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Release of DNA from Polyelectrolyte Multilayers Fabricated Using ‘Charge-Shifting’ Cationic Polymers: Tunable Temporal Control and Sequential, Multi-Agent Release

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Abstract

We report an approach to the design of multilayered polyelectrolyte thin films (or ‘polyelectrolyte multilayers’, PEMs) that can be used to provide tunable control over the release of plasmid DNA (or multiple different DNA constructs) from film-coated surfaces. Our approach is based upon methods for the layer-by-layer assembly of DNA-containing thin films, and exploits the properties of a new class of cationic ‘charge-shifting’ polymers (or amine-functionalized polymers that undergo gradual changes in net charge upon side-chain ester hydrolysis) to provide control over the rates at which these films erode and release DNA. We synthesized two ‘charge-shifting’ polymers (polymers **1** and **2**) containing different side chain structures by ring-opening reactions of poly(2-alkenyl azlactone)s with two different tertiary amine functionalized alcohols (2-dimethylaminoethanol and 3-dimethyl-1-propanol, respectively). Subsequent characterization revealed large changes in the rates of side chain ester hydrolysis for these two polymers; whereas the half-life for the hydrolysis of the esters in polymer **1** was ~200 days, the half-life for polymer **2** was ~6 days. We demonstrate that these large differences in side chain hydrolysis make possible the design of PEMs that erode and promote the surface-mediated release of DNA either rapidly (e.g., over ~3 days for films fabricated using polymer **2**) or slowly (e.g., over ~1 month for films fabricated using polymer **1**). We demonstrate further that it is possible to design films with release profiles that are intermediate to these two extremes by fabricating films using solutions containing different mixtures of these two polymers. This approach can thus expand the usefulness of these two polymers and achieve a broader range of DNA release profiles without the need to synthesize polymers with new structures or properties. Finally, we demonstrate that polymers **1** and **2** can be used to fabricate multilayered films with hierarchical structures that promote the sequential release of two different DNA constructs with separate and distinct release profiles (e.g., the release of a first construct over a period of ~3 days, followed by the sustained release of a second for a period of ~70 days). With further development, this approach could contribute to the design of functional thin films and surface coatings that provide sophisticated control over the timing and the order of the release of two or more DNA constructs (or other agents) of interest in a range of biomedical contexts.

Supporting Information Available. Details of additional experiments and results related to characterization of polymer side chain hydrolysis, film erosion and DNA release profiles, and cell transfection experiments.

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Keywords

Layer-by-Layer; Polyelectrolyte; Thin Film; DNA Delivery; Surface-Mediated

Introduction

Materials that promote the surface- or substrate-mediated delivery of DNA are of interest in a broad range of fundamental and applied contexts, ranging from the development of platforms for the local delivery of gene-based therapeutics to applications such as tissue engineering and the development of tools for basic biomedical research [1–16]. A key challenge in each of these areas is the development of materials that can be used to functionalize surfaces and interfaces in ways that permit both spatial and temporal control over the release of DNA. Approaches that rely on the adsorption of DNA or the coating of surfaces with DNA-containing thin films provide a reasonably straightforward basis for exerting spatial control over DNA delivery (e.g., an implant coated with a DNA-containing thin film can be used to localize delivery to the vicinity of an implant site, etc.). In contrast, methods that can be used to control the timing with which one or more DNA constructs are released – that is, to achieve desired levels of temporal control – have generally been more difficult to develop. This paper reports a step toward these broader goals through the design of ultrathin polyelectrolyte-based films that erode gradually in physiologically relevant environments and provide tunable control over the release of DNA (or multiple different DNA constructs) from film-coated surfaces.

The work reported here makes use of methods developed for the layer-by-layer assembly of multilayered polyelectrolyte thin films (or ‘polyelectrolyte multilayers’, PEMs) [17]. Numerous past studies have demonstrated that these methods can be used to fabricate thin films and coatings with compositions and thicknesses that can be controlled systematically on the nanometer-scale [17–24]. The ability to control the structures and properties of these materials, combined with the ability to incorporate a broad range of agents of therapeutic interest, has fueled efforts to exploit PEMs in the context of a broad range of biomedical and biotechnological applications, including the design of thin films, capsules, and reservoirs for the controlled release of small-molecule and macromolecular therapeutics [15,19,21–26]. Of particular interest to this current study, several groups have reported the design of PEMs that release plasmid DNA from surfaces and promote the surface-mediated delivery of DNA into solution or to cells [27–34]. Many of these approaches have made use of cationic polymers that are degradable (e.g., either hydrolytically [27–30], enzymatically [31,32], or reductively [33,34] degradable) or other methods [35–37] to promote disruptions in electrostatic interactions and facilitate film erosion and the release of DNA. These and other approaches to the fabrication of DNA-containing PEMs have been reviewed recently [15].

We recently reported an alternative approach to the disruption of DNA-containing PEMs that is based on the incorporation of so-called ‘charge-shifting’ polyelectrolytes (that is, polyelectrolytes that do not degrade, but instead undergo gradual changes in net charge – e.g., from positively charged to negatively charged – when incubated in aqueous environments) [38–40]. Our group and others have demonstrated that it is possible to design ‘charge-shifting’ polymers, and exert control over the rates and extents of changes in charge state, by incorporating hydrolyzable side chains into the structures of cationic or anionic polymers [38–47]. Several different approaches to the design of cationic ‘charge-shifting’ polymers have been reported, largely in the context of developing new materials for the polyplex-mediated delivery of DNA (e.g., to provide control over the disassembly of cationic polymer/DNA complexes in solution or in cells) [41–47].

