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Polymer Multilayers that Promote the Rapid Release and Contact Transfer of DNA

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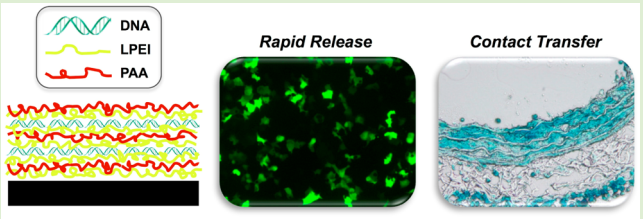
ABSTRACT: We report a layer-by-layer approach to the fabrication of thin polymer-based multilayers that release DNA rapidly in physiologically relevant environments. This approach exploits the properties of a weak anionic polyelectrolyte [poly(acrylic acid); PAA] to disrupt ionic interactions and promote disassembly in coatings that otherwise erode slowly. We investigated this approach using multilayers fabricated from plasmid DNA and linear poly(ethylenimine) (LPEI), a model synthetic cationic polymer used widely for DNA delivery. LPEI/DNA multilayers erode and release DNA slowly over ∼4 days when incubated in PBS buffer. In contrast, substitution of every other layer of DNA with PAA lead to thin films that released DNA rapidly, with >60% being released in the first 5 min. These quick-release coatings release bioactive DNA and can be used to fabricate uniform coatings on a variety of objects, including the tips of inflatable balloon catheters. We demonstrate that these coatings can promote high levels of cell transfection in vitro and the robust contact transfer and expression of DNA in vascular tissue in vivo using a rat model of vascular injury. These materials provide useful alternatives to multilayers and other coatings that promote the prolonged release of DNA. More broadly, approaches that depart from the use of degradable polymers to promote film erosion create opportunities to design new gene delivery coatings using a broader range of polymer-based building blocks designed for other gene delivery applications. With further development, this approach could thus provide a new and useful platform for the rapid contact transfer of DNA to cells and tissues of interest in a range of fundamental and applied contexts.



INTRODUCTION

Thin films, coatings, and matrices that provide control over the release of DNA from surfaces have the potential to serve as platforms for the local delivery of DNA in therapeutic contexts and are also useful as tools for basic biomedical research. Many different approaches, including (i) the encapsulation of DNA or polymer/DNA complexes (polyplexes) into bulk polymer[1−3] and (ii) the physical adsorption or tethering of DNA or polyplexes onto surfaces,[4−9] have been developed for this purpose. These methods can provide spatial and temporal control over the release, delivery, and expression of DNA in vitro and in vivo, but they often provide limited control over the extent to which these or other important parameters and properties can be manipulated or tuned to meet the needs of specific applications.

Our group and others have pursued alternative approaches to the surface-mediated delivery of DNA by exploiting "layer-by-layer" methods of assembly[10−13] that permit the incorporation of plasmid DNA into ultrathin polymer-based "multilayers".[14] These DNA-containing multilayers are fabricated by the alternating deposition of DNA with thin layers of cationic polymers and are, therefore, comprised of intimate mixtures of DNA and cationic polymer agents that can promote the internalization and processing of DNA by cells. This approach also offers other practical advantages when compared with methods mentioned above, including (i) the ability to precisely tune the loading of DNA by changing the number of DNA layers deposited,[15,16] (ii) precise control over the amounts and locations of different DNA constructs by sequential deposition of multiple different plasmids,[17−22] and (iii) the ability to faithfully and conformally coat the surfaces of topologically complex substrates, including interventional medical devices[23,24] with ultrathin and mechanically compliant coatings.

One challenge confronting the design of DNA-containing multilayers for potential gene delivery applications lies in designing assemblies that undergo physical erosion and release their DNA on appropriate time scales (e.g., to achieve sustained release, rapid release, sequential release, or exert tunable temporal control). Many different types of multilayers have been developed to address these needs, with most approaches placing an emphasis on the incorporation of degradable groups (e.g., hydrolytically or reductively degradable groups) that can be used to disrupt ionic interactions, promote film disassembly, and enable DNA release.[14,25] These approaches and other

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biomedical applications of degradable and DNA-containing multilayers have been reviewed recently.[14,23−28] Many of these past studies have focused on the design of coatings that promote the gradual and continuous release of DNA for prolonged periods. The work reported here was motivated by the potential clinical utility of conformal multilayer coatings that promote the rapid release or rapid transfer of DNA in contexts that are inherently time-limited, such as in vascular interventions[29,30] or where short-duration transfer is otherwise useful or desirable.[31]

Here, we report DNA-containing multilayers that release DNA rapidly upon exposure to physiologically relevant media. This approach builds from recent work in our group demonstrating that depositing layers of the weak polyelectrolyte poly(acrylic acid) (PAA; a biocompatible polyanion; Figure 1A) during assembly can be used to design DNA-

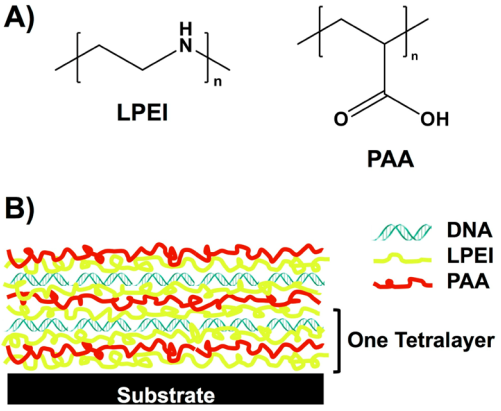

Figure 1. (A) Chemical structures of linear poly(ethylenimine) (LPEI) and poly(acrylic acid) (PAA). (B) Schematic illustration showing the repeating "tetralayer" structure of the (LPEI/PAA/LPEI/DNA)$_x$ multilayer films investigated in this study. Films were fabricated either directly on bare, untreated substrates (shown) or, in some cases, on substrates pre-coated with a thin polyelectrolyte multilayer base layer (not shown; see text).

containing multilayers that disassemble rapidly at physiological pH (through a mechanism that involves increases in the ionization of the PAA layers and an increase in net anionic charge upon exposure to physiological pH; this change in ionization results in changes in ionic interactions within the films that can promote more rapid film disassembly).[32] In that study, we used films fabricated from DNA, PAA, and a hydrolytically degradable cationic poly(β-amino ester) (PBAE) to demonstrate proof of concept. Those PAA-containing coatings were found to release DNA up to 24× faster than films designed using DNA and degradable PBAEs alone (over ∼3 h as compared to ∼3 days).[32] We note, however, that exploiting changes in the ionization of a weak polyelectrolyte to drive film disruption, rather than relying on the incorporation of a degradable polymer, also creates opportunities to integrate a broader range of functional polymers into these assemblies, including many non-degradable cationic polymers and other anionic weak polyelectrolytes commonly used to promote DNA delivery, into "quick-release" coatings that could promote more rapid and efficient cell transfection.

