Articles

Release of Plasmid DNA from Intravascular Stents Coated with Ultrathin Multilayered Polyelectrolyte Films

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Materials that permit control over the release of DNA from the surfaces of topologically complex implantable devices, such as intravascular stents, could contribute to the development of new approaches to the localized delivery of DNA. We report the fabrication of ultrathin, multilayered polyelectrolyte films that permit both the immobilization and controlled release of plasmid DNA from the surfaces of stainless steel intravascular stents. Our approach makes use of an aqueous-based, layer-by-layer method for the assembly of nanostructured thin films consisting of alternating layers of plasmid DNA and a hydrolytically degradable polyamine. Characterization of coated stents using scanning electron microscopy (SEM) demonstrated that stents were coated uniformly with an ultrathin film ca. 120 nm thick that adhered conformally to the surfaces of stent struts. These ultrathin films did not crack, peel, or delaminate substantially from the surface after exposure to a range of mechanical challenges representative of those encountered during stent deployment (e.g., balloon expansion). Stents coated with eight bilayers of degradable polyamine and a plasmid encoding enhanced green fluorescent protein (EGFP) sustained the release of DNA into solution for up to four days when incubated in phosphate buffered saline at 37 °C, and coated stents were capable of mediating the expression of EGFP in a mammalian cell line without the aid of additional transfection agents. The approach reported here could, with further development, contribute to the development of localized gene-based approaches to the treatment of cardiovascular diseases or related conditions.

Introduction

Interventional cardiology, including methods for the delivery of antiproliferative drugs from the surfaces of intravascular stents,[1−5] has contributed broadly to clinical treatments for coronary artery disease and related conditions. The advance of gene-based approaches to the treatment of cardiovascular ailments has progressed more slowly than approaches based on the localized delivery of small-molecule drugs.[6,7] This is due, in part, to a lack of effective and minimally invasive methods for the localized delivery of DNA to vascular tissues. New materials that provide spatial and temporal control over the delivery of DNA from the surfaces of topologically complex devices such as stents would make possible gene-based approaches to the treatment of cardiovascular disease or the prevention of complications that arise from interventional procedures.[6,7] We report here a layer-by-layer approach to the fabrication of ultrathin DNA-containing polyelectrolyte films that permit both immobilization and localized release of plasmid DNA from the surfaces of stainless steel intravascular stents.

The work reported here builds upon a series of recent studies demonstrating that intravascular stents can be used as platforms for the localized delivery of DNA.[6,8−14] This past work has focused largely on the encapsulation of plasmid DNA in thin films of degradable polymer[8,10−13] or the tethering of viruses to collagen-coated stents[9] or bare metal stents.[14] In the long term, methods for nonviral gene delivery have the potential to be safer than methods based on the use of viruses.[15,16] However, past studies on stent-mediated delivery of plasmid DNA have made use of relatively thick (micrometer-scale) films using polymers that have been observed to lead to inflammatory responses in vivo.[17] In addition, conventional methods for the bulk encapsulation of DNA involve the use of organic solvents, and these methods provide limited control over DNA loading and the spatial distribution of encapsulated DNA. The development of ultrathin films that combine the ability to localize DNA at a surface with the ability to control release profiles and promote subsequent internalization would constitute a significant advance and make possible new approaches to localized gene delivery.

The approach reported here is based on layer-by-layer methods[18−20] for the incorporation of DNA constructs into ultrathin, multilayered polyelectrolyte films.[21−26] We recently reported that multilayered polyelectrolyte films fabricated from plasmid DNA and hydrolytically degradable polyamines permit spatial and temporal control over the release of DNA from surfaces.[27,28] For example, multilayered films 100 nm thick fabricated from degradable poly(β-amino ester) **1** and plasmid DNA encoding enhanced green fluorescent protein (EGFP) erode gradually and release DNA over a period of one to two days when incubated in PBS buffer.[27] The DNA released from these films remains transcriptionally active, and we have

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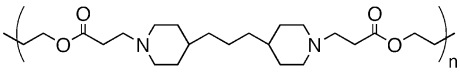
demonstrated that planar objects (e.g., glass slides) coated with these ultrathin films can be used to mediate the localized and contact-mediated transfection of cells in serum-containing cell culture media.[28] These results suggest the basis of an approach that could be used to localize the delivery of plasmid DNA from the surfaces of topologically complex implantable materials and devices.



