Published in final edited form as:
Biomacromolecules. 2013 May 13; 14(5): 1696–1704. doi:10.1021/bm4005222.

Polyelectrolyte Multilayers Promote Stent-Mediated Delivery of DNA to Vascular Tissue

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Abstract

We report an approach to deliver DNA to vascular tissue *in vivo* using intravascular stents coated with degradable, DNA-containing polyelectrolyte multilayers (PEMs). Ionically-crosslinked multilayers ~120 nm thick were fabricated layer-by-layer on the surfaces of balloon-mounted stainless steel stents using plasmid DNA and a hydrolytically degradable poly(β-amino ester) (polymer **1**). Characterization of stents coated using a fluorescently end-labeled analog of polymer **1** revealed film erosion to be uniform across the surfaces of the stents; differential stresses experienced upon balloon expansion did not lead to faster film erosion or dose dumping of DNA in areas near stent joints when stents were incubated in physiologically relevant media. The ability of film-coated stents to transfer DNA and transfect arterial tissue *in vivo* was then investigated in pigs and rabbits. Stents coated with films fabricated using fluorescently labeled DNA resulted in uniform transfer of DNA to sub-endothelial tissue in the arteries of pigs in patterns corresponding to the locations and geometries of stent struts. Stents coated with films fabricated using polymer **1** and plasmid DNA encoding EGFP resulted in expression of EGFP in the medial layers of stented tissue in both pigs and rabbits two days after implantation. The results of this study, combined with the modular and versatile nature of layer-by-layer assembly, provide a polymer-based platform that is well suited for fundamental studies of stent-mediated gene transfer. With further development, this approach could also prove useful for the design of non-viral, gene-based approaches to preventing complications that arise from the implantation of stents and other implantable interventional devices.

Introduction

Coronary artery disease is a leading cause of death in the United States.[1] This condition is characterized by a buildup of plaque in coronary arteries that leads to restricted blood flow to heart muscle, and treatment often requires surgical intervention.[2,3] Balloon angioplasty

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Supporting Information Available: Alternate versions of Figures 4 and 5 displayed without dashed lines indicating the locations of internal and external elastic lamina. This material is available free of charge via the Internet at:

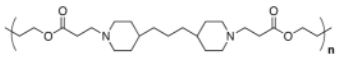
and the implantation of stents have become the preferred interventions for many patients, but post-operative restenosis (a re-blocking of the artery caused by arterial collapse or neointimal hyperplasia) occurs in ~20% of patients treated using these methods.[4,5] Drug-eluting stents coated with polymer films that release small-molecule antiproliferative drugs (e.g., paclitaxel and rapamycin) can reduce the risk of restenosis further (to ~10%).[6–8] However, drug-eluting stents also prevent healthy re-endothelialization. As a result, patients treated with drug-eluting stents must often remain on long-term therapy with anticoagulants to reduce risks of thrombosis.[9–11] New therapeutic approaches that prevent or reduce restenosis without preventing re-endothelialization could reduce these risks and further improve patient outcomes. Several groups have sought to address these and other challenges by developing approaches to the stent-mediated delivery of nucleic acid-based agents, such as DNA or siRNA, to vascular tissue.[12–23] These gene-based methods could offer levels of control over responses to vascular injury that are difficult to achieve using current small-molecule drugs.[17,19,24]

In a series of seminal studies, Levy and coworkers demonstrated the release of plasmid DNA from stents coated with degradable thin films of poly(lactic-co-glycolic acid) (PLGA) or denatured collagen.[12,15] That work demonstrated the feasibility and potential therapeutic value of localized, stent-mediated delivery of DNA to arterial tissue *in vivo*. Other groups have continued to develop non-viral approaches to stent-mediated delivery by incorporation of naked DNA or DNA complexes into thin polymer-based films.[13,16–19,22] Several of these approaches use fabrication methods that result in relatively thick polymer coatings or create webs of excess film suspended between stent struts, and, in general, more work will be required to address important issues associated with controlling the rate of release of DNA and incorporating polymers or other materials that can help promote more effective internalization of DNA by cells. The work reported here takes a step toward addressing these and other important design issues by using nanotechnology-based ‘layer-by-layer’ methods to deposit ultrathin layers of cationic polymers and DNA on the surfaces of stents.

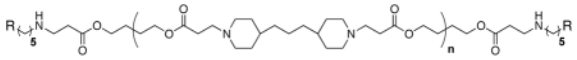
Layer-by-layer assembly is a versatile method for preparing polymer-based films by the alternating adsorption of ‘layers’ of oppositely charged polymers (‘polyelectrolytes’) on surfaces.[25–31] Adsorption of each polymer layer results in a reversal of surface charge that supports the subsequent adsorption of another oppositely charged layer; iterative adsorption leads to the stepwise assembly of ultrathin, ionically-crosslinked ‘polyelectrolyte multilayers’ (PEMs; e.g., ~10–100 nm thick). This method has been used in many past studies to incorporate and release plasmid DNA (an anionic polyelectrolyte),[30] and it is very well suited to the fabrication of thin films that conform faithfully to the surfaces of objects with complex geometries typical of medical devices, including stents.[32–35] In the context of gene delivery, these multilayered films combine layers of DNA with alternating layers of cationic polymers (a class of materials used widely for non-viral DNA delivery[36–38]) that could help promote more efficient internalization and processing of DNA by cells (e.g., by releasing DNA as an aggregate bound to a cationic polymer delivery agent, as opposed to simply releasing unbound, ‘naked’ DNA).[39] In a more general context, several past studies have exploited this layer-by-layer approach to design PEMs relevant to the treatment of cardiovascular diseases and conditions.[32,34,40–47]

The work reported here builds on past reports from our group and others demonstrating that PEMs fabricated using plasmid DNA and hydrolytically degradable poly(β-amino ester)s[34,39,46–53] (e.g., polymer **1**) can be used to promote direct, surface-mediated delivery of DNA to cells.[34,39,46,47,49,52] Of relevance to this current study, we reported that polymer **1** and plasmid DNA can be used to fabricate ultrathin polymer **1**/DNA multilayers on the surfaces of stents.[34] As a step toward the potential application of this ‘multilayered’ approach to stent-mediated gene delivery, this current study sought to characterize the

physical and biological behaviors of these polymer-based materials in more complex environments and determine the extent to which these ultrathin films could be used to promote stent-mediated transfer of DNA to arterial tissue *in vivo* when implanted into the arteries of pigs and rabbits.