Recent studies have also demonstrated that the properties of ‘charge-shifting’ polymers can be exploited to promote time-dependent changes in the electrostatic interactions within PEMs and, as a result, provide control over the release of DNA or other payloads [38–40,43]. Of particular relevance to this current study, we reported recently that ‘charge-shifting’ polymer **1** can be used to design PEMs that promote the release of transcriptionally active plasmid DNA from film-coated surfaces over extended time periods (e.g., for up to three months using polymer/DNA films ~100 nm thick) [38]. The long-term release profile of this system (e.g., as compared to the much faster release profiles of PEMs fabricated using hydrolytically degradable polyamines) [15] was made possible, at least in part, by the relatively slow rate at which the ester side chain functionality of polymer **1** hydrolyzes in physiologically relevant media (and thus the relatively slow disruption of the electrostatic interactions in these materials) [38].

This study sought to investigate the influence of the structure of this ‘charge-shifting’ cationic polymer on rates of film erosion and the subsequent release of DNA from film-coated surfaces. Here, we describe the synthesis and characterization of a second ‘charge-shifting’ polymer (polymer **2**), a derivative of polymer **1** that contains a shorter linker between the tertiary amine side chain functionality and the amide/ester linkage to the polymer backbone. We demonstrate that this small change in structure leads to large changes in the rate of hydrolysis of the side chains of this polymer, and that DNA-containing films fabricated using polymer **2** erode and release DNA at rates that are significantly faster than films fabricated using polymer **1** (e.g., over periods of days v. weeks or months). The availability of these two ‘charge-shifting’ polymers provides opportunities to design more sophisticated and tunable films using blends or combinations of these two different materials. In the sections below, we also demonstrate that different combinations of these two polymers can be used to (i) provide tunable control over the release of DNA from surfaces, and (ii) design ultrathin PEMs that promote the staged or sequential release of two different DNA constructs with separate and distinct release profiles. Our results provide an alternative to the design of thin films fabricated using degradable cationic polymers and extend the range of functionality that can be designed into this class of materials in ways that could prove useful in a range of different biomedical contexts.

Materials and Methods

Materials

2-Dimethylaminoethanol, 3-dimethylamino-1-propanol, N,N-dimethylethylene diamine, and 1,8 diazabicyclo[5.4.0]-undec-7-ene (DBU) were purchased from Acros Organics (Morris Plains, NJ). 2-Vinyl-4,4-dimethylazlactone (VDMA) was a generous gift from Dr. Steven M. Heilmann (3M Corp. Minneapolis, MN). Sodium acetate buffer was purchased from Lonza (Rockland, ME). Test grade n-type silicon wafers were purchased from Si-Tech, Inc.

(Topsfield, MA). Phosphate-buffered saline (PBS, pH = 7.4, 137 mM NaCl) was prepared by diluting commercially available concentrate (EM Science, Gibbstown, NJ) and adjusting the pH to 7.4 with 1.0 M HCl or NaOH. Polymers **1** and **3** were synthesized as described previously [38]. Plasmid DNA [pEGFP-N1 (encoding enhanced green fluorescent protein; EGFP) or pDsRed2-N1 (encoding red fluorescent protein; RFP) (4.7 kb), > 95% supercoiled] was obtained from Elim Biopharmaceuticals, Inc. (San Francisco, CA). All materials were used as received without further purification unless noted otherwise. Deionized water (18 MO) was used for washing steps and to prepare buffer and polymer solutions. Compressed air used to dry films and coated substrates was filtered through a 0.4 μ m membrane syringe filter.

General Considerations

^1H nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AC+ 300 (300.135 MHz) spectrometer. Chemical shift values are given in ppm and are referenced with respect to residual protons from solvent. 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). Gel permeation chromatography (GPC) was performed using a GPCmax-VE2001 Solvent/Sample module (Viscotek Corp., Houston, TX), and two PlusPore Organic GPC Columns (Polymer Laboratories, Amherst, MA) in series. THF was used as the eluent at a flow rate of 1.0 mL/min. Data were collected using the RI detector of a Viscotek TDA 302 triple detector array and processed using the OminiSEC 4.5 software package. Molecular weights and polydispersities of polymers are reported relative to monodisperse polystyrene standards. Silicon substrates (e.g., 0.7×3.5 cm) used for the fabrication of multilayered films were cleaned with deionized water and dried under a stream of filtered compressed air. Surfaces were then activated by etching in oxygen plasma for 5 min (Plasma Etch, Carson City, NV) prior to film deposition. Optical thicknesses of films deposited on silicon substrates were determined using a Gaertner LSE ellipsometer (632.8 nm, incident angle = 70°). Data were processed using the Gaertner ellipsometer measurement program. Relative thicknesses were calculated assuming an average refractive index of 1.577 for the films. Thicknesses were determined in at least 5 different standardized locations on each substrate and are presented as an average (with standard deviation) for each film. All films were dried under a stream of filtered compressed air prior to measurement. UV absorbance values for solutions used to determine film release kinetics were recorded on a Beckman Coulter DU520 UV/vis spectrophotometer (Fullerton, CA). Fluorescence microscopy images used to evaluate EGFP or RFP expression in transfection experiments were recorded using an Olympus IX70 microscope and analyzed using the Metavue version 4.6 software package (Universal Imaging Corp.). Data were stored in single channel, 12-bit TIF format. Additional image processing was limited to false coloring and uniform scaling.