Polymer **1**

In the context of controlled release, the layer-by-layer approach described above offers several potential advantages relative to conventional methods for the bulk encapsulation of DNA[8,10−13] or other methods recently reported for the immobilization and release of DNA from surfaces.[29−32] First, layer-by-layer fabrication offers a straightforward mechanism for control over the amount of DNA incorporated into a film by control over the number of layers of polymer and DNA deposited.[27] Second, the process used to fabricate these films is entirely aqueous and does not involve the exposure of the DNA or the film itself to organic solvents that could remain in these materials after fabrication. Third, because the DNA in these multilayered assemblies is packaged between alternating layers of cationic polymer (a class of materials that is used broadly to transfect cells),[15,16] these thin films could potentially be designed to participate in the packaging, presentation, and internalization of DNA by cells. Finally, the scope of objects that can be coated with multilayered films is broad[19] and includes materials such as stainless steel[33−35] and other materials used to construct implantable devices.[36−41] Of particular relevance to the work reported here, Thierry et al. recently described the fabrication of multilayered films on the surfaces of nickel−titanium alloy wires and disks and the use of these films for the release of sodium nitroprusside, a nitric oxide donor used clinically to prevent restenosis, to vascular tissue.[42]

This current study sought to determine whether polyamine **1** and DNA could be used to fabricate erodible multilayered assemblies on the surfaces of stainless steel intravascular stents and, subsequently, whether these ultrathin films could withstand a range of mechanical forces typically associated with stent deployment. We report here that multilayered films ca. 120 nm thick fabricated from polyamine **1** and plasmid DNA encoding green fluorescent protein can be deposited homogeneously and conformally onto the surfaces of stainless steel stents using an entirely aqueous layer-by-layer fabrication procedure. Further, we demonstrate that these ultrathin coatings remain intact and substantially defect free after stent expansion and exposure to other physical challenges associated with stent deployment. Finally, we demonstrate that the DNA released from stents coated with these materials is able to mediate the transfection of mammalian cells in vitro. The results reported here could, with further development, contribute to the design of stent-mediated approaches to the localized delivery of DNA to vascular tissues.

Materials and Methods

Materials. Poly(sodium 4-styrenesulfonate) (SPS, MW = 70 000) and sodium acetate buffer were purchased from Aldrich Chemical Co. (Milwaukee, WI). Linear poly(ethylene imine) (LPEI, MW = 25 000) was obtained from Polysciences, Inc. (Warrington, PA). Polymer **1** was prepared as previously reported[43] and isolated by precipitation into hexane. Clinical-grade plasmid DNA [pEGFP-N1 (4.7 kb), >95% supercoiled] was obtained from the Waisman Clinical BioManufacturing Facility at the University of Wisconsin−Madison. Test grade n-type silicon wafers were purchased from Si-Tech, Inc. (Topsfield, MA). An assortment of 316L stainless steel coronary stents and deployment systems from Cordis (Miami, FL), Medtronic (Shoreview, MN), and Boston Scientific (Natick, MA) were obtained from the Cardiovascular Physiology Core Facility at the University of Wisconsin. Stents and catheter balloons were provided in sterile packages and had nominal diameters ranging from 2 to 5 mm and lengths ranging from 8 to 30 mm. Stent and balloon assemblies employed over-the-wire type guidewires, and expansion was performed using a standard inflation device and three-way valve with air or deionized water as the expansion fluid. Phosphate buffered saline was prepared by dilution of commercially available concentrate (EM science, Gibbstown, NJ). Deionized water (18 MΩ) was used for washing steps and to prepare all buffer and polymer solutions. All buffers and polymer solutions were filtered through a 0.2 μm membrane syringe filter prior to use unless noted otherwise. Compressed air used to dry films and coated stents was filtered through a 0.4 μm membrane syringe filter. All materials were used as received without further purification unless noted otherwise.