Polymer **1**



Polymer $\mathbf{1}_{\mathbf{TMR}}$; R = Tetramethylrhodamine (TMR)

Materials and Methods

Materials and General Considerations

Linear poly(ethyleneimine) (LPEI, M_w = 25,000) was purchased from Polysciences, Inc. (Warrington, PA). Sodium poly(styrene sulfonate) (SPS, M_w = 70,000) was purchased from Aldrich (Milwaukee, WI). Polymer **1** (M_n = 16,000) was synthesized as previously described.[54] A fluorescently end-labeled analog of polymer **1** (polymer $\mathbf{1}_{TMR}$) was synthesized by the conjugation of tetramethylrhodamine cadaverine (TMR-cad) to an acrylate end-functionalized derivative of polymer **1**, as described recently.[55] Plasmid DNA constructs encoding enhanced green fluorescent protein (pEGFP-N1, 4.7 kb, > 90% supercoiled) or firefly luciferase (pCMV-Luc, 6.2 kb, > 90% supercoiled) were purchased from Elim Biopharmaceuticals, Inc. (San Francisco, CA). Concentrated phosphate-buffered saline (PBS) and sodium acetate buffer were purchased from EMD Chemicals (Gibbstown, NJ) and Lonza (Rockland, ME), respectively. An assortment of balloon-mounted, 316L stainless steel stents manufactured by Cordis (Bridgewater, NJ) or Boston Scientific (Natick, MA) were obtained from the Cardiovascular Physiology Core Facility at the University of Wisconsin. Legend V 316L stainless steel stents (3.0 mm diameter, 10 mm length) mounted on balloon catheters were purchased from Relisys Medical Devices (Hyderabad, India). Goat anti-GFP was purchased from Abcam (Cambridge, MA). Donkey anti-goat IgG conjugated to Alexa Fluor 594 was purchased from Invitrogen (Carlsbad, CA). Label-IT TM-rhodamine nucleic acid labeling kits were purchased from Mirus Bio, LLC (Madison, WI) and used according to the manufacturer's instructions (labeling density = 1 label:200 bp). Deionized water (18 MΩ) was used to prepare all buffers and polymer solutions.

Preparation of Polyelectrolyte Solutions

Solutions of LPEI and SPS used for fabricating LPEI/SPS precursor layers were prepared at 20 mM (with respect to the molecular weight of the polymer repeat unit) in 26 mM aqueous sodium chloride. LPEI solutions had 8 mM HCl added to aid polymer solubility. Solutions of polymer **1** and polymer $\mathbf{1}_{TMR}$ were prepared at 5 mM (with respect to the molecular weight of the polymer repeat unit) in 100 mM sodium acetate buffer (pH 4.9). All synthetic polymer solutions were filtered and transferred into 1.5 mL centrifuge tubes (sterilized under UV irradiation in a Class II Biological Safety Cabinet for 15 minutes) using sterile 0.2 μm pore nylon syringe filters. Plasmid DNA solutions were prepared at 1 mg/mL in 100 mM sodium acetate buffer (pH 4.9). The stock sodium acetate buffer used to dilute the DNA was sterilized by filtration with 0.2 μm nylon syringe filters prior to diluting the DNA. Neither stock nor diluted solutions of DNA were filtered prior to use.

Fabrication of Multilayered Films on the Surfaces of Stainless Steel Stents

The fabrication of polymer **1**/DNA and polymer $\mathbf{1}_{TMR}$/DNA films on the surfaces of stainless steel stents was performed using a dipping protocol optimized for the fabrication of these films on silicon and glass substrates and used previously to fabricate these films on stents.[34,48] Films were fabricated on the surfaces of stents mounted and crimped onto catheter balloon deployment systems. Prior to the fabrication of polymer **1**/DNA films, the stents were coated with 10 layer pairs (or 'bilayers') of a multilayered film composed of LPEI and SPS (~20 nm thick, fabricated with a top layer of SPS) to provide a sufficiently charged surface for the adsorption of polymer **1**, as previously described.[34,48] Polymer **1**/DNA films were fabricated on these precursor layers by: (1) immersing the stents in a solution of polymer **1** for 5 minutes, (2) rinsing the stents in 100 mM sodium acetate buffer (pH = 4.9) for 1 minute, followed by a second 1 minute rinse in fresh sodium acetate, (3) immersing the stents in a solution of DNA for 5 minutes, and (4) rinsing the stents in the manner described above. This process was repeated until the desired number of polymer **1**/DNA bilayers (typically eight) was adsorbed. After fabrication, the outside surfaces of the stents were rinsed briefly with sterile, filtered deionized water, and allowed to air dry. For stents intended for implantation in rabbits or pigs, this coating procedure was carried out entirely within a sterile Class II biological safety cabinet (BSC). In addition, the inside surfaces of the guiding catheters (through which guide wires are passed during stent implantation) were rinsed with sterile, filtered deionized water and dried using suction to prevent the formation of polymer or salt deposits that could prevent free passage of the guide wire through the catheter. Stents were replaced in their sterile packaging and stored in a BSC (typically overnight) prior to use in surgical implantation experiments. For experiments in which fluorescently labeled DNA was incorporated into the film structure (see text), 25% of the DNA (by weight) used to prepare aqueous dipping solutions was labeled with TMR (the remainder of the plasmid in these solutions was unlabeled). For experiments in which poly $\mathbf{1}_{TMR}$ was incorporated into the film structure, 10% of the polymer (by weight) used to prepare the aqueous dipping solutions was fluorescently labeled.