As a step toward these goals, we sought to characterize the ability of this "weak polyelectrolyte approach" to destabilize multilayers formed from DNA and linear poly(ethylenimine) (LPEI), a model non-degradable polyamine used widely as a basis for the design of DNA delivery systems (Figure 1A)[33,34] and investigated as a potential film component in preliminary experiments described in our past study on degradable polymers.[32] We report here that multilayers fabricated from alternating layers of plasmid DNA, PAA, and LPEI erode rapidly upon exposure to physiological media, and that substrates coated with these quick-release coatings can be used to promote the efficient surface-mediated transfection of cells in vitro. With a view to developing new device/coating combinations that can promote the rapid transfer of DNA from the surfaces of interventional devices, we also investigated the ability of these coatings to promote the contact-transfer of DNA to vascular tissue in vivo using film-coated inflatable balloon catheters. Our results reveal this approach to promote the robust transfer and local expression of plasmid DNA encoding the reporter gene β-galactosidase (β-gal) in a rat model of carotid artery injury.

MATERIALS AND METHODS

Materials. Linear poly(ethylene imine) (LPEI, MW = 25000) and poly(acrylic acid) (PAA, MW = 90000) were purchased from Polysciences Inc. (Warrington, PA). Sodium poly(styrenesulfonate) (SPS, MW = 70000) was obtained from Aldrich (Milwaukee, WI). Plasmid DNA encoding enhanced green fluorescent protein [pEGFP-N1 (4.7kb), > 95% supercoiled], β-galactosidase [pCMV-β-gal (7.2 kb), 85% supercoiled], and luciferase (pCMV-Luc; 6.2 kb) were purchased from Elim Biopharmaceuticals, Inc. (San Francisco, CA). Test grade n-type silicon wafers were obtained from Silicon, Inc. (Boise, ID). Stainless steel wire mesh (type 316; 12 in. × 12 in.; wire diameter = 0.0036 in.; width of opening = 0.0046 in.) was obtained from MSC Industrial Supply Co. (Melville, NY). Fogarty arterial embolectomy catheters (2-French diameter) were purchased from Edwards Lifesciences, LLC (Irvine, CA). For experiments requiring fluorescently labeled DNA, a tetramethylrhodamine (TMR) Label-IT nucleic acid kit was purchased from Mirus Bio Corporation (Madison, WI) and used according to the manufacturer's instructions. X-Gal staining kits were purchased from Genlantis (San Diego, CA). Solutions of sodium acetate buffer (VWR, West Chester, PA) and phosphate-buffered saline (PBS; EM Science, Gibbstown, NJ) were prepared by diluting commercially available concentrate. Sodium acetate buffer (100 mM, pH = 4.9) was used for all rinsing steps during film fabrication and the preparation of polymer and DNA solutions. Dulbecco's modified Eagle medium (DMEM) culture medium used in in vitro cell culture experiments was purchased from Invitrogen (Carlsbad, CA). COS-7 cells used for in vitro transfection experiments were purchased from the American Type Culture Collection (ATCC, Manassas, VA). All materials were used as received unless otherwise noted. Solutions of LPEI and PAA used to fabricate polymer multilayers were filtered through a 0.2 μm nylon membrane syringe prior to use.

General Considerations. Silicon and mesh substrates (∼3.5 cm × 0.5 cm) were cleaned with acetone, ethanol, methanol, and deionized water for 5 min prior to film fabrication. The optical thicknesses of films fabricated on silicon substrates were determined using a Gaertner LSE Stokes ellipsometer (632.8 nm, incident angle = 70°). Thicknesses were measured in at least five locations and the data were processed using the Gaertner Ellipsometer Measurement Program software package to calculate relative thicknesses by assuming an average refractive index of 1.58 for the multilayered films. Prior to ellipsometric measurements the films were dried with filtered, compressed air using a 0.2 μm membrane syringe filter. The amount of DNA released from the multilayered films during incubation in PBS was quantified by recording UV−vis absorbance values at a wavelength of 260 nm (corresponding to the absorbance maximum of double-stranded DNA) using a DU 520 UV−vis spectrophotometer (Beckman Coulter, Fullerton, CA). Fluorescence microscopy and phase contrast microscopy images were acquired using either an Olympus IX70 fluorescence microscope, Nikon Eclipse E600 micro-

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scope, or a Ti−U Eclipse fluorescence microscope using Metavue 7.1.2.0, cellSens, or Nikon Elements software packages, respectively.

Preparation of Polyelectrolyte Solutions. Solutions of LPEI and PAA (5 mM with respect to the repeat unit molecular weight of the polymer) were prepared using 100 mM sodium acetate buffer (pH 4.9). Solutions of plasmid DNA were prepared at 1 mg/mL in 100 mM acetate buffer (pH 4.9) but were not filtered prior to use. For experiments requiring fluorescently labeled DNA, labeled DNA was added to a solution of unlabeled DNA to give a 20% (w/w) labeled/unlabeled plasmid solution.

Fabrication of Multilayered Films. Films were deposited layer-by-layer on planar silicon substrates, stainless steel mesh substrates, and inflatable embolectomy catheter balloons according to the following protocol: (1) substrates were immersed in a solution of LPEI for 5 min, (2) substrates were removed and immersed in two wash baths of 100 mM acetate buffer for 1 min each, (3) substrates were then immersed in a solution of anionic polymer (either PAA or DNA; as appropriate, see below) for 5 min, and (4) substrates were rinsed again in the manner described above. This cycle was repeated until the desired number of cationic and anionic polymer layers were deposited. This general procedure was used to fabricate films having the following general structure: $[\text{LPEI}/(\text{PAA or DNA})/\text{LPEI}/\text{DNA}]_x$, where "$x$" denotes the number of polymer "tetralayers" deposited. For films fabricated on silicon substrates coated with base layers (see text), thin multilayers composed of 10 bilayers of LPEI and SPS were deposited prior to the assembly of DNA-containing tetralayers using methods described previously.[15,32] For experiments using balloon catheters, film-coated balloons were rinsed with 18 MΩ deionized water, allowed to air-dry following the final rinse step, and then stored in their original packaging in a dry, dark location prior to use. All films were fabricated and stored at ambient room temperature.