General Considerations. Silicon substrates (e.g., 0.5 cm × 3.0 cm) were cleaned with acetone, ethanol, methanol, and water, and then dried under a stream of compressed air passed through a 0.2 μm filter. Surfaces were then activated by etching in an oxygen plasma for 5 min (Plasma Etch, Carson City, NV). Ellipsometric thicknesses of films deposited on silicon substrates were determined using a Gaertner LSE Stokes Ellipsometer (632.8 nm, incident angle = 70°). Data were processed using the Gaertner Ellipsometer Measurement Program software package. Relative thicknesses were calculated assuming an average refractive index of 1.55 for the multilayered films. UV−visible absorbance values used to determine plasmid release kinetics were recorded on a Beckman Coulter DU520 UV−vis spectrophotometer (Fullerton, CA). Absorbance values were recorded at a wavelength of 260 nm in triplicate for all samples. For characterization of surface morphology by scanning electron microscopy (SEM), an accelerating voltage of 3 kV was used to obtain images on a LEO DSM 1530 scanning electron microscope. Samples were coated with a thin layer of gold using a sputterer (30 s at 45 mA, 50 mTorr) prior to imaging.

Preparation of Polyelectrolyte Solutions. Solutions of polymer **1** used for dipping (5 mM with respect to the molecular weight of the polymer repeat unit) were prepared in sodium acetate buffer (100 mM, pH = 5.0) and filtered through a 0.2 μm membrane syringe filter prior to use. 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 50 mM NaCl solution in 18 MΩ water. LPEI solutions contained 5 mM HCl to aid polymer solubility. Solutions of plasmid DNA were prepared at 1 mg/mL in sodium acetate buffer and were not filtered prior to use.

Fabrication of Multilayered Films. Films fabricated from polymer **1** and plasmid DNA (pEGFP-N1) encoding enhanced green fluorescent protein were fabricated using a dipping protocol optimized for the deposition of this plasmid/polymer system on planar substrates.[27,28,44] Polymer/DNA films were fabricated on either planar silicon substrates or stainless steel stents mounted and crimped on catheter balloon deployment

systems. Prior to the fabrication of polymer/DNA films, substrates were precoated with 10 bilayers of a multilayered film composed of LPEI and SPS (ca. 20 nm thick, terminated with a topmost layer of SPS) to ensure a suitably charged surface for the adsorption of polymer **1**, as previously described.[27,28,44] Polymer **1**/DNA layers were deposited on these foundation layers either manually or by using an automated dipping robot (Model DR-3, Riegler & Kirstein GmbH, Berlin, Germany) using an alternating dipping procedure. Briefly: (1) Substrates were submerged in a solution of polymer **1** for 5 min, (2) substrates were removed and immersed in a wash bath of 100 mM sodium acetate buffer (pH = 5.0) for 1 min followed by a second wash bath for 1 min, (3) substrates were submerged in a solution of plasmid DNA for 5 min, and (4) substrates were rinsed in the manner described above. This cycle was repeated until the desired number of polymer and DNA layers (typically eight) had been deposited. Stents coated using this procedure were dried under a stream of filtered compressed air and were either used immediately or stored in a vacuum desiccator until use. All films were fabricated at ambient room temperature.

Mechanical Manipulation and Imaging of Coated Stents. For experiments designed to evaluate the effects of stent expansion on film integrity and release profiles, balloon-mounted coated stents (fabricated and prepared as described above) were attached to a syringe or standard inflation device, and a pressure of 6−10 atm was applied by injecting air or deionized water into the balloon. The inflation pressure was maintained for 30 s, followed by aspiration of the fluid and removal of the stent from the balloon assembly. To characterize film integrity upon exposure to other mechanical forces typically encountered during stent deployment, coated stent/balloon assemblies were passed along a guide wire through the septum and shaft of an arterial inducer prior to expansion; the stent was subsequently expanded and removed from the catheter assembly as described above. Stents manipulated using these procedures were either used for characterization by SEM (as described above) or used directly in subsequent erosion and release experiments (as described below).

