the rates at which DNA is released from polymer by varying the mole percentage of methyl esters incorporated into the polymer. Polymers having higher mole percent functionalization (e.g., 80 or 100 mol %) release DNA at shorter times in these experiments than less-substituted polymers (e.g., 40% or 60% functionalized). Polymers having particularly low percent substitution (e.g., 20%) did not release DNA within the limitations of the ethidium bromide detection assays used in this investigation.

These data are consistent with a mechanism in which the gradual introduction of anionic character to these polyamines (via gradual side chain hydrolysis) introduces destabilizing interactions that promote the dissociation of polymer from DNA. Higher levels of backbone substitution result in a more extensive reduction of net positive charge upon hydrolysis and promote the disruption of DNA/polymer complexes more rapidly than for less substituted polymers. The synthetic approach used here should also be applicable to the derivatization of hyperbranched PEI or other polycationic materials functionalized with primary or secondary amines. In the longer term, cationic polymers with structures that can be tuned to disrupt DNA/polycation complexes over a broad range of times could provide a basis for the design of polyelectrolyte assemblies that promote the delivery and/or release of DNA more effectively than conventional materials.

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Supporting Information Available: An extended version of Figure 3 showing gel electrophoresis data for the complete set of polymers **1a**−**e** and **3a** and LPEI. This material is available free of charge via the Internet at http://pubs.acs.org.

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