Characterization of Film Stability and DNA Release Profiles. Experiments designed to investigate film stability and characterize the release of DNA from multilayered films were performed in the following general manner. Film-coated substrates were submerged in PBS (1 mL, pH = 7.4, 137 mM NaCl) in plastic UV-transparent cuvettes. These samples were incubated at 37 °C and removed at pre-determined intervals for characterization by ellipsometry and UV−vis spectroscopy. For experiments designed to characterize time-dependent changes in film thickness, optical thickness was measured in at least five different pre-determined locations on each substrate. The concentration of DNA released from the films into solution over time was characterized by measuring the UV absorbance of the PBS incubation buffer (at a wavelength of 260 nm, the absorbance maximum of DNA). After each set of measurements, the coated substrates were placed into new cuvettes with fresh aliquots of PBS and returned to the incubator at 37 °C.

Cell Transfection Experiments. COS-7 cells were grown in 24-well plates at an initial seeding density of 100000 cells/mL in 0.5 mL of growth medium (90% Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin). Cells were allowed to grow overnight to approximately 80% confluence, at which point the growth medium was replaced by 0.5 mL of fresh serum-containing growth medium. Stainless steel mesh substrates coated with polymer multilayers were then placed manually into the wells and incubated for 5 h. The meshes were then removed, and growth medium was replaced by 0.5 mL of new serum-containing growth medium. Cells were incubated for an additional 48 h and both fluorescence and phase contrast images were recorded directly using an Olympus IX70 fluorescence microscope. To quantify transfection efficiency, the percentage of cells expressing EGFP was determined by flow cytometry. For these experiments, cells were incubated for 48 h as described above and then washed twice with PBS. Cells were then harvested by treatment with trypsin, collected in microcentrifuge tubes, washed with PBS, and finally resuspended in PBS containing 1% bovine serum albumin (BSA). Cell samples were characterized using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Data were collected for populations of at least 10000 cells and analyzed using WinMDI software (Joe Trotter, Scripps Institute). Results are expressed as the percentage of cells expressing EGFP relative to all cells observed.

General Surgical Procedures. After induction of anesthesia with 2.5% isoflurane, arterial injury was induced in male Sprague−Dawley rats (∼2−3 months old, ∼350 g) by means of carotid balloon angioplasty, as described previously.[29,30,35] Briefly, a longitudinal incision was made in the neck of the rat in order to isolate the left external, internal, and common carotid arteries. To denude the common carotid artery of the endothelial layer, an uncoated angioplasty balloon was inserted into the common carotid artery, inflated to a pressure of 2 atm, and passed three times. Next, a balloon coated with a LPEI/PAA/LPEI/DNA film (either 8 or 32 tetralayers thick, see below) was inserted and inflated until it was observed to expand against the arterial wall (∼2 atm). After a 20 min incubation period, the balloon was deflated and removed from the artery. The external carotid artery was then ligated, blood flow was restored to the common and internal carotid arteries, and the surgical wound was closed layer-to-layer. Animals were sacrificed post-operatively at a predetermined time depending on the time scale of the experiment (see below). All experimental protocols were approved by the Institute Animal Care and Use Committee at University of Wisconsin−Madison (#M002285) and conformed to the Guide for the Care and Use of Laboratory Animals published by the NIH Publication No. 85−23, 1996 revision.

Characterization of in vivo Delivery and Expression of DNA in Rat Carotid Arteries. For fluorescence-based experiments designed to characterize the extent of delivery of DNA to rat arterial tissue immediately following balloon-mediated delivery, the common carotid artery was harvested immediately (i.e., prior to the restoration of blood flow) after treatment with balloons coated with films having the general structure $(\text{LPEI/PAA/LPEI/pCMV-Luc}_{\text{TMR}})_8$. For these experiments, films were fabricated according to protocols outlined above using solutions of DNA containing 20% (w/w) TMR-labeled DNA. Following sacrifice, the common carotid arteries were embedded and frozen in OCT compound and cut into 5 μm sections for analysis by fluorescence microscopy. For experiments designed to characterize β-galactosidase expression, the common carotid artery was treated with balloons coated with films having the general structure $(\text{LPEI/PAA/LPEI/pCMV-}\beta)_{32}$. Balloons coated with films having the general structure $(\text{LPEI/PAA/LPEI/pEGFP})_{32}$ were used as controls. Rats were sacrificed at 3 days and arteries were immediately embedded and frozen in OCT compound and cut into 5 μm sections for X-Gal staining following the manufacturer's instructions. Slides were then characterized using a Nikon Eclipse E800 upright microscope equipped with appropriate filters.

RESULTS

Fabrication of Multilayers Containing DNA, PAA, and LPEI. To fabricate multilayers containing both DNA and PAA (both anionic polyelectrolytes), we adopted a "tetralayer" approach used previously to design films containing layers of DNA, PAA, and a degradable cationic PBAE.[32] This tetralayer approach permits fabrication of films having the general structure $(\text{LPEI/PAA/LPEI/DNA})_x$, where "$x$" denotes the number of repeating tetralayers, by the sequential immersion of substrates into dilute solutions of LPEI, PAA, LPEI again, and then DNA (Figure 1B). We note that this approach also provides a straightforward approach to vary the relative numbers or locations of layers of PAA and DNA deposited in a film (for example, by depositing intermittent tetralayers having the structure (LPEI/PAA/LPEI/PAA) or (LPEI/DNA/LPEI/DNA) during film assembly). For the work reported here, we focused on films having the structure $(\text{LPEI/PAA/LPEI/DNA})_x$, for which the number of layers of PAA and DNA deposited was equal.

In our past study, we reported the behaviors of $(\text{LPEI/PAA/LPEI/DNA})_x$ films fabricated on silicon substrates that were

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first pre-coated with a polyelectrolyte multilayer "base layer" film composed of 10 bilayers of LPEI and the synthetic anionic polymer sodium polystyrenesulfonate (SPS).[32] This thin base layer film [denoted $(LPEI/SPS)_{10}$; ~25 nm thick] was used to permit comparison to the behaviors of hydrolytically degradable $(PBAE/PAA/PBAE/DNA)_x$ films that were the primary focus of that past study. We note here that while DNA-containing multilayers fabricated using PBAEs cannot generally be fabricated on surfaces that are not pre-treated in this way,[15] the presence of these base layers adds substantial complexity to these assemblies and they have been found, in some cases, to participate in, and perhaps promote, the evolution of nanoscale rearrangements that can impact the behaviors of these materials and complicate physicochemical characterization.[36] With the potential for this additional complexity in mind, we performed a first series of experiments to characterize $(LPEI/PAA/LPEI/DNA)_x$ films fabricated on planar silicon substrates (i) with and (ii) without these LPEI/SPS base layers to determine whether they were required to promote robust and uniform film growth in this LPEI/DNA/PAA system.

