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Delivery of Plasmid DNA to Vascular Tissue *in vivo* using Catheter Balloons Coated with Polyelectrolyte Multilayers

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Abstract

We report an approach for the localized delivery of plasmid DNA to vascular tissue from the surfaces of inflatable embolectomy catheter balloons. Using a layer-by-layer approach, ultrathin multilayered polyelectrolyte films were fabricated on embolectomy catheter balloons by alternately adsorbing layers of a hydrolytically degradable poly(β -amino ester) and plasmid DNA. Fluorescence microscopy revealed that the films coated the surfaces of the balloons uniformly. Coated balloons that were incubated in phosphate-buffered saline at 37 °C released ~25 μ g DNA/cm² over 24 hours. Analysis of the DNA by gel electrophoresis showed that the DNA was released in open-circular ('nicked') and supercoiled conformations, and *in vitro* cell transfection assays confirmed that the released DNA was transcriptionally active. Arterial injury was induced in the internal carotid arteries of Sprague-Dawley rats using uncoated balloons, followed by treatment with film-coated balloons for 20 minutes. X-gal, immunohistochemical, and immunofluorescence staining of sectioned arteries indicated high levels of β -galactosidase or enhanced green fluorescent protein (EGFP) expression in arteries treated with film-coated balloons. β -galactosidase and EGFP expression were observed throughout the medial layers of arterial tissue, and around approximately two-thirds of the circumference of the treated arteries. The layer-by-layer approach reported here provides a general platform for the balloon-mediated delivery of DNA to vascular tissue. Our results suggest the potential of this approach to deliver therapeutically relevant DNA to prevent complications such as intimal hyperplasia that arise after vascular interventions.

Keywords

Thin Films; Gene Delivery; Polyelectrolytes; Self-Assembly; Polymers; DNA

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Introduction

Advances in the fields of interventional cardiology and vascular surgery have led to the development of less invasive approaches for the treatment of atherosclerosis, including angioplasty and the implantation of bare metal or drug-eluting intravascular stents. These and other new techniques have saved or improved the lives of millions of patients suffering from coronary artery disease or other vascular diseases [1]. Unfortunately, however, the implantation of stents is not appropriate for the treatment of all atherosclerotic lesions [2], and restenosis caused by complications such as thrombosis and intimal hyperplasia can impact the success of many vascular interventions [3–5]. In total, it is estimated that intimal hyperplasia resulting from interventional procedures leads to the failure of 5 to 30% of all vascular reconstructions. Therefore, the development of new, targeted approaches to prevent intimal hyperplasia after vascular surgery would represent a significant step toward the clinical treatment of atherosclerosis and contribute significantly to the well-being of patients requiring vascular interventions [2].

Intimal hyperplasia is characterized in part by the migration and proliferation of vascular smooth muscle cells, leading to a narrowing of the lumen of an affected artery [6]. Recent animal studies suggest that bone marrow-derived progenitor cells also contribute to intimal lesions [7]. Activation of residential smooth muscle cells and mobilization of progenitor cells are triggered by signals (e.g., growth factors, cytokines, chemokines, and other factors) released at the site of an injured vascular wall during or after interventional procedures. While cell proliferation can be prevented to varying extents by the localized delivery of anti-proliferative small-molecule drugs coated on stents [8–9], the development of protein and gene-based therapies has the potential to target a wider range of underlying cellular mechanisms responsible for triggering activation of smooth muscle cells and progenitor cells and could subsequently provide more effective control of intimal hyperplasia [10]. Experimental approaches based on the delivery of macromolecular therapeutics (e.g., antibodies, soluble receptors, DNA, siRNA, etc.) that can modulate the expression of key regulatory genes and proteins have demonstrated the potential of these approaches to prevent intimal hyperplasia in animal models [2,11–13]. The development of clinical therapies based on these approaches, however, has been limited by the lack of materials and approaches that can be used to locally deliver these macromolecular agents to the vascular wall safely and effectively [4,14].

In general, materials and approaches that have been developed for the localized delivery of conventional small-molecule drugs to the vascular wall (e.g., thin polymer coatings that permit gradual, diffusion-controlled release of small-molecule agents from the surfaces of drug-eluting stents) are not well suited for the release of larger macromolecular drugs such as DNA. As a result, the development of new materials that can be used to localize the delivery of nucleic acid-based therapeutics to the vascular wall will be critical to unlocking the clinical potential of these agents. In addition, approaches that permit delivery of macromolecular drugs directly and locally from the surfaces of interventional devices, such as angioplasty balloons or intravascular stents, could eliminate the need for systemic therapies with these drugs (thus reducing required dosages and the potential for undesired side effects) without increasing the complexity or the number of interventional procedures required [4,14–16]. In this paper, we report a first step toward addressing several of these broader goals by developing methods for the localized delivery of plasmid DNA to vascular tissue using catheter balloons coated with ultrathin polyelectrolyte-based films.