Synthesis of Poly(2-Vinyl-4,4-dimethylazlactone) (PVDMA)

2-Vinyl-4,4-dimethylazlactone (VDMA) was purified by passage through inhibitor removal resin and silica gel prior to polymerization. The initiator 2,2'-azobisisobutyronitrile (AIBN, 14.1 mg, 0.086 mmol) was weighed into a 25 mL round-bottomed flask equipped with a stir bar. Ethyl acetate (12 mL) was added and the solution was purged with N_2 for 10 min. VDMA (3.36 g, 24.2 mmol) was added to the flask via syringe and the N_2 purge was continued for an additional 5 min. The solution was stirred under N_2 at 60°C for 24 hrs, at which point the viscous reaction mixture was cooled to room temperature. CH_2Cl_2 (~5 mL) was added to the flask and the polymer was precipitated into hexanes to produce a white solid. The polymer was redissolved in CH_2Cl_2 and precipitated twice more in hexanes (white solid, 90% yield). $M_n = 52,700$, PDI = 4.3, ^1H NMR (300 MHz, CDCl_3 , 25°C): $\delta =$

1.37 (br s, $(-CH_3)_2$), 1.62–2.1 (br m, $-CH_2CH-$), 2.69 (br s, $-CH_2CH-$). FT-IR (ATR, cm^{-1}): 2980–2900 (C-H), 1820 (lactone C=O), 1672 (C=N).

Synthesis of Polymer 2

PVDMA (1.08 mmol), 2-dimethylaminoethanol (1.62 mmol), and DBU (1.08 mmol) were weighed into a vial and dissolved in THF (5.0 mL), and the resulting mixture was heated to 50 °C in an oil bath. After 48 hrs, the resulting reaction products were concentrated *in vacuo* and precipitated into a hexane/acetone mixture (40 mL; 7:1, v/v). The precipitate was isolated by centrifugation, dissolved in THF, and reprecipitated twice more. The final product was dried under vacuum to yield the product as a white solid. 1H NMR (300.135 MHz, $CDCl_3$, 25 °C): δ = 1.45 (br m, 8H), 2.56 (br m, 8H), 3.42 (br s, 1H), 4.20 (br s, 2H).

Synthesis of Polymer 4

PVDMA (1.08 mmol) and N, N-dimethylethylenediamine (1.62 mmol) were weighed into a vial and dissolved in THF (5.0 mL), and the resulting mixture was heated to 50 °C in an oil bath. After 24 hrs, the resulting reaction products were concentrated *in vacuo* and precipitated into a hexane and acetone mixture (40 mL; 5:3, v/v). The precipitate was isolated by centrifugation, dissolved in THF, and reprecipitated twice more. The final product was dried under vacuum to yield the product polymer as a white solid. 1H NMR (300.135 MHz, $CDCl_3$, 25°C): δ = 1.49 (br s, 8H), 2.22 (br m, 8H), 2.95 (br s, 1H), 3.27 (br s, 2H).

Characterization of Kinetics of Ester Hydrolysis

1H NMR experiments designed to characterize the hydrolysis of ester functionality in polymers **1** and **2** in aqueous solution were performed in the following manner. Polymer (10 mg) was dissolved in deuterated phosphate buffer (1.0 mL, 0.5 M, pH = 7.2). 3-(Trimethylsilyl)-1-propanesulfonic acid sodium salt (2 mg) was added as an internal standard, and this solution was transferred to a glass NMR tube. The NMR tube was incubated at 37 °C and removed periodically for analysis by 1H NMR spectroscopy. The change of the resonance corresponding to the methylene protons adjacent to the ester functionality (at 4.2 ppm) to a resonance at 3.7 ppm after hydrolysis was monitored, and the extent of hydrolysis was determined by integrating these signals versus the trimethylsilyl protons of the internal standard.

Preparation of Polyelectrolyte Solutions

Solutions of polymers **1–4** used for dipping (5 mM with respect to the molecular weight of polymer repeat units) were prepared in sodium acetate buffer (100 mM, pH = 5). To investigate a range of different solution compositions, we also prepared four different polymer solutions comprised of 10, 25, 50, and 75 mol % polymer **2** (with the remainder comprised of polymer **1**; solutions were 5 mM with respect to total polymer repeat units). DNA solutions (1 mg/mL) used for the deposition of polymer/DNA layers were prepared in sodium acetate buffer (100 mM, pH = 5).

Fabrication of Multilayered Films

Multilayered films were fabricated on planar silicon substrates manually using an alternating dipping procedure according to the following general protocol: 1) Substrates were submerged in a solution of polyamine for 5 min, 2) substrates were removed and immersed in an initial water bath for 1 min followed by a second water bath for 1 min, 3) substrates were submerged in a solution of DNA for 5 min, and 4) substrates were rinsed in the manner described above. This cycle was repeated until the desired number of polymer/DNA layers (or ‘bilayers’) was reached. Hereafter, we describe films fabricated in this manner using the

notation (X/DNA)_n, where “X” refers to the polyamine used and “n” denotes the number of polyamine/DNA bilayers deposited. For films fabricated from mixtures of polyamines, additional subscripts are used to denote the mol% of each polyamine in the solutions used to fabricate the films. For example, a film 8 bilayers thick fabricated from a 50 mol% mixture of polymer **1** and polymer **2** is denoted as (1₅₀2₅₀/DNA)₈. Films were either used immediately or dried under a stream of filtered, compressed air and stored in a vacuum desiccator until use. All films were fabricated at ambient room temperature.