Characterization of the Release of DNA and Polymer 1 from Film-Coated Stents

Balloon-mounted stents used to characterize DNA release profiles were coated with polymer $\mathbf{1}_{TMR}$/DNA films eight bilayers thick using methods described above. Fluorescence microscopy was used to characterize the uniformity of the coating on the surface of the stents. The stents were subsequently expanded by inflating the balloon to a pressure of 10 atm using deionized water and a standard inflator. The balloons were then deflated and the stents were carefully removed. The films were imaged again using fluorescence microscopy to characterize the influence of stent expansion on the uniformity of the films (e.g., to characterize the presence or absence of flaking and peeling, etc.). These stents were then submerged and incubated in PBS (pH = 7.4) at 37 °C in 1.5 mL microcentrifuge tubes. At predetermined intervals, the stents were removed from solution and the absorbance of the solution was measured at a wavelength of 260 nm (the absorbance maximum of DNA) using a DU 520 UV/visible spectrophotometer (Beckman Coulter, Brea, CA). At each time point, the stents were rinsed gently with deionized water and dried under a stream of filtered air, and fluorescence microscopy was used to characterize changes in the intensity and uniformity of the polymer $\mathbf{1}_{TMR}$/DNA films as a function of incubation time. After each measurement, the stents were placed in fresh PBS and incubated at 37 °C.

Implantation of Film-Coated Stents in Pigs and Rabbits

All experiments involving animals were conducted in accordance with the NIH Guide for the Use and Care of Laboratory Animals, and using protocols approved by the University of

Wisconsin Institutional Animal Care and Use Committee (protocol #M01839). Swine (35–50 kg) were pre-medicated with a drug cocktail composed of Telazol (4–6 mg/kg IM), Xylazine (2 mg/kg, IM) and Atropine (0.05 mg/kg IM), Thiopental (10–15 mg/kg IV), intubated, and anesthetized with isoflurane (1–5%). New Zealand white rabbits (3–3.5 kg) were pre-medicated with ketamine (30–50 mg/kg, IM), intubated, and anesthetized with isoflurane (1.5–2%). All animals were placed on a heating pad to prevent a drop in body temperature upon administration of anesthesia. After right carotid artery cut-down, an appropriately sized introducer sheath was positioned in the right carotid artery. The animals were treated with heparin (10–300 units/kg IV) initially and every hour thereafter (10–150 units/hr for rabbits, 500–3000 units/hr for pigs). A standard 0.014 inch diameter guide wire was positioned in the femoral artery (for pigs) or the femoral or iliac artery (for rabbits) using angiography. Coated or uncoated stents, mounted on balloon catheters, were advanced over the guide wire to the site of implantation. The stents were inflated to an appropriate pressure (6–14 atm, depending on the sizes of the stent and artery). In a typical experiment (n = 3 for both pigs and rabbits), animals received from 4 to 6 stents, consisting of: 2 to 4 stents coated with polymer **1**/pEGFP films, and one each of a bare metal (uncoated) stent and a stent coated with a polymer **1**/pCMV-Luc film. The number of stents with films containing pEGFP varied and was adjusted as necessary based on the exact positioning of the stents in each animal. Stents were implanted such that control stents (e.g., bare metal stents or stents coated with a polymer **1**/pCMV-Luc films) were implanted proximally to stents coated with films containing pEGFP, to prevent potential artifacts associated with distal transport of DNA by blood flow. After stent implantation, all devices were removed and the wound was closed in layers.

Characterization of Dissemination of DNA in Porcine Femoral Arteries in vivo

Experiments designed to characterize the dissemination of DNA to arterial tissue in the femoral arteries of pigs were conducted by implanting stents coated with films fabricated using DNA fluorescently labeled with TMR. Stents were implanted as described above. Two days after stent implantation, the pigs were euthanized using Beuthanasia-D (1 cc/10 lb). The stented sections of artery were removed and the tissue was processed in one of two ways. In the first method, the stent and artery were cut lengthwise with scissors, and the stent was removed. The opened artery was placed on a glass microscope slide to allow imaging of the inside (luminal) surface of the artery. The surface of the artery was imaged using an Olympus IX70 fluorescence microscope equipped with a mercury vapor lamp and a Texas Red filter cube (Chroma Technology Corp.; Bellows Falls, VT; exciter: 560/55, emitter: 645/75, beamsplitter: 595LP). Images were acquired using the MetaVue version 7.1.2.0 software package, and analyzed using Adobe PhotoShop and ImageJ (NIH; Bethesda, MD). In the second tissue processing method, the stents were removed and the intact vessels were cross-sectioned into equal portions prior to snap freezing in O.C.T. Sections were cut at a thickness of 5 microns and characterized immediately using a fluorescence microscope.

Characterization of Cell Transfection in vivo

Experiments designed to evaluate the ability of stents coated with polymer **1**/DNA films to promote transgene expression in vascular tissue were conducted as follows. Stents [either (i) uncoated, or (ii) coated with films fabricated using unlabeled plasmid DNA encoding enhanced green fluorescent protein or firefly luciferase] were implanted in the femoral or iliac arteries of pigs and rabbits as described above. Two days after stent implantation, the animals were euthanized with Beuthanasia-D (1 cc/10 lb). The stented sections of artery were removed and fixed overnight in 10% neutral buffered formalin. The stents were removed and the remaining vessels were cross-sectioned into equal segments and embedded in paraffin blocks. Samples were then sectioned at a thickness of 5 micrometers and either

stained with hematoxylin & eosin (H&E) or submitted for immunofluorescence staining using an anti-GFP antibody. Paraffin sections were deparaffinized and rehydrated through a graded alcohol series to deionized water. Tissue sections were blocked to prevent nonspecific antibody binding with 10% bovine serum albumin in PBS for 1 hour. Tissue sections were incubated overnight in goat anti-GFP primary antibody (Abcam; diluted 1:1,500) at 4 °C. Sections were then washed with TBS/tween. GFP expression was characterized using immunofluorescence methods by incubating tissue sections with a donkey anti-goat IgG conjugated to Alexa Fluor 594 (Invitrogen; diluted 1:1,000) for 35 minutes. The sections were then characterized by fluorescence microscopy.