The approach reported here is based on methods developed for the layer-by-layer deposition of multilayered polyelectrolyte films (or ‘polyelectrolyte multilayers’, PEMs) on surfaces [17–18]. These methods are entirely aqueous and can be used to fabricate ultrathin films

(e.g., from ~10 nm to several hundred nm thick) using a broad range of positively and negatively charged polymers, including biomacromolecules such as proteins, viruses, and DNA. Numerous past studies have demonstrated that these materials can be used as platforms for the release of small-molecule drugs and macromolecular therapeutics. Examples of these approaches and other biomedical applications of these thin-film materials, including the general design of PEMs for the delivery of DNA [19], have been reviewed comprehensively and are not discussed in detail here [19–22]. Of particular relevance to the work reported here, however, we note that several recent studies by our group and others have demonstrated that PEMs fabricated using plasmid DNA can be used to promote the surface-mediated delivery of DNA to cells [19,23–30]. In addition, we note that other groups have reported the design of PEMs that promote virus-mediated cell transfection [31] and assemblies that promote the internalization of other nucleic acid-based agents (e.g., siRNA) to cells [32].

In the context of DNA delivery, layer-by-layer approaches to the fabrication of PEMs offer several potential practical advantages as compared to conventional approaches to the immobilization and release of DNA from thin films of bulk polymer. First, DNA can be directly incorporated into multilayered assemblies (e.g., as a ‘layer’), providing opportunities to precisely control the loading of DNA within a film (e.g., by changing the number of layers of DNA deposited during fabrication). The modular nature of layer-by-layer assembly can also be exploited to incorporate multiple layers of different therapeutic agents to design thin films and coatings that release multiple agents simultaneously or sequentially [23,26,29,33–35], and to incorporate auxiliary agents (e.g., cationic polymers [36–38]) that could facilitate the delivery and cellular internalization of DNA. In addition, the aqueous procedures used for film fabrication do not require the use of organic solvents that could compromise the biological function of the DNA or harm the surfaces of devices on which they are deposited. Finally, layer-by-layer assembly leads to uniform surface coatings that conform faithfully to surfaces with complex or irregular shapes and microscale dimensions, such as those typical of interventional devices and implants [25,39–44].

The work described here builds on past reports from our group demonstrating that PEMs fabricated using plasmid DNA and a hydrolytically degradable cationic polymer (polymer **1**) can be used to promote the release [45–47] and surface-mediated delivery [23,25,48] of DNA to cells *in vitro*. These past studies have also demonstrated that the layer-by-layer approach used to assemble these materials can be used to fabricate ultrathin DNA-containing films (e.g., 100 nm thick) on the surfaces of interventional devices, such as intravascular stents [25]. This current study sought to determine whether this approach could be used to coat the surfaces of inflatable embolectomy catheter balloons with thin, DNA-containing PEMs and promote localized transgene expression in the vascular wall *in vivo* using a rat model of balloon-induced arterial injury used previously for studies of intimal hyperplasia.

Polymer 1

Materials and Methods

Materials and General Considerations

Linear poly(ethylene imine) (LPEI, MW = 25,000) was purchased from Polysciences, Inc. (Warrington, PA). Sodium poly(4-styrenesulfonate) (SPS, MW = 70,000) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Commercial polyelectrolytes were used as received without further purification. Concentrated sodium acetate buffer was purchased from Lonza (Rockland, ME). Polymer **1** ($M_n = 16,000$) was synthesized as described previously [49]. Plasmid DNA encoding enhanced green fluorescent protein [pEGFP-N1 (4.7 kb), > 90% supercoiled] or β -galactosidase [pCMV- β (7.2 kb), 85% supercoiled] were purchased from Elim Biopharmaceuticals, Inc. (San Francisco, CA). TM-Rhodamine Label-IT nucleic acid labeling kits were purchased from Mirus Bio Corporation (Madison, WI) and used according to the manufacturer's instructions (labeling density = 1 label:200bp). Fogarty arterial embolectomy catheters (2-French diameter) were purchased from Edwards Lifesciences, LLC (Irvine, CA). Goat anti-GFP IgG was purchased from AbCam (Cambridge, MA). 4 Plus Biotinylated mouse anti-goat IgG and 4 Plus streptavidin-horseradish peroxidase conjugate were purchased from Biocare Medical (Concord, CA). DAB substrate kit was purchased from Thermo Scientific (Rockford, IL). Alexa Fluor 594 donkey anti-goat IgG was purchased from Invitrogen (Carlsbad, CA). X-gal staining kit was purchased from Genlantis (San Diego, CA). Deionized water (18 M Ω) was used to prepare all buffers and polymer solutions, unless otherwise noted. Phosphate-buffered saline (PBS) was prepared by diluting commercially available concentrate (EMD Chemicals, Gibbstown, NJ). Fluorescence and optical microscopy images were analyzed using Adobe Photoshop and ImageJ software (National Institutes of Health).