Characterization of Film Erosion and DNA Release Profiles

Experiments designed to characterize film erosion and DNA release profiles were performed in the following general manner: Film-coated substrates were placed in a plastic UV-transparent cuvette and 1.0 mL of PBS was added to cover the film-coated portion of the substrate. The samples were incubated at 37 °C and removed at predetermined intervals to be characterized by ellipsometry. Films were rinsed under deionized water and dried under a stream of filtered compressed air prior to measurement. Values of optical thickness were determined in at least 5 different predetermined locations on the substrate and the sample was returned immediately to the buffer solution. For experiments designed to monitor the concentration of DNA released into solution, UV absorbance readings (at 260 nm, the absorbance maximum of DNA) were made directly on solutions used to incubate these samples.

Cell Transfection Assays

COS-7 cells were grown in 96-well plates at an initial seeding density of 12,000 cells/well in 200 µL of growth medium (90% Dulbecco's modified Eagle's medium, 10% fetal bovine serum, penicillin 100 units/mL, streptomycin 100 mg/µL). Cells were grown for 24 h, at which time 50 µL of a Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and plasmid mixture was added directly to the cells according to the general protocol provided by the manufacturer. The Lipofectamine 2000/plasmid transfection milieu was prepared by mixing 30 µL of the DNA solutions collected at each time point during release experiments (arbitrary concentrations but constant volumes) with 30 µL of diluted Lipofectamine 2000 reagent (24 µL stock diluted into 976 µL of water). Fluorescence images used to characterize levels of gene expression were acquired after 48 h.

Results and Discussion

Synthesis and Characterization of ‘Charge-Shifting’ Cationic Polymer 2

The ‘charge-shifting’ polymer used in our past studies (polymer **1**) was synthesized by the ring-opening addition of the hydroxyl group of 3-dimethylamino-1-propanol to the reactive, azlactone-functionalized polymer poly(2-vinyl-4,4-dimethylazlactone) (PVDMA), as shown in Scheme 1 [38]. This polymer has a side chain containing a tertiary amine group positioned 3 carbons away from the hydrolyzable amide/ester linker to the backbone of the polymer. One practical advantage of adopting this PVDMA-based approach is that it is modular and can be used to synthesize multiple different structurally-related polymers. For example, we demonstrated recently that PVDMA can be used as a reactive template for the parallel synthesis of a small library of structurally related cationic polymers having different side chain functionality but uniform (or nearly uniform) molecular weights and polydispersities (PDIs) [48]. This template-based approach facilitated characterization of differences in the abilities of these polymers to deliver DNA to cells in ways that could be understood to arise primarily from differences in the side chain structures of these polymers (e.g., as opposed to differences in molecular weight or PDI, etc.). We sought to use this reactive template approach to synthesize new ‘charge-shifting’ polymers that could be used

to provide predictable control over the erosion of polyelectrolyte multilayers fabricated from plasmid DNA.

To evaluate the feasibility of this approach, we synthesized side-chain functionalized polymer **2** by the ring-opening addition of the hydroxyl group of 2-dimethylaminoethanol to a sample of PVDMA having a molecular weight and PDI ($M_n = 52,700$; $PDI = 4.3$) identical to that used to synthesize polymer **1** (Scheme 1). The structure of polymer **2** places the tertiary amine functionality located in the side chains of the polymer 2 carbons away from the amide/ester linker to the backbone of the polymer (as opposed to 3 carbons away, as in the structure of polymer **1**). Characterization of the loss of ester functionality in solutions of polymer **2** incubated in deuterated phosphate buffer (pH 7.4, 37 °C) revealed a large difference in the rate of side chain ester hydrolysis as compared to polymer **1**.

Whereas the half-life for the hydrolysis of the esters in polymer **1** was ~200 days (in good agreement with results reported in past studies) [38] the half-life for the hydrolysis of the side chains of polymer **2** was ~6 days (see Figure S1, Supporting Information). These results demonstrate that a relatively small change in the distance of the tertiary amines from the amide/ester linker in the backbone of these polymers can result in a large change in side chain hydrolysis. This difference in rates of hydrolysis could result from a combination of two influences: (i) differences in the hydrophobicity of the side-chains of these two polymers, and (ii) differences in the mechanisms for the hydrolysis of the esters in these two polymers (e.g., the potential for an intramolecularly-assisted hydrolysis mechanism for polymer **2**, made possible by the two-carbon linker of this polymer, that could result in a more rapid hydrolysis as compared to polymer **1**, for which such intramolecular assistance would be less likely). It is also possible that these differences in ester hydrolysis could result from differences in the extent of inductive electron withdrawal upon protonation of the tertiary amine groups of these two polymers.

Characterization of Multilayered Films Fabricated from Plasmid DNA and Polymers **1** and **2**

We performed a series of experiments to determine whether polymer **2** could be used to fabricate multilayered films using a plasmid DNA construct (pEGFP-N1) encoding enhanced green fluorescent protein (EGFP), and, subsequently, whether differences in the structures of polymers **1** and **2** could be exploited to design films with different physical erosion and DNA release profiles. For these and all other experiments described below, films were fabricated on planar silicon substrates to facilitate characterization of film growth and erosion using ellipsometry. Figure 1 shows a plot of the optical thicknesses of films having the general structures $(\mathbf{1}/\text{pEGFP})_8$ and $(\mathbf{2}/\text{pEGFP})_8$ with respect to the numbers of polymer/DNA layers (referred to hereafter as ‘bilayers’) deposited. Inspection of these data reveals that the optical thicknesses of these films increased in a manner that was linear after the deposition of the first 2 bilayers to yield films with reasonably similar thicknesses (~80 nm for films fabricated using polymer **1** and ~100 nm for films fabricated using polymer **2**) after the deposition of 8 bilayers. The growth profiles of $(\mathbf{1}/\text{pEGFP})_8$ films were similar to those reported in past studies [38].