Evaluation of Plasmid Release Kinetics. Experiments designed to investigate the erosion and release profiles of multilayered polymer **1**/plasmid DNA stent coatings were performed in the following general manner: Film-coated stents (Boston Scientific Express2 Monorail; 3.5 mm × 24 mm; coated and then balloon expanded as described above) were placed in a plastic UV-transparent cuvette containing phosphate buffered saline (PBS, pH 7.4, 137 mM NaCl) in an amount sufficient to cover the stent. The samples were incubated at 37 °C and removed at predetermined intervals to permit characterization of the incubation milieu by UV−visible spectrophotometry. After each measurement, 25 μL of buffer was removed for characterization by gel electrophoresis, and stents were placed back into the cuvette and returned to the incubator.

Cell Transfection Assays. For experiments designed to evaluate release profiles and the integrity of released plasmid, COS-7 cells were grown in 96-well plates at an initial seeding density of 12 000 cells/well in 200 μL of growth medium (90% Dulbecco's modified Eagle's medium, 10% fetal calf serum, penicillin 100 units/mL, streptomycin 100 μg/mL). Cells were grown for 24 h, at which time a 50 μL mixture of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and plasmid solution was added directly to the cells according to the manufacturer's protocol. The lipid/plasmid transfection milieu 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 reagent (24 μL of stock diluted into 976 μL of serum-free culture medium). Fluorescence images were taken after 48 h using an Olympus IX70 microscope and analyzed using the Metavue version 4.6 software package (Universal Imaging Corporation). For experiments designed to evaluate the ability of coated stents to transfect cells in culture directly (i.e., in the absence of a secondary transfection agent): COS-7 cells were grown in six-well plates at an initial seeding density of 150 000 cells/ml in 3.0 mL of growth medium (identical to the composition defined above). Cells were allowed to grow overnight to approximately 80% confluence and a stent coated with eight bilayers of a multilayered polymer **1**/DNA film (Medtronic AVE S670; 4 mm × 18 mm; coated and then balloon expanded as described above) was placed manually into the wells on top of and in direct contact with the cells. Cells were incubated for an additional 48 h, and both fluorescence and phase contrast images were recorded directly using an Olympus IX70 fluorescence microscope. Percentages of cells expressing EGFP were determined using the Metavue version 4.6 software package (Universal Imaging Corporation) and the Adobe Photoshop software package (Adobe Systems, Incorporated). The number of EGFP positive cells was counted in at least four different locations. The percentage of cells transfected was reported as an average relative to all cells in the corresponding phase contrast images.

Results and Discussion

Multilayered polyelectrolyte films have been investigated broadly in the context of biomedical and biotechnological applications.[36] Numerous past studies have demonstrated that DNA (an anionic polyelectrolyte) can be used to fabricate ultrathin multilayered films layer-by-layer on surfaces using a variety of different natural and synthetic cationic polymers.[21−26] The work reported here is founded upon recent reports from our group demonstrating that hydrolytically degradable synthetic polyamines, such as polymer **1**, can be used to fabricate films that erode gradually and release anionic polymers, including DNA, when incubated in physiologically relevant media.[27,28,44−49] On the basis of this work, we sought to determine (1) whether polymer **1** and plasmid DNA could be used to fabricate erodible multilayered coatings on the surfaces of stainless steel intravascular stents, (2) whether these ultrathin films were capable of withstanding mechanical forces associated with stent expansion and deployment, and (3) whether stents coated with these materials were capable of mediating the transfection of cells in vitro.

Fabrication of Erodible Films on the Surfaces of Intravascular Stents. One attribute of the layer-by-layer procedure used to fabricate multilayered polyelectrolyte films is that this procedure permits the fabrication of ultrathin films that conform faithfully to the surfaces of topologically complex objects.[19,20,50] In principle, any solid surface that can be wetted by dilute, aqueous solutions of polyelectrolyte can be coated with a homogeneous and conformal multilayered film. We fabricated multilayered films on the surfaces of surgical grade 316L stainless steel intravascular stents using polymer **1**, a plasmid construct (pEGFP-N1) encoding EGFP, and an alternate dipping protocol optimized previously for the deposition of polymer **1**/DNA films on planar silicon and quartz substrates.[27,28,44] In all cases, stents were precoated with a thin multilayered film (ca. 20 nm thick) composed of sodium poly(styrene sulfonate) (SPS) and linear poly(ethylene imine) (LPEI) to provide a charged surface suitable for the adsorption of polymer **1**. To investigate a range of different fabrication procedures, films were fabricated on (1)