Preparation of Polyelectrolyte Solutions

Solutions of LPEI and SPS used for the fabrication of LPEI/SPS precursor layers (20 mM with respect to the molecular weight of the polymer repeat unit) were prepared using a 26 mM NaCl solution and filtered using a 0.2 μ m pore nylon syringe filter prior to use. LPEI solutions contained 5 mM HCl to aid polymer solubility. Solutions of polymer **1** (5 mM with respect to the molecular weight of the polymer repeat unit) were prepared using 100 mM sodium acetate buffer (pH = 4.9) and were filtered using a 0.2 μ m pore nylon syringe filter prior to use. Solutions of plasmid DNA were prepared at 1 mg/mL in filtered 100 mM sodium acetate buffer (pH = 4.9), but were not filtered prior to use. For experiments requiring fluorescently labeled DNA, unlabeled plasmid was mixed with 20% (w/w) of plasmid labeled with TM-Rhodamine.

Fabrication of Multilayered Films

Prior to the fabrication of DNA-containing films, the embolectomy catheters were pre-coated with 10 bilayers of a multilayered film composed of LPEI and SPS (~20 nm thick, terminated with a topmost layer of SPS), as previously described for the fabrication of polymer **1**/DNA films on silicon, glass, or stainless steel substrates [23,25,45]. Polymer **1**/DNA layers were then deposited as follows: 1) Substrates were submerged in a solution of polymer **1** for 5 minutes, 2) substrates were removed and immersed in a wash bath of 100 mM sodium acetate buffer (pH = 4.9) for one minute followed by a second wash bath of sodium acetate buffer for one minute, 3) substrates were submerged in a solution of DNA for 5 minutes, and 4) substrates were rinsed in the manner described above. This cycle was repeated until the desired number of bilayers (typically 16) had been deposited. Following the final rinse step, the substrates were rinsed briefly with deionized water (18 M Ω) and dried under a stream of filtered air. Embolectomy catheters were either used immediately or stored in a dark location prior to use. All films were fabricated and stored at ambient room temperature. For catheters coated using fluorescently labeled DNA, the presence and

condition of the multilayered films on the surface of the balloon were characterized visually using an Olympus IX70 fluorescence microscope. Images were acquired using the Metavue 7.1.2.0 software package.

Characterization of Film Erosion and DNA Release Profiles

Experiments designed to evaluate the loading and release of DNA from films fabricated on embolectomy catheter balloons were conducted in the following general manner. Each coated balloon and an adjacent ~1 inch length of catheter was cut from the end of the full length catheter and placed in PBS (1 mL; pH = 7.3, 137 mM NaCl) in a plastic UV-transparent cuvette. The cuvette was capped, sealed with Parafilm, and incubated at 37 °C. The balloon was removed from solution at predetermined intervals to permit characterization of the incubation solution. A DU520 UV-visible spectrophotometer (Beckman Coulter, Brea, CA) was used to measure the absorbance of the solution at 260 nm (the wavelength corresponding to the maximum absorbance of double-stranded DNA). The balloon was then placed in a new cuvette containing fresh PBS and incubated at 37 °C until the next measurement. Cuvettes containing DNA released into PBS were stored at 4 °C for characterization by gel electrophoresis and use in *in vitro* cell transfection experiments as described below.

Gel Electrophoresis

Agarose gel electrophoresis was used to evaluate the integrity and conformation of DNA released into PBS during the *in vitro* erosion experiments described above. Samples of plasmid DNA released from coated balloons (25 µL) were mixed with a loading buffer (2.5 µL of a 50:50 (v/v) glycerol:water mixture) and analyzed on a 1% agarose gel (20 mM HEPES, pH 7.2, 80V, 90 min). DNA bands were visualized by ethidium bromide staining.

Characterization of Cell Transfection in vitro

COS-7 cells (American Type Culture Collection, Manassas, VA) were seeded into 96-well plates at 15,000 cells/well in 200 µL of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 U/mL of penicillin and 100 µg/ml of streptomycin. After the cells had reached 80% confluence (~24 hours after seeding), the medium was aspirated and replaced with fresh medium, and 50 µL of a Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and plasmid DNA mixture was added to each well. The Lipofectamine 2000/plasmid DNA mixture was prepared by mixing 25 µL of the plasmid solution collected at each time point during release experiments (arbitrary concentrations but constant volumes) with 25 µL of diluted Lipofectamine 2000 (24 µL diluted into 976 µL Opti-MEM I Reduced Serum Medium). Fluorescence microscopy images used to characterize expression of EGFP were acquired after 48 h using an Olympus IX70 fluorescence microscope and the MetaVue version 7.1.2.0 software package.