Results and Discussion

We selected PEMs fabricated using polymer **1** for use in this investigation for several reasons. First, our past study demonstrated that polymer **1** and plasmid DNA can be used to fabricate uniform and conformal PEMs (~120 nm thick) on the surfaces of stainless steel stents, and that these coatings can withstand simple mechanical challenges associated with stent deployment (e.g., passage through the shaft of an arterial introducer and balloon expansion, as characterized by scanning electron microscopy (SEM)).[34] Second, this simple model system has been well characterized both *in vitro* and from a physicochemical standpoint.[34,48,49,51,56–58] Finally, recent studies by our group[46,47] and others[52] demonstrate that polymer **1**/DNA films can be used to promote surface-mediated transgene expression *in vivo*, including to vascular tissue in rats after short-term administration using film-coated inflatable catheter balloons.[46,47] These past results suggested that PEMs fabricated using polymer **1** would be well suited for short-term investigations of stent-mediated delivery.

For all studies described below, films were assembled layer-by-layer directly on the outer surfaces of stainless steel stents crimped onto inflatable polymer balloons, and were fabricated to contain eight layer pairs (referred to hereafter as ‘bilayers’) of plasmid DNA and either (i) polymer **1** or (ii) a fluorescently end-labeled derivative of polymer **1** (polymer $\mathbf{1}_{TMR}$; to permit characterization of film erosion using fluorescence-based methods).[39,55] This approach to fabrication resulted in the deposition of thin, conformal films on the outer surfaces of the stents as well as exposed portions of the balloon assemblies that were not covered by the crimped stents. On the basis of our past studies[34] (and independent characterization of otherwise identical films fabricated on reflective silicon substrates), we estimate the films investigated here to be ~120 nm thick.

Characterization of the Erosion of Films on Balloon-Expanded Stents

Our past study demonstrated that polymer **1**/DNA films do not crack, peel, or delaminate substantially from stent surfaces after expansion in air, as determined by SEM characterization of dry expanded stents.[34] It is possible, however, that physical stresses experienced by portions of film located near stent joints could lead to changes in structure that influence film erosion and release profiles after introduction to aqueous environments (and possibly lead to more rapid release or ‘dose dumping’ of DNA at these locations). To characterize changes in the uniformity of these coatings during incubation and film erosion (e.g., when the stents are submerged), we coated stents with films fabricated using polymer $\mathbf{1}_{TMR}$ and a plasmid DNA construct encoding enhanced green fluorescent protein (EGFP). This permitted simultaneous characterization of DNA release (by measurements of solution absorbance) and film erosion (using fluorescence microscopy) during incubation. Figure 1 shows the DNA release profile for a stent coated with a polymer $\mathbf{1}_{TMR}$/DNA film that was balloon-expanded and incubated in PBS at 37 °C. These results reveal DNA (~20 μg) to be released gradually from the surface of the stent over a period of ~3 days. This profile is consistent with DNA release profiles reported in past studies of films fabricated using

unlabeled polymer **1**[34,48,51] and suggests that the use of polymer $\mathbf{1}_{TMR}$ in the studies reported here does not lead to large changes in film behavior.

Figure 2 shows fluorescence microscopy images of a film-coated, balloon-expanded stent (A) prior to, and after (B) 5 hours or (C) 12 hours of incubation in PBS. Red fluorescence in the image in panel A reveals both the joints and the struts of the stent to be coated uniformly prior to incubation. The images in panels B and C reveal time-dependent decreases in fluorescence intensity that are consistent with gradual film erosion (e.g., Figure 1) and the loss of polymer $\mathbf{1}_{TMR}$ from the stent surface. Further inspection reveals these decreases in fluorescence to occur in a manner that is generally uniform in areas on and around both the joints and struts of the stent. These results demonstrate that potential changes in local film structure that could arise during expansion and physical manipulation do not result in large differences in film erosion (e.g., substantially faster erosion or complete dose dumping) in areas on or around stent joints.

The images in Figure 2 also reveal, within the limits of inspection at this magnification, that forces experienced during stent expansion do not result in large-scale cracking, peeling, or delamination of film in other locations during incubation and erosion (that is, we did not observe large patches devoid of fluorescence during these experiments, as would result if large portions of film were to delaminate or fall off during incubation). We note that while small punctate areas of brighter red fluorescence are also observed in each of these images, these features were generally distributed uniformly over the entire surface of the stent, and they decreased in intensity over time in a manner that was also generally uniform across the stent surface.

Characterization of Stent-Mediated Dissemination of DNA in the Arteries of Pigs

To characterize the ability of film-coated stents to release and transfer DNA to arterial tissue *in vivo*, we conducted a series of stent implantation experiments using films fabricated using polymer **1** (unlabeled) and fluorescently labeled plasmid DNA. For these experiments, 25% of the DNA (by weight) used to prepare aqueous dipping solutions used for film fabrication was labeled with TMR (the remainder of the plasmid was unlabeled). Stents coated with these films were then implanted in the femoral arteries of pigs. Two days after implantation, stented sections of artery were harvested and tissue samples were prepared for characterization by either (i) cutting the arteries and stents lengthwise with scissors, carefully removing the stent, and placing the artery on a microscope slide to allow imaging of the inside (luminal) surface of the whole artery, or (ii) by removing the stent and then freezing and sectioning the artery to provide cross-sectional views. Figures 3A–B show images of the luminal surface of an artery after removal of a film-coated stent and reveal ‘zig-zag’ patterns of red fluorescence that correspond closely to the strut geometries of the stents used in this experiment.

These results demonstrate (i) that these ultrathin polyelectrolyte-based films were able to survive processes associated with implantation (including, in this experiment, mechanical challenges during passage through the vasculature and contact with blood and tissue prior to expansion), and (ii) that they released DNA locally and uniformly to arterial tissue with which they were placed in contact. We note that these images also show lower levels of background red fluorescence in areas of tissue surrounding the patterns transferred by contact with stent struts. This background fluorescence arises from normal arterial tissue autofluorescence that was also observed in un-stented control arteries.