Figure 2 shows a plot of optical film thickness versus the number of polymer tetralayers deposited for films having the structure $(LPEI/PAA/LPEI/pEGFP)_8$ (closed circles; fabricated with PAA layers) or $(LPEI/pEGFP/LPEI/pEGFP)_8$ (open circles; fabricated without PAA layers) on LPEI/SPS base layers 25 nm thick. Inspection of these data reveals both films to exhibit similar and approximately linear growth profiles, and that both films reach thicknesses of ~300 to 350 nm after the deposition of 8 tetralayers (or 32 individual polymer layers). These results demonstrate that substitution of eight layers of DNA with eight layers of PAA does not change film growth profiles or overall film thickness substantially. For comparison, Figure 2 also shows growth profiles for $(LPEI/PAA/LPEI/pEGFP)_8$ films (closed squares) and $(LPEI/pEGFP/LPEI/pEGFP)_8$ films (open squares) fabricated on bare silicon substrates (no base layers). These films increased in thickness more slowly than films fabricated on base layers for the first 2–3 tetralayers and then grew in a manner that was approximately linear to yield films with overall thicknesses of ~200 to 250 nm after the deposition of 8 tetralayers. These results again suggest that "swapping" every other layer of DNA with PAA does not have a substantial impact on overall film growth. These results also demonstrate that LPEI/SPS base layers are not required to promote robust film growth.

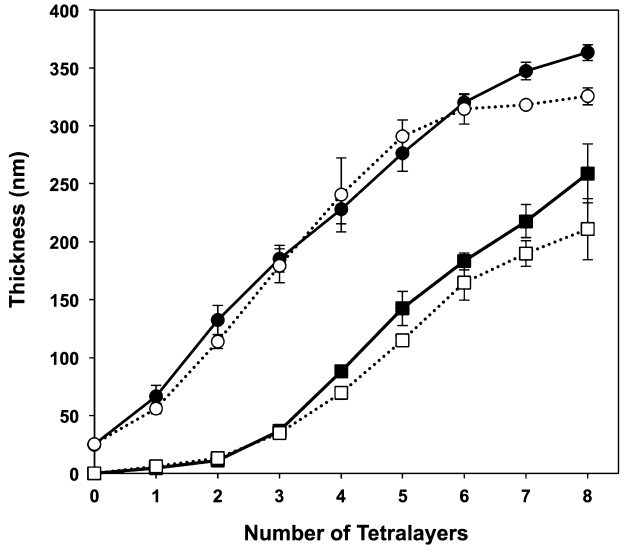

Figure 2. Plot of film thickness as a function of the number of tetralayers deposited for films fabricated on silicon substrates. Symbols correspond to average optical thickness values (with standard deviations) measured during the fabrication of three different films having the structure $(LPEI/DNA/LPEI/DNA)_8$ (□), $(LPEI/PAA/LPEI/DNA)_8$ (■), $(LPEI/SPS)_{10}(LPEI/DNA/LPEI/DNA)_8$ (on base layers; ○), and $(LPEI/SPS)_{10}(LPEI/PAA/LPEI/DNA)_8$ (on base layers; ●). Measurements of film thickness were made in at least five different locations on each film. LPEI/SPS base layers, where used in these experiments, were ~25 nm thick (see text).

Characterization of Film Stability and the Release of DNA. To characterize the impact of PAA and the influence of base layers on the stability and DNA release profiles of these PAA-containing films in physiologically relevant media, we incubated silicon substrates coated with $(LPEI/PAA/LPEI/pEGFP)_8$ and $(LPEI/pEGFP/LPEI/pEGFP)_8$ films (fabricated with or without base layers) in PBS at 37 °C. Figure 3A shows a plot of the amount of DNA released into solution, normalized to the total amount of DNA released from each film, as a function of time. Inspection of these data reveals $(LPEI/pEGFP/LPEI/pEGFP)_8$ films (open symbols) to release DNA into solution gradually over a period of 4–5 days and that the presence of base layers does not influence release profiles significantly (compare open circles and open squares). In contrast, films containing PAA released DNA much more rapidly (with >60% of DNA released in the first 5 min and the remainder being released over the following ~24 h; Figure 3A, closed symbols). This characteristic release profile, consisting of a period of rapid release followed by a short period during which the remaining DNA was released more slowly, differed substantially from that of films that did not contain PAA (open symbols) and was unaffected by the presence or absence of base layers applied prior to fabrication (compare closed circles and closed squares).

The influence of film architecture and the presence of base layers on the total amount of DNA released from these films is shown in Figure 3B. In general, films fabricated with layers of PAA released less DNA than films fabricated without PAA (for example, for films fabricated on base layers, $(LPEI/PAA/LPEI/pEGFP)_8$ films released 14.10 ± 2.01 $\mu g/cm^2$ of DNA, while $(LPEI/pEGFP/LPEI/pEGFP)_8$ films, which contained eight additional layers of DNA, released 25.55 ± 2.47 $\mu g/cm^2$ of DNA). The presence of base layers had a measurable impact on the amount of DNA released from $(LPEI/pEGFP/LPEI/pEGFP)_8$ films (Figure 3B, right), but the amount of DNA released from PAA-containing films did not vary significantly regardless of the presence or absence of base layers (Figure 3B, left).

PAA-Containing Rapid-Release Coatings Promote Surface-Mediated Transfection in vitro. We performed a series of experiments to characterize the ability of objects coated with the rapid-release PAA-containing films characterized above to promote the transfection of mammalian cells in vitro. In these and all other studies described below, we used films fabricated in the absence of base layers (see Discussion). For these experiments, films were fabricated on the surfaces of stainless steel wire mesh substrates suitable for direct placement into cultures without harming cells.[37] Film-coated substrates were placed in cultures of COS-7 cells growing in serum-containing medium for 5 h and then removed; cells were incubated for another 2 days before characterization of EGFP