Characterization of in vivo Delivery and Expression of DNA to Rat Carotid Arteries

Male Sprague-Dawley rats weighing ~300 g underwent angioplasty of the left common carotid artery as described elsewhere [13]. Briefly, a longitudinal incision was made in the neck of the rat, and the left external, internal and common carotid artery was isolated. A 2-French embolectomy catheter balloon was inserted through the external carotid artery into the common carotid artery. Denudation of the endothelial layer was accomplished by 3 passages of the balloon at a pressure of 2 atm. A balloon coated with a polymer 1/DNA film 16 bilayers thick was then inserted into the common carotid artery and inflated to 2 atm for 20 minutes. The coated balloon was deflated and removed from the artery, the external carotid was ligated, and blood flow was restored through the common and internal carotid arteries. The wound was closed layer to layer. The animals were then sacrificed at

postoperative day 3. The arteries were then harvested by perfusion fixation with 4% paraformaldehyde at a physiologic pressure of 100 mmHg. All experiments involving animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health, NIH Publication No. 85-23, 1996 revision. Approval from the Institutional Animal Care and Use Committee at University of Wisconsin Madison was granted (#M02285).

Evaluation of Gene Expression in Arterial Tissue

The common carotid arteries were embedded and frozen in OCT compound and cut into 6 μm sections. To evaluate EGFP expression, immunohistochemical staining using goat anti-GFP antibody was performed as described previously [13,50]. Immunofluorescent staining was also performed with donkey anti-goat Alexa 594 (Invitrogen, CA). For the evaluation of β -galactosidase expression, X-Gal staining was performed following the manufacturer's instructions. Slides were then visualized with a Nikon Eclipse E800 upright microscope with equipped with appropriate filters. Images were analyzed using Adobe Photoshop and ImageJ software.

Results and Discussion

Layer-by-Layer Assembly of DNA-Containing Films on the Surfaces of Embolectomy Catheter Balloons

We have demonstrated in several past studies that hydrolytically degradable cationic polymers (such as polymer **1**) can be used to fabricate ultrathin PEMs that erode and promote the surface-mediated release of DNA [23,25,34,45,47–48,51–53]. These past studies have demonstrated that the hydrolytically degradable ester functionality in polymer **1** plays an important role in promoting film disassembly [47,53], and that changes in the side-chain or backbone structures of these polymers can be used to achieve tunable control over film erosion profiles and design films that release DNA or other agents over a broad range of times (e.g., ranging from several days to several weeks) [34,51–52]. In this study, we sought to determine whether this layer-by-layer approach could be used to fabricate DNA-containing PEMs on the surfaces of catheter balloons, and, subsequently, whether balloons coated in this manner could be used to promote the delivery of DNA directly to vascular cells *in vivo*. We selected Fogarty embolectomy catheter balloons (2-French diameter) for use in the work described here because they are appropriately sized for use with rodents and have been used in several past studies of intimal hyperplasia using a rat model [13]. As noted above, several different hydrolytically degradable polymers have been demonstrated to promote the disassembly of DNA-containing PEMs and tune DNA-release profiles. We selected polymer **1** as a model polymer for use in the work described here for several reasons: (i) the physical erosion profiles, DNA-release profiles, and general physicochemical behaviors of films fabricated using polymer **1** are more well characterized than those of PEMs fabricated using other hydrolytically degradable polymers [23,25,45–48], (ii) past reports have demonstrated that this polymer **1**/DNA system can be used to promote the direct, surface-mediated delivery of DNA to cells *in vitro* [23,25], and (iii) films fabricated using polymer **1** conform well to the surfaces of complex medical devices (such as intravascular stents) and have been demonstrated to be able to withstand basic mechanical challenges (such as balloon expansion) typically associated with the deployment of these devices [25].

We conducted an initial series of experiments to determine whether DNA-containing PEMs could be fabricated on the surfaces of flexible, polymer-based catheter balloons. For these experiments, multilayered films composed of polymer **1** and plasmid DNA (referred to hereafter as 'polymer **1**/DNA films') were fabricated on the surfaces of uninflated Fogarty

embolectomy catheter balloons using an alternate dipping protocol similar to that used previously for the fabrication of these films on planar silicon and glass substrates. As described in our previous studies, the balloons were first pre-coated with 10 layer pairs (or 'bilayers') of linear poly(ethylene imine) (LPEI) and sodium poly(styrene sulfonate) (SPS) to provide a suitable surface for the adsorption of polymer **1** and DNA [45]. For these initial studies, films were fabricated using a plasmid construct (pEGFP-N1) encoding enhanced green fluorescent protein (EGFP) labeled with tetramethylrhodamine (TMR) to permit inspection and characterization of the presence and uniformity of the films using fluorescence microscopy. Figure 1A shows a representative fluorescence microscopy image of a balloon after the deposition of 16 bilayers of polymer **1** and DNA. The presence of red fluorescence distributed over the surface of the balloon demonstrates the presence of the DNA-containing films. Further inspection of this image reveals that this film is generally uniform and devoid of large-scale defects or uncoated patches (the striped appearance of the film is an artifact of imaging and arises from the presence of a metallic spring inside the balloon assembly and is not a feature of the film itself). Figure 1B shows an image of a balloon coated with a film fabricated using unlabeled DNA and demonstrates that the fluorescence in the image in Figure 1A does not arise from the balloon, polymer **1**, or the DNA in these assemblies.