Figures 3C–D show cross-sectional images of arterial tissue two days after implantation of a film-coated stent (stents were removed prior to processing; see discussion above and Materials and Methods for additional details). In these images, green fluorescence

corresponds to autofluorescence arising from elastic tissue in the artery that was also present in bare metal stent (no-film) controls; red fluorescence again shows the location of fluorescently labeled DNA. We draw several conclusions on the basis of these results. First, these images reveal red fluorescence to be localized in sub-endothelial areas of the arterial wall. Second, although the stents used in these experiments were removed prior to tissue processing, the localization of high levels of red fluorescence near indentations in the arterial wall suggests that the transfer of DNA occurred locally and primarily in areas that were in direct contact with film-coated stent struts. These results demonstrate that this polyelectrolyte-based approach can be used to deliver DNA into the relatively thick porcine arterial wall.

Finally, we note again that the protocol used to coat the stents used here also resulted in the deposition of polymer **1**/DNA films on exposed portions of the balloons on which the stents were crimped. We reported recently that inflatable catheter balloons (i.e., without stents) coated uniformly with polymer **1**/DNA films could be used to transfer DNA and promote circumferential transgene expression in the balloon-injured arteries of rats.[46,47] The highly localized nature of the red fluorescence observed in Figures 3C and 3D (and, specifically, the absence of red fluorescence in other surrounding areas of the tissue), suggest that balloon-mediated transfer of DNA did not occur to a significant extent in these pig-based experiments. The high levels of circumferential gene expression observed in our past studies on balloon-transfer in rats likely resulted from the fact that, in those experiments, film-coated balloons were expanded and left in direct contact with injured tissue for 20 minutes.[46,47] In contrast, the balloons used in stent-mediated experiments in this current study were inflated for only 15–20 seconds prior to deflation and removal. In addition, we note that the arterial tissue of rats is significantly thinner than that of pigs, and the tissue treated in those past experiments was balloon-injured prior to experiment.[46,47] In a broader context, we note that layer-by-layer assembly is also very well suited for coating the surfaces of expanded stents[34] that could subsequently be crimped onto bare balloon assemblies after fabrication. This approach, while technically straightforward, was not adopted here to minimize potential influences that crimping procedures could have on the structures or behaviors of the films used in these proof-of-concept experiments.

Characterization of Stent-Mediated Transfection in Pigs

To characterize the ability of film-coated stents to promote localized transfection of arterial tissue, we conducted a series of experiments in which film-coated stents (fabricated using unlabeled polymer and unlabeled plasmid encoding EGFP) were implanted in the femoral arteries of pigs. Two days after stent implantation, the stented arteries were harvested, fixed, embedded in paraffin, and sectioned for analysis. Levels of normal, tissue-derived green autofluorescence (as noted above and visible in Figure 3) complicated direct characterization of green EGFP expression in these samples using fluorescence microscopy. We therefore used indirect immunofluorescence staining to characterize the presence and location of EGFP in tissue samples arising from these experiments.

Figures 4A–B show fluorescence microscopy images of cross-sections of arteries stained with goat anti-GFP and donkey anti-goat IgG conjugated to Alexa Fluor 594; red fluorescence reveals the locations of the fluorescently labeled secondary antibody (and, thus, regions in which EGFP was expressed). To determine the extent to which areas of red fluorescence corresponded to specific staining of EGFP-expressing cells, sections of arteries in which control stents were implanted [either bare metal (uncoated) stents (4C) or stents coated with an otherwise identical film fabricated using polymer **1** and plasmid DNA encoding firefly luciferase (pCMV-Luc; 4E–F)] were also stained with anti-GFP. The levels of red fluorescence observed in the media of these control sections and other sections of

untreated arteries (not stented; Figure 4D) were lower than levels observed in the media of arteries treated with stents coated with polymer **1**/pEGFP films. This suggests that the red fluorescence observed in Figures 4A–B results from specific staining of cells expressing EGFP, and that it is does not arise from either (i) the implantation of the stents themselves, or (ii) the polymer or DNA used to fabricate the films. We note that while the images in Figure 4A–B also reveal the presence of red fluorescence in intimal layers (i.e., the thin layer of cells on the luminal sides of the arteries), some non-specific staining in the intima of was also observed in control arteries (e.g., Figure 4C,F).

The results above demonstrate that stents coated with these ultrathin polymer **1**/DNA films can promote localized transfection of porcine arteries *in vivo*. Further inspection of the images in Figures 4A–B reveals EGFP expression to be confined primarily to the medial layer of the arterial wall, and that expression is, in general, patchy and not uniformly distributed around the circumference of the artery (consistent with strut-mediated, as opposed to balloon-mediated, transfer of DNA, as discussed above). These results are consistent with the localized transfer and relatively limited transport of DNA – and the subsequent internalization and successful processing of DNA by cells – over the 48-hour time course of these experiments. The EGFP-positive staining in these images extends over areas that are larger than the more confined areas of red fluorescence observed for the strut-mediated transfer of fluorescently labeled DNA shown in Figure 3. The reasons for this are not completely understood, although we note here that the images in Figure 4 reveal the location of EGFP, not the location of DNA itself as shown in Figure 3, and that the tissue samples used in these two experiments were processed in different ways prior to imaging.