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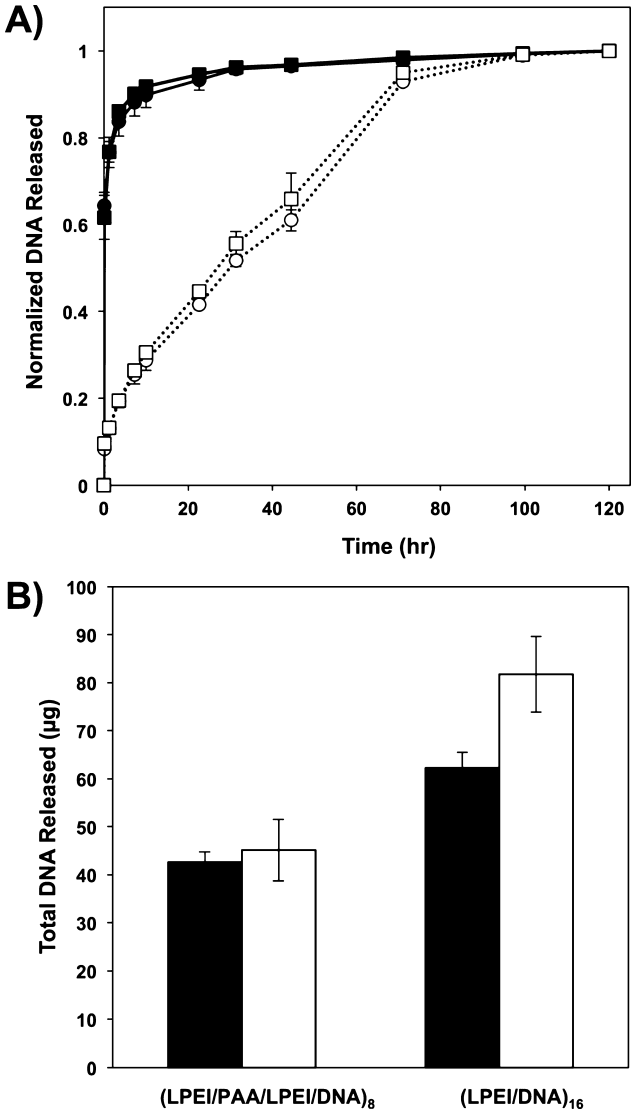

Figure 3. (A) Plot showing DNA release profiles for substrates coated with films having the structure $(LPEI/DNA/LPEI/DNA)_8$ (□), $(LPEI/PAA/LPEI/DNA)_8$ (■), $(LPEI/SPS)_{10}(LPEI/DNA/LPEI/DNA)_8$ (on base layers; ○), and $(LPEI/SPS)_{10}(LPEI/PAA/LPEI/DNA)_8$ (on base layers; ●) upon incubation in PBS buffer (pH 7.4) at 37 °C. Measurements were made in triplicate; data represent the average values with standard deviation. (B) Comparisons of the total amount of DNA released from the films in (A) deposited on bare silicon substrates (black bars) or on substrates pre-coated with LPEI/SPS base layers (white bars).

expression using fluorescence microscopy and flow cytometry (see Materials and Methods for additional details of these experiments).

Figure 4A,B shows representative fluorescence and phase contrast micrographs of COS-7 cells treated with a mesh substrate coated with a $(LPEI/PAA/LPEI/pEGFP)_8$ film. The image in Figure 4A shows high levels of green EGFP-associated fluorescence, demonstrating that DNA is released from these films in a form that is transcriptionally active and that these rapid release coatings can promote significant levels of transgene expression in vitro without the use of additional transfection agents (at levels of ~30%; vide infra). Figure 4C,D shows the results of a similar experiment using mesh substrates coated with PAA-containing films 16 tetralayers thick $[(LPEI/PAA/LPEI/pEGFP)_{16}]$. These thicker films, which contained twice as many layers of DNA as the eight-tetralayer films used in Figure 4A,B, promoted higher levels of transfection (~50% of cells transfected; vide infra). Expression of EGFP was not confined or localized to cells in direct contact with (or growing in the immediate vicinity of) the film-coated substrates, as observed in past studies on the substrate-mediated transfection of cells using glass slides coated with hydrolytically degradable multilayers.[17] Rather, we observed the expression of EGFP to be well distributed across the culture well (Figure 4E), consistent with the rapid release and dissemination of DNA from these substantially more porous and open mesh substrates.[16,37]

To quantify the relationship between the number of DNA layers deposited and levels of transfection promoted by film-coated mesh substrates, we performed a series of otherwise identical in vitro experiments using mesh substrates coated with $(LPEI/PAA/LPEI/pEGFP)_x$ films 0, 2, 4, 8, and 16 tetralayers thick and used flow cytometry to quantify differences in transgene expression in larger populations of cells. Figure 5 shows a plot of the average percentages of cells expressing EGFP relative to the total number of cells as a function of the number of LPEI/PAA/LPEI/pEGFP tetralayers deposited. These results reveal a correlation between levels of transfection and the number of tetralayers, demonstrating that transfection efficiency can be modulated or tuned by control over the amount of DNA deposited during fabrication. For films 4, 8, and 16 tetralayers thick, the percentage of cells transfected increased linearly with the number of tetralayers deposited. Mesh substrates coated with films 16 tetralayers thick promoted surface-mediated transfection in ~50% of cells using this model in vitro system.

Characterization of Film-Coated Balloon Catheters and Transfer of DNA to Arterial Tissue. Figure 6A shows a fluorescence microscopy image of a portion of the inflatable tip of an embolectomy balloon catheter coated with an LPEI/PAA/LPEI/DNA film eight bilayers thick. The films in these experiments were fabricated using a plasmid construct encoding firefly luciferase (pCMV-Luc) fluorescently labeled with the fluorophore tetramethylrhodamine ($pCMV\text{-}Luc_{TMR}$) to permit characterization of film uniformity and the transfer of DNA to arterial tissue in subsequent experiments. Inspection of the image in Figure 6A reveals fluorescence to be distributed uniformly over the surface of the balloon. Figure 6B shows an image of the same balloon after insertion and inflation in the carotid artery of a rat for 20 min (the balloon was removed prior to imaging; dotted white lines are shown to guide the eye and identify the location of the non-fluorescent balloon). This image reveals a striking decrease in fluorescence intensity on the surface of the balloon, consistent with the substantial and nearly complete removal of DNA upon the insertion, inflation, and contact-transfer of DNA to arterial tissue. Further evidence in support of the contact-transfer of DNA is shown in Figure 6C,D, which shows low and high magnification fluorescence microscopy images of cross sections of the artery treated in this experiment (tissue was harvested after removal of the balloon and prior to restoring blood flow). Inspection of these images reveals bright red DNA-associated fluorescence around the circumference of the inner surface of the arterial wall.