We next sought to characterize and compare the stabilities of films fabricated from polymers **1** and **2** in physiologically relevant environments. Figure 2A shows a plot of decreases in the optical thicknesses of DNA-containing films fabricated using polymers **1** and **2** (expressed as percentages of original film thickness) as a function of time upon incubation in PBS at 37 °C. Inspection of these data reveals large differences in film erosion profiles that correlate to differences in the structures of these two polymers. The thickness of the film fabricated from polymer **1** (Figure 2A, closed squares) did not decrease significantly over the first ~15 days, and then decreased gradually up to ~30 days. This general behavior (observation of a lag phase followed by a period of slow film erosion) is similar to the behavior of polymer **1**/

DNA films reported previously (albeit over a shorter overall time scale in this current study) [38]. In contrast, films fabricated from polymer **2** (Figure 2A, closed diamonds) decreased in thickness rapidly, and almost completely, within the first three days of incubation in PBS. These results demonstrate that the rates of erosion of these polymer/DNA films are influenced significantly by differences in the chemical structures of polymers **1** and **2**.

During the film erosion experiments described above, we also characterized the surface-mediated release of plasmid DNA into solution. Figure 2B shows a plot of increases in solution absorbance (measured at 260 nm, the absorbance maximum of DNA) as a function of time for films fabricated using polymer **1** or polymer **2** (closed squares and closed diamonds, respectively). The release of DNA from the polymer **1**/DNA film exhibited a lag phase over the first ~15 days, followed by the gradual release of DNA for up to ~30 days. However, the release of DNA from the polymer **2**/DNA film was complete over a period of ~3 days. These differences in DNA release profiles correlate well with the differences in rates of film erosion shown in Figure 2A.

We interpret the large differences in film behavior and release rates shown in Figure 2 to arise chiefly from the large differences in rates of ester side chain hydrolysis (and, as a result, the 'charge-shifting' natures) of polymers **1** and **2**. For example, polymer **2**, which hydrolyzes more rapidly than polymer **1** in solution, would be expected to undergo a shift in net charge more rapidly, and, as a result, lead to more rapid changes in charge/charge interactions within a multilayered film. To probe further the nature of the disassembly processes, and to establish a link between side chain ester hydrolysis and film erosion more clearly, we conducted an additional series of experiments using films fabricated from DNA and polymers **3** and **4**. Polymers **3** and **4** are identical in structure to polymers **1** and **2**, with the exception that the side chain functionality of these polymers is linked to the polymer backbone through an amide/amide linker rather than an amide/ester linker. Polymers **3** and **4** were synthesized by the ring-opening addition of the primary amine groups of 3-dimethylamino-1-propylamine and N,N-dimethylethylenediamine, respectively, to PVDMA having a molecular weight and PDI identical to that used to synthesize polymers **1** and **2**, using methods similar to those reported in our past studies [38]. Films fabricated from polymer **3** did not decrease in thickness or release measurable amounts of DNA into solution for periods up to 30 days [38]. Films fabricated from polymer **4** were also observed to be stable, and did not decrease in thickness or release measurable amounts of DNA for at least 3 days (see Figure S2, Supporting Information). Because the amide/amide functionality of polymers **3** and **4** should not hydrolyze readily under the conditions used in this study (pH = 7.4, 37 °C), the results of these additional experiments provide support for the view that the erosion and release of DNA from films fabricated using polymers **1** and **2** are dictated largely by differences in the rates of hydrolysis of the side chains of these two polymers, and not by other factors (e.g., changes in ionic strength, etc.) arising from the incubation and handling of these materials.

Characterization of Multilayered Films Fabricated from DNA and Blends of Polymers **1** and **2**

The results described above demonstrate that it is possible to design ‘charge-shifting’ polymers that can be used to fabricate multilayered films that erode and release DNA over either short (e.g., 3-day) or extended (e.g., 30-day) periods. We next sought to develop approaches that could be used to expand the range of release profiles that are accessible using these materials (for example, to design films with erosion profiles that are intermediate to these two extremes, or to develop approaches that could be used to exert tunable control over the release of DNA over this range). One approach to the design of such films would be to synthesize new ‘charge-shifting’ polymers with ester-hydrolysis rates that are intermediate to those of polymers **1** and **2**. Although this approach is technically feasible in view of the modular design of our synthetic approach described above (Scheme 1), we report here a different approach to the design of films fabricated using mixtures and blends of polymers **1** and **2**.