The results of this study, combined with the modular nature of layer-by-layer assembly, provide a platform well suited for fundamental studies of stent-mediated gene transfer. This study sought to characterize the ability of polymer **1**/DNA films to promote stent-mediated transgene expression in vascular tissue *in vivo* and establish proof of concept over an initial two-day implantation period. Our current results thus do not permit conclusions regarding the potential of these polymer films to promote expression over longer time periods, and additional characterization of this and other parameters will be necessary to further evaluate the therapeutic potential of this approach. In this broader context, levels and time-courses of expression of potential therapeutic genes are likely to depend upon a number of additional factors. For example, in addition to cell-based challenges associated with delivering DNA to vascular tissue, there are physiological/surgical considerations (e.g., thickness of the arterial wall, extent of existing disease or injury, stent strut design) as well as many material properties (e.g., polymer structure, film thickness, DNA loading, and release profile) that provide opportunities to evaluate this approach further and achieve specific therapeutic outcomes (e.g., to prevent restenosis or promote more effective re-endothelialization using therapeutic DNA constructs). Past studies demonstrate that this layer-by-layer approach can be used to vary the loading or extend and manipulate release profiles of DNA-containing PEMs by varying the structures of the cationic polymers used to fabricate these erodible assemblies.[34,50,59–61] Our past studies also suggest that the cationic polymers in these coatings could play important roles in promoting the internalization and processing of DNA (e.g., by releasing DNA as an aggregate bound to cationic polymer, rather than as ‘naked’ DNA).[34,39,61] The results of this current study do not shed light on the extent to which polymer and DNA remain associated after release *in vivo*. However, the juxtaposition of polymer **1** and DNA in these coatings, combined with the ability to incorporate ‘layers’ of other, more conventional cationic gene delivery polymers that could address specific barriers to intracellular delivery, suggest opportunities to design ‘multilayered’ coatings that promote more effective stent-mediated transfer and expression of DNA in therapeutic contexts.

Characterization of Stent-Mediated Transfection in Rabbits

Finally, as a first step to investigate the potential influence of arterial anatomy on cell transfection and gene expression, we conducted an additional series of *in vivo* stent implantation experiments using rabbits. We selected rabbits for use in these studies because the vascular walls of rabbits are significantly thinner than those of pigs, and rabbits have been used in several past studies on stent-mediated DNA delivery.[13,16,18,22] Stents coated with films composed of eight bilayers of polymer **1** and the pEGFP-N1 plasmid (identical to those used above in studies in pigs) were implanted in the femoral and iliac arteries of New Zealand White Rabbits. Two days after implantation, stented arteries were harvested and fixed, the stents were removed, and the tissue was embedded in paraffin and sectioned for analysis. Cross-sections of tissue arising from these experiments were characterized using indirect immunofluorescence staining methods similar to those described above.

Figure 5 shows fluorescence microscopy images of tissue sections arising from these experiments. Compared to the results of experiments using pigs described above, we observed EGFP expression in the medial layers of stented arteries to be spread over larger areas around the circumference of the arteries (Figures 5A–B; see red fluorescence). Levels of red fluorescence in sections of un-stented tissue or tissue treated with control stents coated with films fabricated using pCMV-Luc (Figures 5C–D) were again lower than those observed in tissue treated with films fabricated using pEGFP. Overall, these differences in transfection in pigs and rabbits are likely to result, at least in part, from significant physiological differences in the structures of the arteries of these animals. As noted above, the arterial walls of rabbits are thinner than those of pigs; procedures associated with balloon expansion and stent implantation in rabbits could thus result in higher levels of arterial injury that allow DNA greater access to vascular tissue.

Summary and Conclusions

We have demonstrated that stents coated with PEMs fabricated using degradable polymer **1** can be used to promote stent-mediated transfer of DNA to arterial tissue in pigs and rabbits. Implantation of stents coated with films fabricated using fluorescently labeled plasmid DNA resulted in uniform, localized transfer of DNA to sub-endothelial tissue in pigs in patterns that corresponded to the locations and geometries of the stent struts. Additional fluorescence microscopy studies using polymer $\mathbf{1}_{TMR}$ demonstrated that film erosion was uniform across the surfaces of the stents, and that differential mechanical stresses experienced during expansion do not result in faster film erosion or dose dumping of DNA near stent joints. Immunofluorescence staining of arteries treated with film-coated stents fabricated using DNA encoding EGFP revealed localized expression of EGFP in the medial layers of stented tissue in both rabbits and pigs two days after implantation. Our results demonstrate that PEM-coated stents are capable of promoting localized transgene expression *in vivo* in two animal models that are used widely for the pre-clinical evaluation and testing of drug- and gene-eluting stents. The results of this proof-of-concept study, combined with the modular and versatile nature of the layer-by-layer assembly process used here, provide a polymer- and nanotechnology-based platform that is well suited for fundamental studies of stent-mediated gene transfer. With further development, this approach could also prove useful for the development of new non-viral gene therapy approaches to preventing complications that arise from the implantation of stents and other implantable interventional devices.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support was provided in part by the National Institutes of Health (R01 EB006820). This work made use of shared facilities supported, in part, by the National Science Foundation through a grant to the Materials Research Science and Engineering Center (MRSEC) at the University of Wisconsin (DMR 1121288). E.M.S. was supported in part by a 3M Foundation Graduate Research Fellowship. The authors are grateful to Guoqing Song, Larry F. Whitesell, and Matthew R. Wolff for assistance with surgical procedures and for many helpful discussions.

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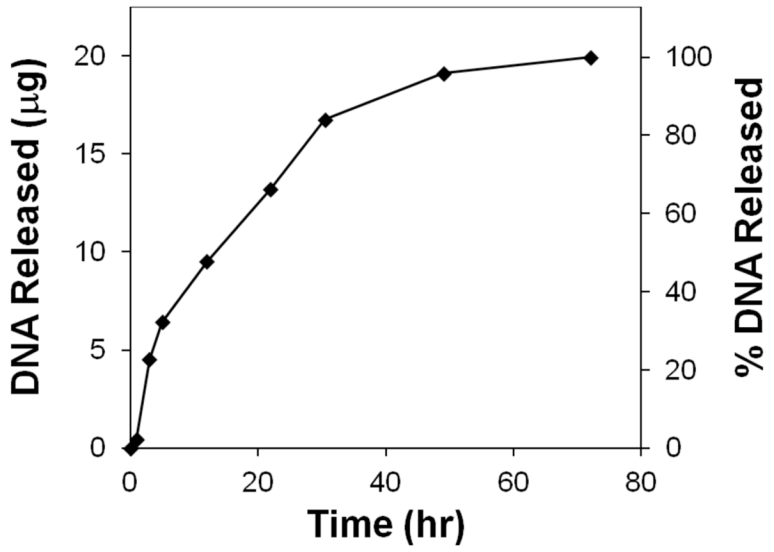

Figure 1.
Plot of DNA released vs. time for a stent coated with a polymer $\mathbf{1}_{TMR}$/pEGFP film 8 bilayers thick incubated in PBS at 37 °C. Error bars are shown but are in most cases smaller than the symbols used.