Film-Coated Balloon Catheters Promote Contact-Mediated Transfection in Arterial Tissue. We conducted a final series of experiments to characterize the ability of our PAA-containing coatings to promote localized transgene

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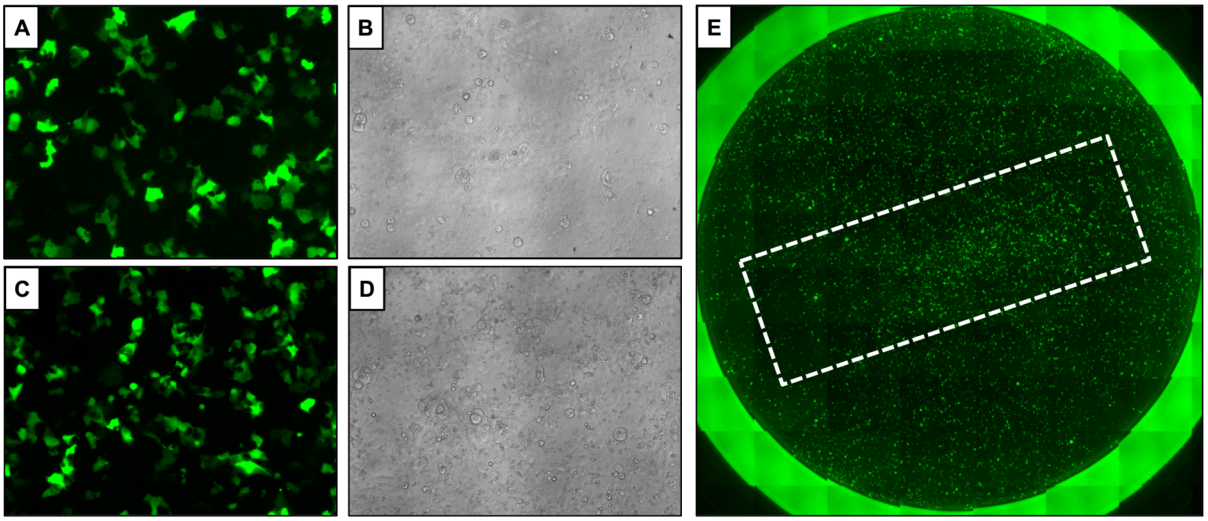

Figure 4. (A–D) Representative fluorescence (A, C) and phase contrast (B, D) microscopy images (10×) showing EGFP expression in monolayers of COS-7 cells incubated in the presence of stainless steel mesh substrates coated with films having the structure $(LPEI/PAA/LPEI/DNA)_8$ (A, B) or $(LPEI/PAA/LPEI/DNA)_{16}$ (C, D); substrates were removed prior to imaging (see text). (E) Series of adjacent low magnification (4×) fluorescence microscopy images showing expression of EGFP in a confluent population of COS-7 cells in the well of a standard six-well tissue culture plate. The large bright green circle is the circular wall of the culture well (diameter = 35 mm); the dotted white rectangle marks the approximate location of the film-coated mesh.

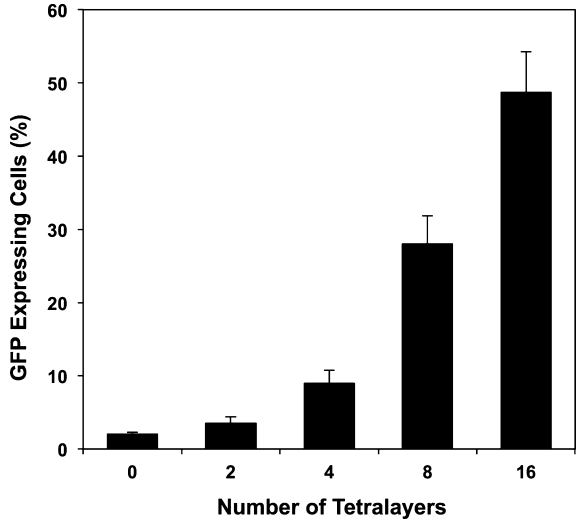

Figure 5. Percentage of COS-7 cells expressing EGFP 48 h after treatment with stainless steel mesh substrates coated with $(LPEI/PAA/LPEI/DNA)_x$ films 0, 2, 4, 8, or 16 tetralayers thick. Data are expressed as the average percentage (with standard deviation) of EGFP-positive cells relative to the total cell numbers in each experiment. Experiments were performed in triplicate.

expression in arterial tissue using the rat model of carotid artery injury used above and methods used in past studies[29,30] to characterize the balloon-mediated transfer of DNA using hydrolytically degradable multilayer coatings. For these experiments, we used catheter balloons coated with films having the general structure $(LPEI/PAA/LPEI/pCMV\text{-}\beta\text{-}gal)_{32}$. These films were fabricated using plasmid DNA encoding β-gal to facilitate characterization of transgene expression in arterial tissue using X-gal staining; we used films 32 tetralayers thick to maximize the amount of DNA available for transfer in these proof of concept experiments (balloon catheters coated with films having this structure released ~23 μg of DNA when incubated in PBS buffer). For these in vivo experiments, balloon catheters coated with $(LPEI/PAA/LPEI/pEGFP)_{32}$ films containing the pEGFP plasmid were used as controls. Film-coated balloons were inserted into rat carotid arteries denuded of endothelium for 20 min and then deflated and removed. Rats were sacrificed 3 days after surgery, and cross sections of balloon-treated tissue were characterized using X-gal staining and optical microscopy (see Materials and Methods and other past studies for details of surgical procedures and tissue characterization).

Figure 7A,B shows low and high magnification microscopy images of cross sections of arterial tissue treated with a balloon coated with a $(LPEI/PAA/LPEI/pCMV\text{-}\beta\text{-}gal)_{32}$ film. The blue precipitate observed in this tissue indicates the expression of β-gal as revealed by X-gal staining. These images reveal intense blue staining to be located uniformly around the circumference of the artery and present in both the medial layers and a portion of the adventitial layers of the tissue. Figure 7C,D shows cross sections of a control artery treated with a balloon coated with a $(LPEI/PAA/LPEI/pEGFP)_{32}$ film (the image was also acquired after treatment with X-gal). No significant blue staining was observed in the medial or adventitial layers of this control artery, confirming that the localized expression of β-gal in arteries treated with balloons coated with the $(LPEI/PAA/LPEI/pCMV\text{-}\beta\text{-}gal)_{32}$ films does not arise from non-specific effects associated with the LPEI or PAA components of these coatings.

DISCUSSION

Our group and others have demonstrated that polyelectrolyte multilayers containing DNA can be used to exert both spatial and temporal control over the release or contact-transfer of DNA [e.g., by placing film-coated objects directly in contact with cells (spatial control) or by changing the structures of degradable polymer components to tune film erosion rates (temporal control)].[14,23−28] These past studies have demonstrated that fabrication using hydrolytically degradable cationic polymers is a useful approach to design multilayers that release DNA gradually (e.g., over days, weeks, or months).[14,27,28] We