Several past studies have reported on the layer-by-layer assembly of multilayered films using solutions comprised of blends of structurally different polyelectrolytes [49–52]. For example, Debreczeny *et al.* demonstrated that varying the compositions of dipping solutions of two different polyanions [poly(L-glutamic acid) (PGA) and poly(L-aspartic acid) (PAA)] could be used to control the relative amounts of PGA and PAA incorporated into films fabricated using poly(L-lysine) as a cationic component [49]. Caruso and coworkers have also demonstrated approaches to the design of polyelectrolyte multilayers using dipping solutions comprised of mixtures of either sodium (polystyrene sulfonate) (SPS) and poly(acrylic acid) [50] or SPS and single-stranded DNA [51]. We also reported recently on the fabrication and characterization of multilayered films fabricated using SPS and solutions composed of defined mixtures of two different but structurally related degradable polyamines [52]. The results of these past studies demonstrate that the use of solutions containing blends of polyelectrolytes can be used to (i) control the relative compositions of different polyelectrolytes incorporated into a PEM [49–52] and, to varying extents, (ii) provide tunable control over the resulting materials properties (such as rates of film erosion and the release of incorporated polyanions [52]). One additional practical advantage of this blend-based approach is that it can be used to expand the usefulness of a limited pool of polyelectrolytes, and reduce or eliminate the need to design and synthesize new polymers with new properties for any given new application or desired film property.

We conducted a series of experiments to determine whether control over the compositions of solution blends of polymers **1** and **2** could be exploited to tune the erosion and release profiles of DNA-containing films. To investigate a range of different solution compositions, we initially prepared two different polymer solutions comprised of 25 mol% and 50 mol% polymer **1** (with the remainder comprised of polymer **2**; solutions were 5 mM with respect to total polymer repeat units). For films fabricated from mixtures of polymer **1** and **2**, additional subscripts are used to denote the mole percentage of each polyamine in the solutions used to fabricate the films. For example, a film 8 bilayers thick fabricated from a 50 mol% mixture of polymers **1** and **2** is denoted as $(1_{50}2_{50}/\text{pEGFP})_8$. As considered in further detail below, this notation is used to refer to the compositions of the solutions used to fabricate these films and is not meant to imply the relative amounts of polymers **1** or **2** that may actually be incorporated into a film during fabrication.

We fabricated four polymer/DNA films 8 bilayers thick using polymer solutions comprised of either polymer **1** exclusively, polymer **2** exclusively, or solutions comprised of defined 25/75 or 50/50 mixtures of polymer **1** and **2**, respectively. Characterization of the erosion and release profiles of these four films revealed that the behaviors of films fabricated using blends of polymers **1** and **2** at these two compositions were almost identical to those

observed for films fabricated using polymer **2** exclusively (e.g., release of DNA over ~3 days; see Figure S3, Supporting Information). The specific reasons for this behavior are not clear. However, one possible explanation for these results is that more rapidly eroding polymer **2** could adsorb or be deposited preferentially from solution during film fabrication (thus resulting in differences between the polymer compositions in the films and the compositions of the polymer solutions used to fabricate the films). We note in this context, however, that, given the chemically similar structures of polymers **1** and **2**, the driving force for such preferential deposition is not readily apparent. We note further that because of the similar structures of these polymers, it was unfortunately not possible to determine experimentally the relative amounts of each incorporated during assembly using spectroscopic methods (e.g., FTIR, etc.).

Another possible explanation for the behavior described above is that both polymers **1** and **2** are incorporated into these films at ratios that are similar to the compositions of the solution mixtures, but that the disruption of electrostatic interactions within the films (which is required for the release of DNA) is dominated by the behavior of the more rapidly-hydrolyzed polymer (polymer **2**). To investigate this possibility further, we fabricated two additional sets of (X/pEGFP)₈ films using solutions consisting of mixtures of polymers **1** and **2** with mole percentages of polymer **2** that were significantly lower than those used in the experiments described above (e.g., either 10 mol% or 25 mol%). DNA-containing films fabricated using these solution mixtures increased in thickness linearly and exhibited thicknesses ranging from ~80 to ~120 nm. In contrast to the results above, incubation of these films in PBS revealed large differences in erosion and release profiles. As shown in Figure 2, films fabricated using these solution blends (open circles and closed triangles) exhibited erosion profiles that were intermediate to those fabricated using either polymer **1** or polymer **2** exclusively. Closer inspection of these data reveals that erosion and release profiles were also weighted with respect to the differences in the relative amounts of polymer **2** in the solution mixtures used to fabricate the films. For example, the (1₉₀2₁₀/pEGFP)₈ film, which was fabricated using a solution having a lower mol% of polymer **2**, released DNA more slowly than the (1₇₅2₂₅/pEGFP)₈ film, which was fabricated from a solution comprised of a higher mol% of polymer **2**.

The data in parts A and B of Figure 2, when combined, demonstrate (i) that polymers **1** and **2** can be used to fabricate ultrathin films that release DNA over a period ranging from approximately three days to one month, and (ii) that solution mixtures composed of defined mixtures of these two ‘charge-shifting’ polymers can be used to tune the structures of these films and provide access to intermediate release profiles. As noted above, the modular nature of the synthetic approach used to design polymers **1** and **2** could also be used to synthesize other ‘charge-shifting’ polymers with properties that could also be used to modify or tune further the properties of these films. However, our results demonstrate that methods based on the use of polymer blends can expand the usefulness of a relatively limited pool of polymers (in this case, two polymers) and thereby reduce the need to synthesize new polymers to achieve new surface-mediated DNA release profiles.