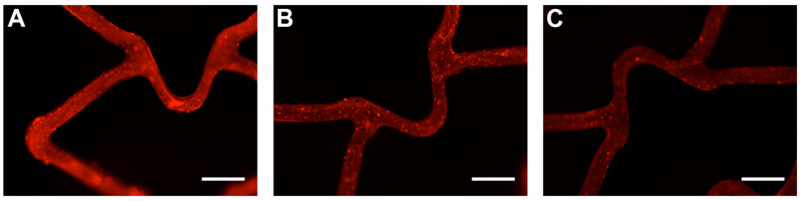

Figure 2.

Fluorescence microscopy images of stainless steel stents coated with polymer $\mathbf{1}_{TMR}$/pEGFP-N1 films eight bilayers thick. Red fluorescence arises from the presence of TMR-labeled polymer **1**. (A) Coated stent after balloon expansion, but prior to incubation, in PBS. (B,C) The same coated stent after incubation in PBS at 37 °C for (B) 5 hours or (C) 12 hours. Scale bars = 500 μm. *[Note for review: Best viewed in color.]*

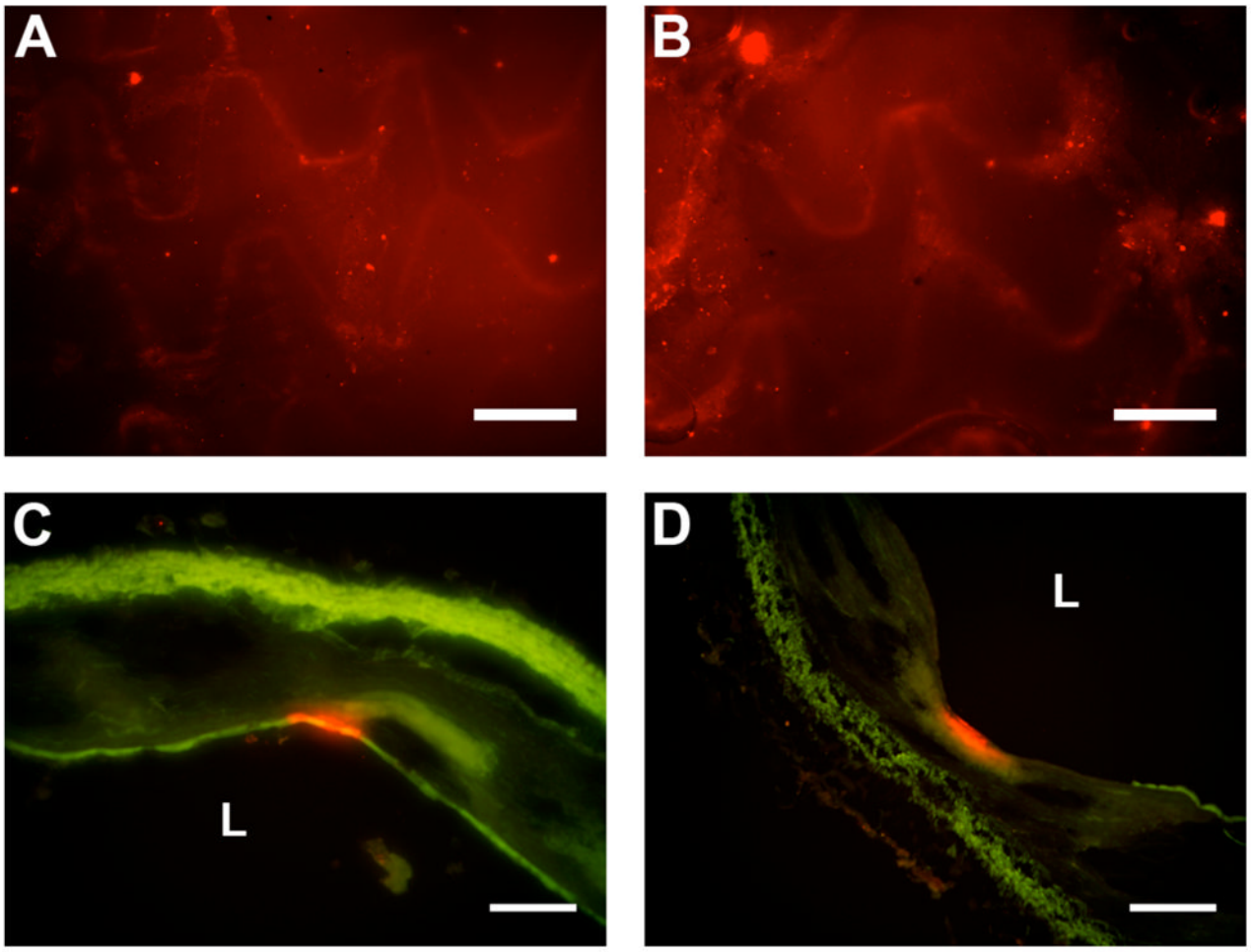

Figure 3.