The thicknesses of PEMs fabricated on planar substrates can be characterized readily using techniques such as ellipsometry and atomic force microscopy. However, the small sizes, cylindrical shapes, and non-reflective nature of the catheter balloons used here prevented the use of these methods to characterize the thicknesses or the growth profiles of the films described above. Our past studies on the growth of polymer **1**/DNA films on planar silicon substrates reveal these films to grow in a linear manner with respect to the number of bilayers of polymer and DNA adsorbed [45]. Past studies on films fabricated on stainless steel also reveal film growth to be largely substrate independent (that is, the thicknesses of films fabricated on stainless steel are similar to those of films fabricated on silicon substrates) [25]. In this context, we note that the optical thickness of a polymer **1**/DNA film 16 bilayers thick fabricated on a planar silicon substrate is approximately 200 nm. We return to a consideration of film thickness and the loading of DNA in films fabricated on the surfaces of these polymer balloons again in the discussion below.

Characterization of Film Stability and the Release of DNA in vitro

Our past studies demonstrate that polymer **1**/DNA films fabricated on silicon, glass, and stainless steel release DNA gradually into solution (e.g., over periods ranging from two to four days) when incubated in physiologically relevant media [25,45]. To characterize the release of DNA from films fabricated on the surfaces of polymer balloons, we incubated the uninflated film-coated balloons described above in PBS at 37 °C and characterized the release of DNA into solution using UV absorbance. Figure 2 shows a representative DNA release profile for an embolectomy catheter balloon coated with a polymer **1**/DNA film 16 bilayers thick. Inspection of these data reveals two significant differences in the behavior of polymer **1**/DNA films fabricated on catheter balloons as compared to the results of our past studies. First, the data in Figure 2 demonstrate that over 60% of the DNA in the film is released within the first hour of incubation and that the release of DNA is complete after 24 hours of incubation. This release profile differs from that of films fabricated on the more rigid substrates used in our past studies (which, as described above, sustain the release of DNA over a period of 2–4 days under otherwise identical conditions) [25,45].

The second important difference noted here relates to the loading of DNA in the films fabricated on these balloon-based substrates. Based on the total amount of DNA released in the curve shown in Figure 2, we estimate the initial loading of DNA in the films fabricated on the balloons used in these experiments to be ~25 µg DNA/cm² of coated surface. This

value differs significantly from the values determined for the loading of DNA in otherwise identical 16-bilayer films fabricated on planar silicon substrates ($\sim 5.1 \mu\text{g DNA/cm}^2$; similar to those reported in our past studies) [23]. These striking differences in DNA loading and release profiles suggest that the apparent substrate independence of the properties of these films observed in past studies may not extend to films fabricated on these polymer balloon substrates. The substantially higher DNA loading levels observed for films fabricated on these balloon assemblies also suggest that these films could be substantially thicker than the 200 nm thick films observed for fabrication on silicon substrates, although we note that our current results do not explicitly rule out other possibilities (such as changes in surface roughness that could lead to differences in the surface area available for coating during film assembly). We note further that while the reasons for the more rapid release profiles observed here are not clear, films that release DNA more rapidly are of potential value for applications (such as the balloon-mediated delivery of DNA to vascular tissue *in vivo*, as described below) that are time-limited and/or require short delivery times rather than prolonged or sustained release.

We performed additional experiments to characterize the physical and functional integrity of the DNA released during the experiments described above. Figure 3A shows the results of agarose gel electrophoresis characterization of aliquots of DNA-containing solutions removed at different time points during the incubation of a film-coated balloon in PBS. The data in lanes 2–8 reveal that DNA is released from these films in an open-circular or supercoiled conformation, consistent with the results of our past studies of films fabricated on more rigid substrates (for comparison, lane 1 shows a sample of supercoiled plasmid obtained from a stock solution) [45,48]. These data demonstrate that the DNA is not substantially degraded during incorporation and release from these films. In addition, we note that the relative intensities of the different DNA bands in the gel as a function of time are consistent, in general, with the DNA release profile shown in Figure 2 (that is, the majority of the DNA is released during the first hour, followed by the slower release of the remaining DNA). Figures 3B and 3C show the results of an *in vitro* cell transfection assay conducted using COS-7 cells and samples of DNA released from the surfaces of balloons fabricated using polymer **1** and unlabeled plasmid DNA encoding EGFP (see Materials and Methods for additional details related to these *in vitro* experiments). The observation of high levels of EGFP expression in the cells in these experiments demonstrates that the DNA released from these films is released in a form that remains capable of promoting transgene expression in mammalian cells. These results are also consistent with the results of our past studies [23,25,45,48] and provide a platform from which to characterize the ability of these film-coated balloons to promote the delivery of DNA to vascular tissue *in vivo*.