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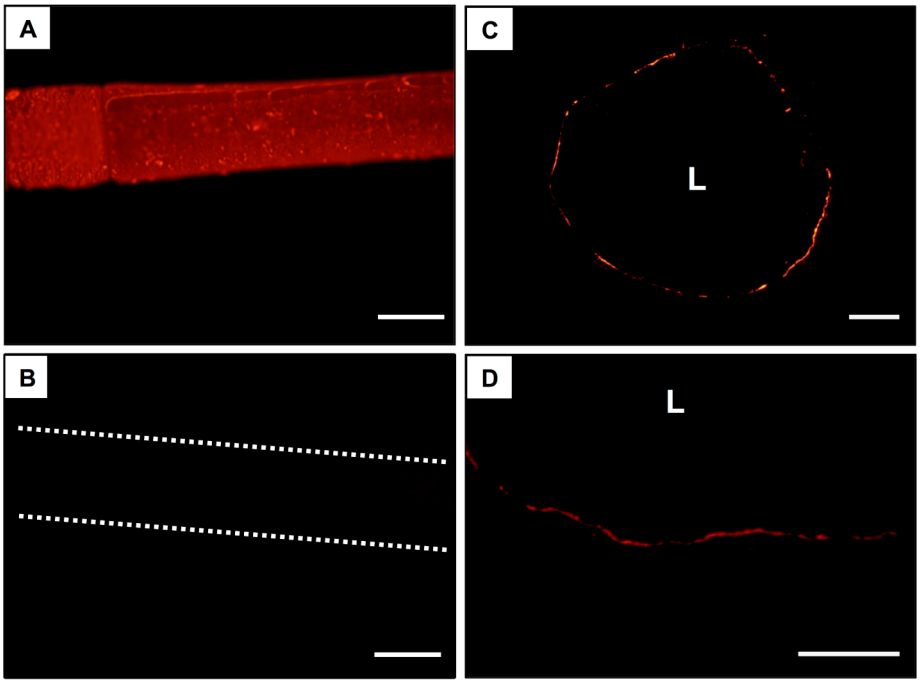

Figure 6. (A, B) Representative fluorescence microscopy images of the inflatable tip of a balloon catheter coated with a film having the structure $(LPEI/PAA/LPEI/DNA_{TMR})_8$ fabricated using fluorescently labeled DNA after fabrication (A) and after insertion and inflation in the carotid artery of a rat for 20 min (B; balloon was deflated and removed prior imaging; white dotted lines mark the location of the edges of the non-fluorescent balloon). (C, D) Low (C) and high (D) magnification fluorescence images of cross sections of the carotid artery of a rat after treatment with a balloon coated with a $(LPEI/PAA/LPEI/DNA_{TMR})_8$ film; arteries were harvested prior to the restoration of blood flow (see text; the designation "L" indicates the location of the lumen of the vessel). Scale bars = 500 μm in (A) and (B) and 200 μm in (C) and (D).

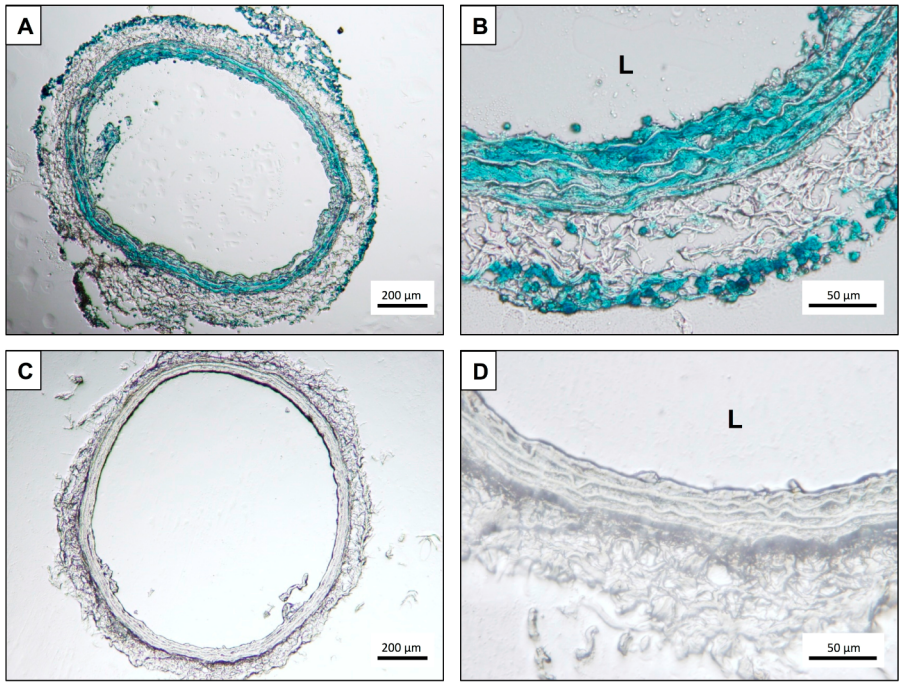

Figure 7. Representative low (A, C) and high (B, D) magnification optical microscopy images of cross sections of carotid arteries of a rat 3 days after treatment with catheter balloons coated with films having the structure $(LPEI/PAA/LPEI/pCMV\text{-}\beta)_{32}$ (A, B) or $(LPEI/PAA/LPEI/pEGFP)_{32}$ (C, D) after staining with X-gal. The designation "L" indicates the location of the lumen of the vessels. Scale bars = 200 μm in (A) and (C) and 50 μm in (B) and (D).

have found it more difficult, in general, to use this "degradable polymer approach" alone to design coatings that release DNA rapidly (e.g., over several seconds or several minutes).[38] Multilayers that promote the rapid release or transfer of DNA from film-coated devices would be broadly useful and, in many cases, critical in contexts for which dwell time is restricted (such as in balloon-mediated vascular interventions)[29,30] and in other applications (such as microneedle-base delivery to the

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skin)[31] where short-duration transfer is desirable. The 'weak polyelectrolyte approach reported here departs from the use of degradable polymers and provides alternative, pH-based means to promote the short-term instability of DNA-containing multilayers in physiological media.[32] This approach thus provides new opportunities to design films that promote the rapid release and rapid contact-transfer of DNA.

We used the synthetic polyamine LPEI as a model cationic polymer for this study for several reasons. First, this polymer is commercially available and used widely for the design of non-viral gene delivery systems;[33,34] the presence of LPEI in DNA-containing multilayers could thus, in principle, be leveraged to address intracellular barriers to cell transfection and improve levels of contact-mediated gene expression. Second, and more relevant to the specific goals of this study, multilayers fabricated from plasmid DNA and this non-degradable polyamine do not themselves release DNA rapidly when incubated in physiological media (e.g., Figure 3A). Finally, initial results communicated in our past study demonstrated that integration of PAA into LPEI/DNA multilayers could be used to promote rapid release of DNA upon exposure of these materials to physiologically relevant media.[32] In this current study, we sought to (i) characterize factors that influence the fabrication and stability of LPEI/DNA multilayers containing intermittent layers of PAA and (ii) evaluate the utility of these PAA-containing films as coatings for the rapid release and surface-mediated transfer of DNA in vitro and in vivo. To facilitate characterization of these materials in the context of these broader biological goals, we used films fabricated using transcriptionally active reporter plasmids encoding either EGFP or β-gal.