Fluorescence microscopy images of arteries in which stents coated with polymer **1**/pEGFP-TMR films eight bilayers thick were implanted for two days. All images were acquired after removal of the stents. (A,B) Individual images of the inside (luminal) surfaces of an artery in the stented area of the tissue; the brighter red zig-zag patterns indicate the locations of fluorescently labeled DNA. (C,D) Cross-sections of stented arterial tissue prepared by freezing the tissue in O.C.T. Green fluorescence corresponds to the autofluorescence of elastic tissue in the artery. Red fluorescence corresponds to the fluorescence of TMR-labeled DNA delivered from the surface of the stent. In panels C–D, the designation 'L' indicates the location of the artery lumen. Scale bars are 500 μm for images in A–B and 50 μm for images in C–D. *[Note for review: Best viewed in color.]*

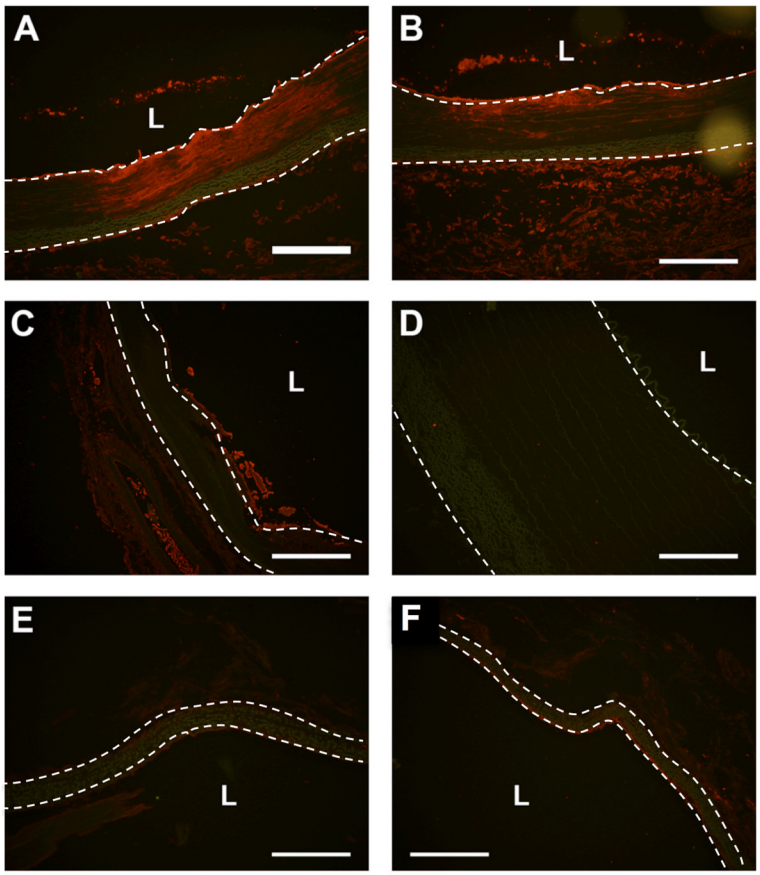

Figure 4.

(A–F) Fluorescence microscopy images of cross-sections of porcine arteries stained with anti-GFP and a red fluorescent secondary antibody. (A–B) Cross-sections of femoral arteries in which stents coated with polymer **1**/pEGFP films eight bilayers thick were implanted for two days. (C) Cross-section of femoral artery in which an uncoated stent was implanted. (D) Un-stented section of carotid artery. (E–F) Cross-sections of femoral arteries in which stents coated with polymer **1**/pCMV-Luc films eight bilayers thick were implanted for two days. Faint green fluorescence visible in some of these images arises from tissue autofluorescence and red fluorescence arises from the presence of the Alexa Fluor 594-conjugated secondary antibody and indicates areas of tissue in which EGFP is expressed. In all images, the designation 'L' indicates the location of the artery lumen, and the approximate locations of the internal and external elastic lamina defining the boundaries of the media are indicated by white dashed lines. An alternate version of this figure displayed without these white dashed lines is included as Figure S1 of the Supporting Information. Scale bars are 75 μm. *[Note for review: Best viewed in color.]*

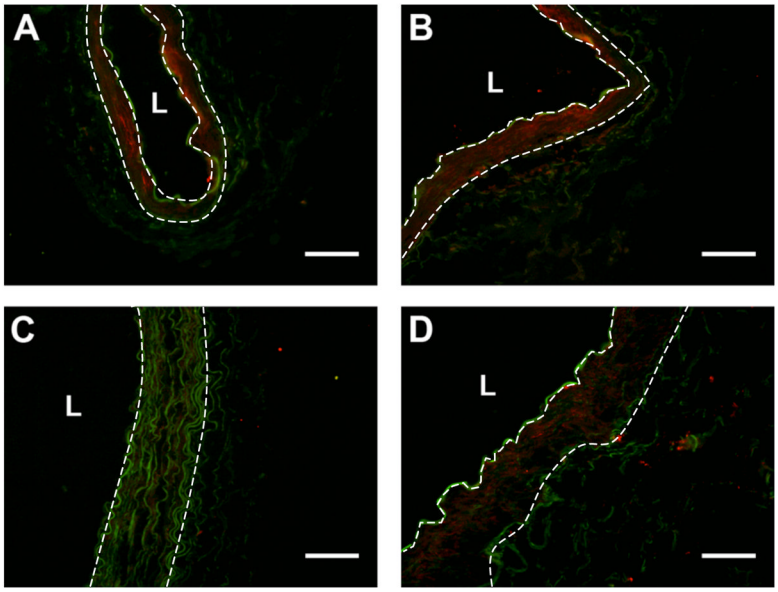

Figure 5.

Fluorescence microscopy images of cross-sections of arterial tissue from the femoral and iliac arteries of rabbits in which stents were implanted for two days. Images were collected after removal of the stents. All polymer **1**/DNA films were eight bilayers thick. (A–B) Cross-sections of arteries in which stents coated with polymer **1**/pEGFP films were implanted; tissue was stained with anti-GFP. (C) Un-stented section of artery stained with anti-GFP. (D) Cross-section of arteries in which a stent coated with a polymer **1**/pCMV-Luc film was implanted; tissue was stained with anti-GFP. Green fluorescence in these images arises from autofluorescence of elastic tissue in the artery and red fluorescence arises from the presence of the Alexa Fluor 594-conjugated secondary antibody used to detect EGFP expression. In all images, the designation 'L' indicates the location of the artery lumen, and the approximate locations of the internal and external elastic lamina defining the boundaries of the media are indicated by white dashed lines. An alternate version of this figure displayed without these white dashed lines is included as Figure S2 of the Supporting Information. Scale bars are 100 μm for images in A, B, and D and 50 μm for the image in C. *[Note for review: Best viewed in color.]*