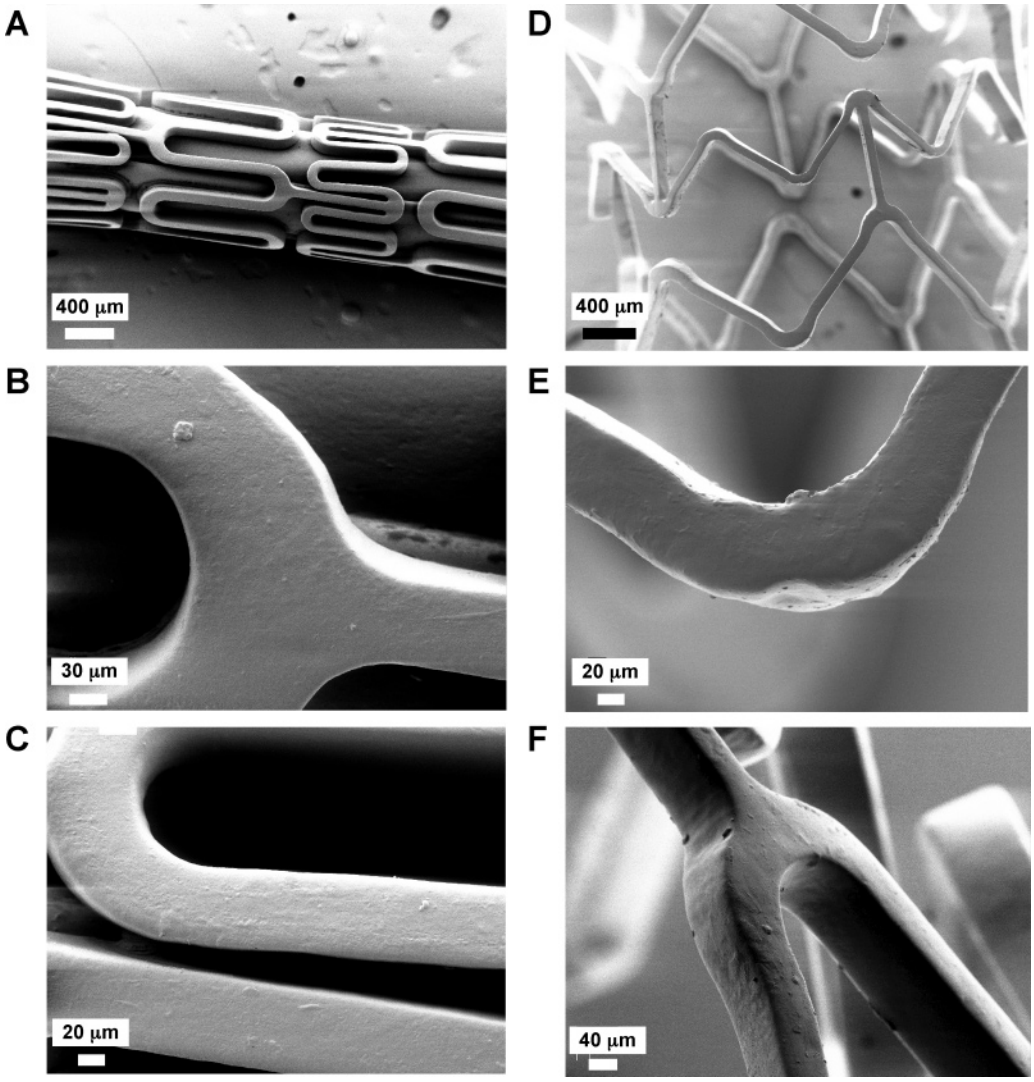


Figure 1. Scanning electron microscopy images of stainless steel intravascular stents coated with multilayered films fabricated from eight bilayers of polymer **1** and a plasmid DNA construct (pEGFP-N1) encoding enhanced green fluorescent protein. Stents were precoated with a thin multilayered SPS/LPEI film (ca. 20 nm thick) prior to the deposition of the DNA-containing films (see text). Images correspond to different magnifications and perspectives of a coated stent imaged as-coated on a balloon assembly (A−C) and after balloon expansion (D−F).

stents that were crimped and premounted on balloon catheter assemblies (as received from the manufacturer) and (2) stents that were expanded and removed from the balloon assemblies prior to coating.