Our results demonstrate that the substitution or "swapping" of every other layer of DNA in a LPEI/DNA multilayer with PAA during layer-by-layer assembly (i.e., films having a repeating $(\text{LPEI/PAA/LPEI/DNA})_x$ "tetralayer" structure; Figure 1) leads to coatings that release DNA rapidly, with ~60% being released within the first 5 min; Figure 3A). This behavior contrasts starkly to that of films fabricated exclusively from LPEI and DNA, which release DNA gradually over a period of ~4 days (Figure 3A). These PAA-containing coatings release DNA that is transcriptionally active and can be used to promote high levels of transfection in mammalian cells in vitro in the presence of serum (e.g., Figure 4) without the addition of auxiliary cell transfection agents. One merit of this overall approach, relative to other methods for the encapsulation of DNA in thin degradable films, is that the amount of DNA incorporated into (and subsequently released from) these coatings can be controlled precisely by controlling the number of layers of DNA deposited during layer-by-layer assembly. Our results demonstrate that this feature can be used to modulate levels of cell transfection in vitro, with the number of transfected cells increasing monotonically from ~4% for coatings two tetralayers thick, to ~50% for films containing 16 tetralayers (the thickest films characterized quantitatively in this study; Figure 5).

Results shown in Figure 7 demonstrate that inflatable balloon catheters coated with LPEI/PAA/LPEI/DNA coatings 32 tetralayers thick can promote high levels of contact-mediated transgene (β-gal) expression in the arterial walls of rats. We used balloon inflation and tissue contact times of 20 min to maximize film transfer in this proof of concept study and to permit comparison to the results of past studies characterizing the contact-transfer of DNA using balloons coated with hydrolytically degradable multilayer coatings.[29,30] However, these new "quick release" coatings provide principles useful for the design of coatings that could enable more rapid contact-transfer of DNA to vascular tissue (e.g., over periods of seconds or minutes) than the degradable coatings used in those past studies. We therefore anticipate that these rapid-release coatings will be useful for the development of new approaches to the contact-transfer and delivery of therapeutic DNA constructs (or other agents) that could mitigate the consequences of arterial injury that occur during angioplasty procedures (e.g., to reduce the occurrences of intimal hyperplasia and restenosis, etc.). As noted above, rapid release of DNA from film-coated objects and devices would also be useful in a broad range of other contexts.

Multilayer coatings fabricated using DNA and hydrolytically degradable cationic polymers investigated in past studies generally require the deposition of thin polyelectrolyte multilayer-based "base layers" on substrates to promote robust film growth.[15−17,29,32] Our current results reveal that this form of substrate preparation or pre-treatment is not necessary to promote robust film growth in this LPEI/PAA/DNA system (Figure 2A). The presence of LPEI/SPS base layers facilitates initial film growth (presumably by providing a more uniform density of anionic charge that promotes the initial adsorption of LPEI) and leads to films that are thicker than those grown on pristine substrates. The presence of base layers does not impact DNA release profiles when these films are subsequently transferred to physiological media (Figure 3). However, in view of the additional complexity that these base layers add to these macromolecular assemblies, we opted to eliminate them in our in vitro and in vivo studies. Our results demonstrate that $(\text{LPEI/PAA/LPEI/DNA})_x$ coatings can be fabricated on a variety of different silicon, stainless steel, and flexible plastic substrates (Figures 2, 4, and 6) and used to promote high levels of transfection in cells and tissue without the use of base layers or other substrate pre-treatment protocols.

Finally, we note again that we used LPEI as a model polymer for these studies based on the fact that it is (i) non-degradable and (ii) used widely as a basis for the design of non-viral DNA delivery systems.[33,34] While our results demonstrate that LPEI/PAA/DNA-based coatings can promote high levels of cell transfection in vitro and in vivo (Figures 4 and 7), this current study does not establish the extent to which the presence of LPEI may (or may not) help promote the transport of DNA or address common intracellular barriers to transfection. Additional studies will be required to characterize the extent to which LPEI may form complexes or aggregates with DNA in solution or promote endosomal escape or other important processes more effectively than other DNA-containing multilayer systems. We note further, however, that the "weak polyelectrolyte approach" used here promotes rapid film disruption through an alternate mechanism (pH-induced changes in the ionization of PAA that promote destabilizing ionic interactions) that does not require the integration of hydrolytically degradable polymers, yet remains compatible with their use. As such, this framework opens the door to new film designs that could leverage the physical properties and biological behaviors of a broad range of other cationic polymers used conventionally to promote the transfer of DNA to cells. We anticipate that the work reported here will help guide the development of more advanced DNA-containing coatings, including films fabricated using other weak polyanions, that could further enhance contact-mediated gene expression.

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SUMMARY AND CONCLUSIONS

We have demonstrated a layer-by-layer approach to the fabrication of thin polyelectrolyte multilayer coatings that release plasmid DNA rapidly from the surfaces of film-coated objects. This approach exploits the pH-dependent ionization of a weak anionic polyelectrolyte to disrupt ionic interactions and promote disassembly in films that otherwise erode slowly in physiological media. Our results demonstrate that the substitution of every other layer of DNA with PAA in LPEI/DNA multilayers leads to thin films that release DNA rapidly, with >60% of DNA being released within the first 5 min of introduction to physiological buffer. These coatings release DNA in a transcriptionally active form and can be used to fabricate uniform coatings on a variety of objects, including the flexible tips of inflatable balloon catheters. The layer-by-layer approach used here can be used to tune the amount of DNA incorporated within a film and, thereby, control levels of surface-promoted transfection that can be achieved. Although not investigated as a part of this study, this approach also provides a straightforward means to control the relative numbers and locations of the layers of PAA that are incorporated. This approach should therefore also provide a useful means to tune film erosion and DNA release profiles further. Finally, our results demonstrate that these quick-release coatings can be used to promote high levels of surface-promoted cell transfection in vitro, as well as the robust contact-transfer and localized expression of DNA in vascular tissue in vivo using a rat model of vascular injury. Promoting rapid film erosion through a mechanism that does not require the use of a degradable cationic polymer creates future opportunities to design DNA-releasing multilayers using a much broader range of polymer-based building blocks, including non-degradable cationic polymers that could promote more efficient cell transfection or improve overall biocompatibility. The materials reported here provide useful alternatives to multilayers and other surface coatings that promote the gradual or prolonged release of DNA. With further development, this approach could thus provide a new and useful platform for the rapid release or contact-transfer of DNA to cells and tissues from objects of interest and potential utility in a range of fundamental and applied contexts.

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Notes

The authors declare no competing financial interest.

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