Fabrication of Multilayered Films that Permit the Sequential Release of Two DNA Constructs

The layer-by-layer approach to film assembly used here can provide control over the relative order in which multiple different agents can be incorporated into the structure of a PEM (for example, by control over the order with which individual ‘layers’ of different agents are deposited) [17,20,22]. PEMs that have reasonably “stratified” internal architectures can thus be designed to provide control over the sequences and time scales with which multiple different agents are released [30,32,39,53,54]. Past studies by our group [30,39] and others [32] have demonstrated that it is possible to use layer-by-layer assembly to fabricate DNA-

containing multilayered films with hierarchical structures can be designed to provide control over the relative orders and time scales with which two different DNA constructs are released from surfaces (that is, to promote the sequential, rather than simultaneous, release of two or more DNA constructs) [30,32,39]. For example, Jessel *et al.* reported the design of DNA-containing films that could be used to control the sequence with which two different DNA constructs were expressed by attached cells (over a period on the order of several hours) [32]. In addition, our group recently reported an approach to the fabrication of PEMs that provide control over the release of two different plasmid DNA constructs into solution with separate and distinct release profiles [39]. This past study made use of films fabricated from two ester-functionalized 'charge-shifting' polymers and reported on films designed to release one DNA construct rapidly (e.g., within one hour), followed by the sustained release of a second DNA construct over a period of several days. We note here that this past study made use of a class of 'charge-shifting' polymers that differs significantly and fundamentally in both structure and function from those used in this current study [39], and that the results of this past study were limited to the design of films that promoted sequential release over relatively short time periods [39]. In contrast to the polymers used in this past study, the relative difference in the time scales of erosion and release of DNA for films fabricated from polymers **1** and **2** is significantly larger. We sought to determine whether this large difference could be exploited to fabricate ultrathin films that provide control over the sequential release of two DNA constructs over significantly longer time periods.

To explore the feasibility of this approach, we fabricated films using the pEGFP-N1 plasmid (as described above) and a second plasmid DNA construct (pDsRed-N1) encoding red fluorescent protein (RFP). Films used in the experiments described below were fabricated to have hierarchical structures composed of (i) four bottommost layers comprised of pEGFP and polymer **1** (which released DNA slowly in the above experiments) and (ii) four topmost layers comprised of pDsRed and polymer **2** (which released DNA rapidly in the experiments described above). Two bilayers composed of polymer **1** and SPS were also deposited as intermediate layers between the DNA-containing layers of these films (e.g., see schematic illustration in Figure 5). Figure 3 shows a plot of optical film thickness with respect to the number of bilayers deposited for a film having the structure $(1/\text{pEGFP})_4(1/\text{SPS})_2(2/\text{pDsRed})_4$. Inspection of these data reveals the optical thickness of the resulting film to be ~280 nm thick.

Figure 4 shows a plot of solution absorbance (at 260 nm, the absorbance maximum of DNA) as a function of time for a film incubated in PBS at 37 °C. These data demonstrate that DNA was released rapidly over a period of the first ~3 days, followed by a second, more gradual stage of release that extended over a period of ~70 days. The initial increase in DNA concentration is consistent with the rapid release of DNA from films fabricated from polymer **2** (e.g., see Figure 2); the subsequent gradual release phase is also consistent with the slower, extended release of DNA from films fabricated using polymer **1**. This overall release profile is thus, in general, consistent with a two-stage, sequential release of these two plasmids from these more complex films. We note, however, that it is not possible to distinguish between the individual release profiles of pEGFP and pDsRed on the basis of measurements of solution absorbance. We performed additional experiments with films fabricated using plasmid constructs labeled with two different fluorescent dyes (e.g., as described in our past studies) [30,39] in an attempt to characterize the individual release profiles for these two different constructs. Unfortunately, the extended times required by these experiments resulted in significant losses of fluorescence intensities and prevented the extraction of meaningful results from these experiments. The results of characterization of the physical erosion profiles of films that provide additional support for two-stage release are included as Figure S4 of the Supporting Information.

We conducted a final set of experiments using films having the structure (1/pEGFP)₄(1/SPS)₂(2/pDsRed)₄ to (i) characterize the structural and functional integrity of the released DNA and (ii) determine whether the two-stage release profiles suggested by the data in Figure 4 could be further confirmed by the observation of differences in the expression profiles of EGFP and RFP in cells treated with solutions of released DNA. Figure 5 shows a series of representative fluorescence micrographs of COS-7 cells 48 hours after treatment with samples of plasmid DNA collected at various time points during the erosion of these films (see Materials and Methods for a complete description of cell transfection experiments) [30,39]. Inspection of data in the left column of Figure 5A (red fluorescence channel) reveals no significant red fluorescence in cells treated with samples of DNA collected after the first 21 hours of incubation. We observed significant levels of RFP expression in cells treated with samples of DNA collected at subsequent time points up to 69 hours (Figures 5B and C). Further inspection of these data (e.g., Figures 5D–H; red channel) reveals very little red fluorescence in cells treated with DNA collected at longer time periods (e.g., from 69 hours up to 75 days). These results are consistent with a film erosion and release profile in which the pDsRed plasmid, which was deposited last to form the topmost layers of the film, is released rapidly and completely after ~3 days of incubation. These data also demonstrate that the pDsRed plasmid that is released remains transcriptionally active.

The right column of Figures 5A–H (green fluorescence channel) show images of the same cells shown in the left column (red channel). Inspection of these data reveals very low levels of EGFP expression in cells treated with samples of DNA collected during the first ~3 days of incubation, followed by a significant and sustained increase in EGFP expression for samples collected at time points ranging from ~3 days up to ~75 days. [Note: The images shown in Figure 5 show eight different representative time points selected, for the sake of clarity of presentation, from a larger set of 29 total time points characterized over the extended 75-day course of this experiment. Figure S5 (parts A, B, C, and D) of the Supporting Information presents a more comprehensive view of the full set of data arising from this extended experiment.]