We fabricated multilayered films composed of eight alternating layers of polymer **1** and pEGFP-N1 (referred to hereafter as eight "bilayers") on the surfaces of stents having a variety of dimensions and strut geometries. We demonstrated previously that the thicknesses of multilayered polymer **1**/DNA films fabricated on silicon increase linearly with respect to the number of bilayers of material deposited, as determined by measuring periodic changes in optical thickness using ellipsometry.[27,44] Ellipsometry is limited, however, by the general requirement that films be deposited on reflective, planar surfaces; this method cannot generally be used to characterize films on nonreflective substrates (e.g., unpolished stainless steel) or on topologically complex surfaces (e.g., stents). To monitor the fabrication process and estimate the thicknesses of films deposited on stents, films were also fabricated on reflective silicon substrates precoated with a 20 nm thick film composed of 10 bilayers of SPS and LPEI, as described above. On the basis of this comparison, we estimate the thicknesses of polymer **1**/DNA films deposited onto stents to be ca. 100 nm. We also deposited films on planar 316L stainless steel substrates to characterize the physical film thickness of scratched films by atomic force microscopy (AFM). Unfortunately, this method of characterization proved to be unreliable, as, in contrast to films deposited on silicon substrates, we were unable to scratch the multilayered films without also scratching the relatively soft stainless steel substrates. The optical thickness values reported above for films deposited on silicon substrates agree reasonably with values of film thickness estimated using scanning electron microscopy, as described below. Past studies demonstrate that polymer **1**/DNA films eight bilayers thick contain DNA at a concentration of approximately 2.7 (±0.8) μg/cm^2, as determined by UV absorbance.[28]

Physical Characterization of Coated Stents. We performed several experiments to characterize the surfaces of film-coated stents both immediately after fabrication and after exposure of the stents to mechanical forces associated with stent placement and deployment. Parts a−c of Figure 1 show representative scanning electron microscopy (SEM) images of a stent that was coated with an eight-bilayer film while crimped and mounted on a balloon assembly. These images demonstrate that the surface of the stent was coated uniformly with a thin film that conformed faithfully to the stent surface, the contours of the stent struts, and the balloon assembly. We did not observe the