Film-Coated Balloons Promote the Delivery of DNA to Vascular Tissue *in vivo*

Past studies have used rodent models of vascular injury to study the underlying mechanisms responsible for triggering intimal hyperplasia. In these studies, arterial injury was induced in the left common carotid artery by inflating an uncoated 2-French balloon catheter in the artery three times, followed by the intraluminal delivery of adenoviral vectors designed to express various signaling proteins in the injured vascular wall [13]. This approach has led to the identification of key proteins responsible for the activation of intimal hyperplasia. However, the development of gene-based therapies targeted toward the treatment of intimal hyperplasia could benefit considerably from new methods that (i) allow the administration of therapeutics directly from the surfaces of coated catheter balloons [4,15–16] (that is, approaches that do not require additional intraluminal obstruction for the delivery of solutions of vectors) and (ii) exploit new non-viral methods of delivery [4,14].

To characterize the ability of catheter balloons coated with polymer **1**/DNA films to promote the direct delivery of DNA to vascular tissue, we conducted a series of experiments using a

modification of the rodent model of vascular injury used previously to study the development of intimal hyperplasia [13]. Our initial experiments were conducted using films 16 bilayers thick fabricated with polymer **1** and a plasmid DNA construct (pCMV- β) encoding β -galactosidase to permit levels of balloon-mediated transgene expression to be characterized in treated vascular tissue using X-gal staining and optical microscopy. Blood flow through the left carotid artery of Sprague-Dawley rats was restricted, and a small incision was made in the external carotid artery to provide access to the artery. After denuding the endothelium of the left common carotid artery using an uncoated balloon inflated to 2 atm (see Materials and Methods for additional details), we inserted a film-coated balloon into the artery, inflated the balloon to a pressure of 2 atm, and allowed the balloon to remain in contact with the artery for 20 minutes. After deflation and removal of the balloon, the external carotid artery was ligated and blood flow was restored through the common and internal carotid arteries. On the third day after this procedure, the treated artery was removed, stained using X-gal, fixed, frozen, and sectioned for characterization of transgene expression.

Figures 4A and 4B show representative images of thin cross-sections of a balloon-treated artery. The blue precipitate visible in these samples is formed by hydrolysis of the X-gal substrate by β -galactosidase and demonstrates that these films were able to promote transgene expression in the injured vascular wall. Inspection of these images reveals bright blue areas that are visible through several layers of the vascular smooth muscle cells that make up the medial layers of tissue and around approximately two-thirds of the circumference of the artery. Further inspection reveals a fainter blue background that is visible around the entire artery. Sections of the contra-lateral carotid artery (Figures 4C and 4D) that were not treated with a balloon show only small, isolated areas of faint blue staining, suggesting that the levels of blue color observed in Figures 4A and 4B arise from the expression of β -galactosidase in the treated artery.

We also performed a series of *in vivo* experiments using balloons coated with films 16 bilayers thick fabricated using polymer **1** and plasmid DNA encoding EGFP. These experiments were conducted in a manner identical to those described above, with the exception that samples of tissue were analyzed using immunohistochemical and immunofluorescence staining rather than X-gal staining (levels of EGFP-derived fluorescence in samples of tissue arising from these experiments were difficult to interpret directly because of high levels of green autofluorescence in the elastic tissue of the artery). The images in Figure 5A–D show the results of immunohistochemical staining of arterial cross sections arising from these experiments; the locations of brown staining, generated by the reaction of horseradish peroxidase with diaminobenzidine (DAB), identify the presence of cells expressing EGFP. The images in Figures 5E–H show the results of additional immunofluorescence staining of these samples (the red fluorescence arising from a fluorescently labeled secondary antibody indicates EGFP-positive cells). The sections of artery treated with a balloon coated with a polymer **1**/pEGFP-N1 film (Figures 5A–B and 5E–F) again show high levels of EGFP expression in the medial layers of tissue. EGFP expression is observed through several layers of medial tissue and around approximately two-thirds of the circumference of the artery. Control sections of artery treated with an uncoated balloon (Figures 5C–D and 5G–H) were negative for EGFP expression. The results of these experiments are consistent with the results of experiments conducted using the pCMV- β plasmid and provide additional confirmation of the relative levels and distributions of transgene expression observed *in vivo*. When combined, these results demonstrate that polymer **1**/DNA films are able to survive the mechanical challenges associated with the insertion and deployment of these film-coated balloons and deliver functional DNA to the vascular wall.