The data in Figure 5, when combined, demonstrate that polymers **1** and **2** can be used to design films that release two different DNA constructs with separate and mutually exclusive release profiles. The results described above cannot be used to establish directly the internal physical structures of these materials. However, the results of physical characterization and the temporal differences in the levels of expression of EGFP and RFP shown in Figure 5 are consistent with a hierarchical structure in which the plasmid construct deposited first comprises the bottommost layers of the film, and the plasmid construct deposited last comprises the topmost layers of the film. The extended time frames over which the two-stage release of DNA can be achieved using polymers **1** and **2** is significantly greater than the two-stage DNA release profiles exhibited by multilayered films reported in past studies [30,32,39]. As such, these materials could prove useful in applications or contexts that require longer release times and/or greater differentiation in the rates of release of DNA and/or other agents or factors.

Summary and Conclusions

We have reported an approach to the design of ultrathin, polymer-based films that erode in physiologically relevant environments and provide tunable control over the surface-mediated release of DNA. We reported on the synthesis and characterization of a new ‘charge-shifting’ polymer (polymer **2**), a derivative of polymer **1** that contains a shorter linker between the tertiary amine side chain functionality and the amide/ester linkage to the polymer backbone. Our results demonstrate that this small change in side chain structure leads to large changes in the rates of side chain hydrolysis, and that these changes in rates of

hydrolysis can be used to fabricate DNA-containing polyelectrolyte multilayers that release DNA relatively rapidly (e.g., over ~3 days, for films fabricated using polymer **2**) or more slowly (e.g., over ~1 month, for films fabricated using polymer **1**). We also demonstrated that these large differences in film erosion and release profiles can be exploited further to design films that provide more sophisticated temporal control over the release of DNA in two different contexts. First, our results demonstrate that solution blends of polymers **1** and **2** can be used to design films that provide tunable control over the release of a single DNA construct from surfaces (e.g., with release profiles that are intermediate to those exhibited by films fabricated using either polymer **1** or polymer **2** exclusively). Second, our results demonstrate that differences in the properties of polymers **1** and **2** can be exploited to fabricate PEMs that promote the sequential release of two different DNA constructs with separate and distinct release profiles (e.g., the rapid release of a first DNA construct followed by the slower, more sustained release of a second DNA construct).

The results of this study provide alternatives to the fabrication of DNA-containing films fabricated using hydrolytically degradable polyelectrolytes and extend the range and scope of functionalities that can be designed into PEMs using these or other 'charge-shifting' cationic polymers reported in past studies. With continued development, this approach could contribute to the design of functional thin films and surface coatings that provide sophisticated control over the timing, the order, and the relative amounts of DNA or other therapeutically relevant agents of interest in a broad range of fundamental and applied contexts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Plot of film thickness v. the number of polymer/DNA bilayers deposited for films fabricated from plasmid DNA and polymer solutions comprised of polymer **1** or **2**, or mixtures of polymers **1** and **2** on planar silicon substrates. Subscripts, where given, denote the mol% of each polymer in the solution mixtures used to fabricate each film (see text).

Figure 2.

(A) Plot of percent film remaining v. time for polymer/DNA films fabricated from polymer **1** (closed squares), polymer **2** (closed diamonds), or mixtures of polymers **1** and **2** (closed triangles and open circles) incubated in PBS (pH = 7.4) at 37 °C. Subscripts, where given, denote the mole percentage of each polymer in the solutions used to fabricate each film. Percent film remaining was determined based on changes in optical film thickness measured using ellipsometry. (B) Plot of percent of DNA released into solution v. time for films characterized in Figure 2A, determined using measurements of UV absorbance (at 260 nm) of the PBS solution used to incubate these films. Error bars are shown but are in some cases smaller than the symbols used to represent the data.

Figure 3.

Plot of ellipsometric thickness v. the number of bilayers for a film having the structure $(\mathbf{1}/\text{pEGFP})_4(\mathbf{1}/\text{SPS})_2(\mathbf{2}/\text{pDsRed})_4$ deposited on a planar silicon substrate.

Figure 4.

Plot of solution absorbance (at 260 nm) v. time showing the release of DNA from films having structure $(\mathbf{1}/\text{pEGFP})_4(\mathbf{1}/\text{SPS})_2(\mathbf{2}/\text{pDsRed})_4$ incubated in PBS at 37 °C.

Figure 5.

Representative fluorescence microscopy images showing relative levels of EGFP (green channel) and RFP (red channel) expressed in COS-7 cells. Cells were transfected using samples of DNA released from a film having the structure $(1/pEGFP)_4(1/SPS)_2(2/pDsRed)_4$; cells were transfected by combining released DNA with Lipofectamine 2000 (transfection agent, see text). The presence, absence, or relative levels of EGFP and RFP observed correspond qualitatively to relative levels of each plasmid released and collected over each of the following time periods: A) 7–21 h, B) 43–55 h, C) 55–69 h, D) 69–77 h, E) 77–91 h, F) 7.7–8.1 d, G) 17.8–27.8 d, H) 53.8–60.8 d. A full set of data including over 20 additional time points can be found in the Supporting Information.

Scheme 1.

Synthesis and hydrolysis of 'charge-shifting' cationic polymers **1** and **2**.