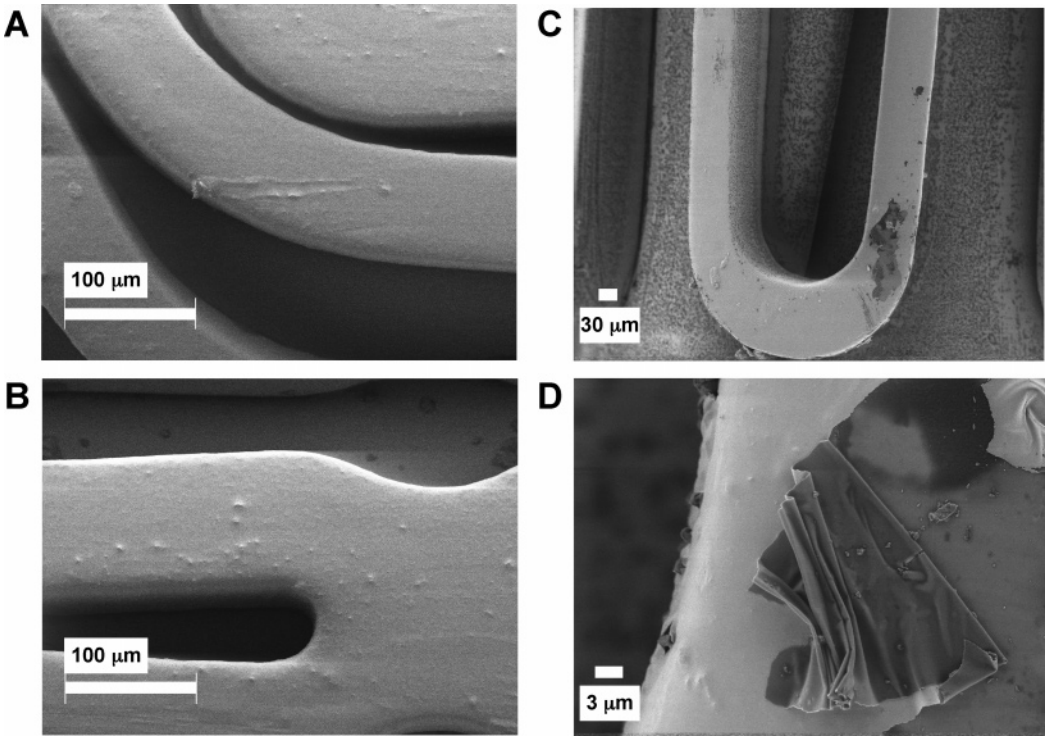


Figure 2. (A−D) Scanning electron microscopy images of intravascular stents coated with multilayered films fabricated from eight bilayers of polymer **1** and a plasmid DNA. Stents were coated while mounted on a balloon assembly and then passed through a silicone septum and arterial inducer prior to imaging (see text). Images correspond to different magnifications and perspectives. The image shown in D shows a magnified view of the delaminated portion of the film shown in C.

presence of "webs" of polymer or film stretched between stent struts or between stents and balloon assemblies, as has been reported previously for the encapsulation of DNA in thicker stent coatings fabricated using conventional materials and coating procedures.[8,10,12] Further inspection of these images demonstrates that these ultrathin coatings are generally smooth and devoid of cracks or other significant defects over large areas of the stent surface.

Stent coatings are subject to a broad range of mechanical challenges during stent placement and deployment. For example, the coating on a stent must be able to pass unharmed through the septum and shaft of an arterial inducer, and the coating must also be compliant enough to withstand expansion of the stent without cracking, peeling, or delaminating from the stent surface. We sought to determine whether the ultrathin polymer **1**/DNA films fabricated above could withstand representative mechanical challenges associated with these procedures. Parts d−f of Figure 1 show representative SEM images of a stent that was coated while mounted on a balloon assembly and then subsequently expanded prior to imaging. Parts e and f of Figure 1 show higher magnification views of two joints at the intersections of stent struts, where the majority of the mechanical forces are concentrated and where the majority of stent deformation occurs upon expansion. These images demonstrate that the multilayered film did not crack, peel, or delaminate and that it remained conformal and adherent to the surface after the balloon expansion procedure.

Figure 2 shows representative images of a stent that was coated while mounted on a balloon assembly and then passed through the silicone septum and the shaft of an arterial inducer prior to imaging. These experiments demonstrated that the polymer **1**/DNA films remained substantially intact, conformal, and adherent to stent surfaces after this procedure. In some cases, we observed small, shallow scratches in the surfaces of the films (Figure 2a). In one case, we also observed small micrometer-scale portions of the film that were peeled from small portions of stent struts (e.g., Figure 2c,d). We cannot discount the possibility that the defects observed in this sample could also have been caused by other physical trauma during the handling of these samples, and additional characterization[40,51,52] will clearly be necessary before a complete and quantitative description of the mechanical properties of these DNA-containing films can be made. However, the images in Figures 1 and 2, when combined, demonstrate that ultrathin multilayered films fabricated from polymer **1** and plasmid DNA remain substantially intact after exposure to physical and mechanical challenges representative of those associated with stent deployment.

We note here that film defects such as the peeling observed in Figure 2d provide an additional means with which to characterize the thicknesses of these ultrathin coatings. The average thicknesses of film fragments were measured to be ca. 120 nm by computer image analysis of high-magnification SEM images of peeled or delaminated films. These values are in reasonable agreement with the optical film thicknesses (ca. 100 nm) estimated by ellipsometry for films fabricated on silicon substrates (as described above).