The images shown in Figures 4 and 5 reveal levels and distributions of tissue transfection to vary around the circumference of the treated arteries. The results of additional experiments also revealed some variability in levels of transfection in arteries collected from different animals treated with otherwise identical film-coated balloons (see Figure S1 of the Supporting Information for additional images and results arising from these experiments). To investigate the origins of these non-uniformities, we conducted a series of experiments designed to characterize (i) the transfer of DNA to the artery from the surface of a balloon and (ii) the influence that restoring blood flow after treatment could have on the amount of DNA transferred to or retained in the arterial wall. For these experiments, catheter balloons were coated with 16 bilayers of polymer 1 and a plasmid construct fluorescently labeled with TMR (similar to the balloon in the image shown in Figure 1) to permit imaging of the location of DNA in treated arterial tissue using fluorescence microscopy. These *in vivo* experiments were performed as described above, with the exception that the treated artery was collected either immediately after removal of the film-coated balloon (i.e., prior to restoring blood flow) or after restoring blood flow to the artery for one hour after treatment.

Figure 6 shows fluorescence microscopy images of cross sections of arteries arising from these experiments; bright areas of red fluorescence correspond to the locations of fluorescently labeled DNA. Figures 6A and 6B correspond to a section of an artery that was removed prior to restoring blood flow, and Figures 6C and 6D show sections of an artery in which blood flow was restored for one hour prior to removal of the artery. In both sets of images, DNA is observed to be distributed non-uniformly around the innermost layers of the artery. The distributions and relative amounts of fluorescence observed in Figures 6A and 6C are similar, suggesting that the restoration of blood flow after balloon-treatment (which could function to dislodge DNA and/or film that is not imbedded firmly into or adhered strongly to the arterial wall) is not likely the primary cause of the non-uniform distributions of transfection observed in Figures 4 and 5. Instead, these images suggest that the non-uniformity of transfection arises from either the nonuniform transfer of DNA or film once the balloon is inflated, or from physical manipulation of the balloon and the artery that occur when the balloon is deflated and removed. We caution, however, that the distributions and amounts of fluorescence visible in the sectioned arteries shown in Figure 6 could also be reduced or otherwise influenced by the physical handling and processing steps associated with the preparation of these tissue samples after removal of the film-coated balloons.

Variations in the transfer of DNA or film to the artery could result from any of several different factors, including (i) the possibility of poor contact between the balloon and the artery after the balloon is inflated, (ii) the incomplete release of DNA from the surface of the balloon during the relatively short 20-minute contact period used in this study, or (iii) cracking, peeling, or other potential damage to the film that could occur upon the inflation and expansion of these film-coated balloons. The likelihood of poor contact between the balloon and artery was minimized by the selection of inflation pressures that cause the artery to be slightly distended by the balloon. To investigate the latter two of these issues, we conducted additional experiments to characterize gross changes in film morphology after the inflation and deflation of balloons coated using fluorescently labeled DNA. For balloons that were inflated and deflated in air prior to characterization, we observed some cracking and wrinkling, but levels of fluorescence were generally uniform over the surfaces of the balloons (data not shown), suggesting that inflation alone does not dislodge large pieces of film. Additional characterization of film-coated balloons after deflation and removal from an artery (e.g., as used in the experiments described above to generate the results shown in Figure 6) revealed low and generally uniform levels of red fluorescence on the surface of the balloon, suggesting that these films had not completely eroded or released all of their DNA during the relatively short 20-minute procedure used in this study.

The observation of residual fluorescence on these balloons is not unexpected in view of the longer times required for the complete release of DNA into solution (e.g., 24 hours; Figure 2) when film-coated balloons were incubated in buffer. We note in this context, however, that physical contact between the film and the arterial wall and other differences in the compositions of PBS and the fluid in the arterial environment could lead to differences in the erosion and release profiles of these films *in vivo*. Finally, we note that the non-uniform distributions in DNA transfer and tissue transfection could also arise from spatial variations in the initial injuries to the vascular wall that were induced prior to the introduction and inflation of the film-coated balloons. Although this final possibility is not addressed explicitly by this study, non-uniform injury could influence the extent to which DNA is transferred and distributed during subsequent treatment with film-coated balloons.

Additional characterization of films fabricated using different cationic polymer structures will be required to optimize the performance of these DNA-containing thin films further as platforms for delivery *in vivo*. In this context, we note again that polymer **1** was selected for investigation in this current study on the basis of its broad use in several past studies, but that numerous other degradable polyamine structures could be used to influence the physicochemical properties and behaviors of these materials (e.g., to either accelerate or prolong the release of DNA, etc.) [34–35,51,54]. In addition, we note that the layer-by-layer approach to assembly used here provides a straightforward means to manipulate the loading of DNA in these materials (or to design coatings that permit the co-delivery of DNA with other agents) by changing the number of DNA layers deposited or by incorporating additional layers of other bioactive agents. Previous *in vitro* studies of transfection mediated by substrates coated with polymer **1**/DNA films suggest that the presence of polymer **1** (a cationic polymer used in past studies to promote the delivery of DNA to cells) [23,25,49] in these films could also contribute to the surface-mediated internalization and processing of DNA by cells, suggesting opportunities for the incorporation of new polymers or additional agents [36–38] that could promote more effective transgene expression *in vivo*. Consideration of each of these related issues is likely to be important for the optimization of these materials for the balloon-mediated delivery of DNA to the vascular wall or for other localized delivery applications. For example, while films that release DNA rapidly (e.g., over ~20 minutes) would be advantageous from the standpoint of the surgical procedures used in this current study, materials that promote more extended release or that prolong gene expression in the arterial wall may ultimately prove necessary to investigate and/or prevent the development of intimal hyperplasia. Experiments designed to identify new polymers and film architectures that provide more rapid and efficient DNA delivery and evaluate the ability of these assemblies to deliver plasmid constructs relevant to fundamental studies of intimal hyperplasia are currently underway.

Conclusions

We have demonstrated that polyelectrolyte multilayers (PEMs) fabricated using plasmid DNA and a hydrolytically degradable cationic polymer can be used to promote the localized transfection of arterial tissue *in vivo*. Inflatable catheter balloons coated layer-by-layer with thin films fabricated using polymer **1** and DNA encoding either EGFP or β -galactosidase promoted localized tissue transfection in the carotid arteries of rats using a rat model of balloon-induced arterial injury used previously for studies of intimal hyperplasia. The results of our experiments demonstrate that these polymer **1**/DNA films are able to survive mechanical challenges associated with the insertion and inflation of these balloons and deliver functional DNA to the vascular wall. Our results provide a proof-of-concept demonstration of localized *in vivo* transfection using this hydrolytically degradable PEM-based approach and suggest the basis of approaches that could, with further development, be used to locally deliver DNA as part of a broader program designed to prevent, mitigate, or

study fundamental aspects of intimal hyperplasia or other complications arising from vascular interventions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Representative fluorescence microscopy images of film-coated catheter balloons. A) A balloon coated with a film having the structure (polymer **1**/pEGFP-TMR)₁₆ fabricated using TMR-labeled DNA. B) A balloon coated with a film having the structure (polymer **1**/pEGFP)₁₆ fabricated using unlabeled DNA. Dashed lines in B indicate the location of the balloon and are included to guide the eye. Scale bars = 500 μ m.

Figure 2. Plot of DNA released vs. time for a catheter balloon coated with a film having the structure (polymer 1/pEGFP)₁₆ incubated in PBS at 37 °C. DNA release is presented as both total amount released and as a percentage of the total DNA released. Error bars are included but are smaller than the symbols used to represent the data.

Figure 3.

A) Results of agarose gel electrophoresis characterization of samples of DNA released from a catheter balloon coated with a film having the structure (polymer **1**/pEGFP-N1)₁₆ as a function of time incubated in PBS. Lane 1: pEGFP-N1 control (5 µg/mL), Lanes 2–8: samples of DNA solutions collected after 1, 3, 6, 12, 24, 36, and 48 hours, respectively. B, C) Fluorescence microscopy images of monolayers of COS-7 cells (95% confluent) transfected using samples of released DNA collected after (B) 3 hours or (C) 12 hours of incubation of a film-coated balloon in PBS. Scale bars = 200 µm.

Figure 4.

Representative microscopy images of rat carotid arteries with X-gal staining. A, B) Rat carotid artery was injured, treated with a (polymer **1**/pCMV- β)₁₆ coated balloon and harvested after 3 days. C, D) Uninjured contra-lateral carotid artery. Scale bars = 500 μ m (A, C), 200 μ m (B, D).

Figure 5.

Representative images of rat carotid arteries stained for EGFP with immunohistochemical staining (A–D) or immunofluorescence staining (E–H). A, B, E, F) Rat carotid arteries were injured and treated with (polymer **1**/pEGFP)₁₆ coated balloons. C, D, G, H) Rat carotid arteries were treated with uncoated balloons as a control. All arteries were harvested after 3 days and stained for EGFP. Scale bars = 500 μ m (A, C, E, G), 200 μ m (B, D, F, H).

Figure 6.

A–D) Fluorescence images of rat carotid artery treated with balloons coated with (polymer **1**/pEGFP-TMR)₁₆. A, B) The carotid artery was treated with a coated balloon and harvested without restoring blood flow. C, D) The carotid artery was treated with a coated balloon and harvested after restoring blood flow through the artery for 1 hour. E, F) Control carotid artery treated with an uncoated balloon after restoring blood flow through the artery for 1 hour. Scale bars = 500 μm (A, C, E), 200 μm (B, D, F).