Characterization of Film Erosion and DNA Release Profiles. The layer-by-layer assembly of multilayered polyelectrolyte films is driven, in large measure, by multivalent interactions (e.g., electrostatic or hydrogen bonding interactions) that occur between oppositely charged polyelectrolytes.[18−20] Owing to the strength of these multivalent interactions, many multilayered polyelectrolyte films are stable in physiological environments. The incorporation of hydrolytically degradable polyamines, such as polymer **1**, into the structure of a multilayered film provides a mechanism with which to trigger or control film erosion in physiological media over periods of time ranging from two days to two weeks.[27,28,44−48] We sought to characterize the physical erosion and DNA release profiles of polymer **1**/DNA films coated onto the surfaces of stainless steel

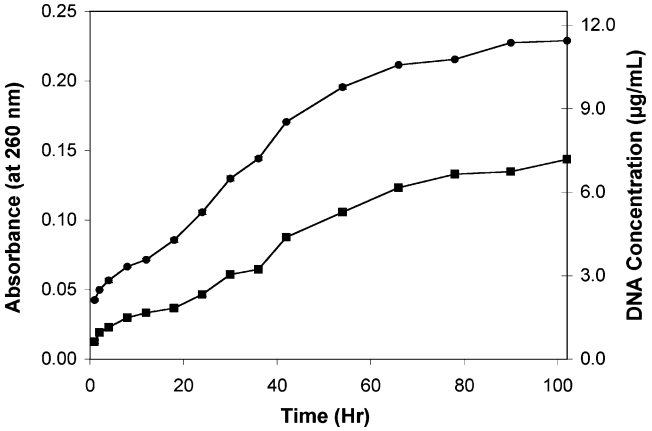


Figure 3. Plot of solution absorbance at 260 nm vs time for two expanded stents coated with either 8 (■) or 16 (●) bilayers of a polymer **1**/DNA film incubated in phosphate buffered saline at 37 °C. Error bars are shown but are smaller than the symbols used to represent absorbance values.

stents. We note in this context that several recent reports describe multilayered films fabricated from naturally occurring polyelectrolytes such as poly(amino acid)s, polysaccharides, or salmon sperm DNA that can be degraded in the presence of enzymes[25,26,38,53,54] and that several other groups have reported multilayered films that erode or disintegrate in physiologically relevant environments.[55−60] In addition, the application of multilayered materials to the controlled release of small molecules and conventional drugs, which can be released by diffusion through multilayered films that need not physically degrade, is also an active and rapidly advancing area of research.[61−65]

Stents coated with polymer **1**/DNA multilayered films were incubated in PBS buffer at 37 °C to characterize film erosion and DNA release profiles. Figure 3 shows a plot of solution absorbance (measured at 260 nm, the absorbance maximum of DNA) versus time for a stent coated with a film eight bilayers thick that was expanded prior to incubation (filled squares; see Materials and Methods for details of incubation procedure). These data demonstrate that following an initial burst that occurs during the first several hours of incubation, absorbance increases in a manner that is approximately linear over a period of up to 65 h. These data suggest that the release of DNA from the surface of the stent is sustained over a period of up to four days under these incubation conditions. Figure 3 also provides an estimate of the cumulative concentration of DNA released during this experiment using values calculated from measured solution absorbance values. On the basis of this calculation, we estimate the cumulative mass of DNA released from this stent coated with eight bilayers of polymer **1** and DNA to be 7.2 μg. It is important to note that this value represents an estimate of total DNA released, as the possibility of changes in volume resulting from evaporation over the course of this experiment and the possible influence of polymer **1** on the absorbance of DNA could introduce error into this conversion. However, we do conclude on the basis of this experiment that DNA is released continuously from the surface of this stent over a period of four days.

Figure 3 also shows a plot of solution absorbance for a second identical stent coated with 16 bilayers of polymer **1** and DNA and then subsequently expanded prior to incubation (Figure 3, filled circles). With the exception of a larger initial burst, the slope and shape of this release curve are similar to those measured for the stent coated with an eight-bilayer film (normalized data not shown). These data demonstrate that it is possible to control the total amount of DNA released from a coated stent by increasing the number of layers of DNA deposited during fabrication. However, several additional points deserve comment. First, we note that the absorbance values measured for the erosion of the 16-bilayer film are not equal to exactly twice the value of the eight-bilayer film, as might be expected if film growth were to proceed in a perfectly linear manner during fabrication. Second, we note that, while the stent coated with the 16-bilayer film released a larger amount of DNA, complete release occurred over the same four-day time period measured for the release of DNA from the stent coated with an eight-bilayer film. These data are consistent with recent observations for the erosion of multilayered films fabricated from polymer **1** and sodium poly(styrene sulfonate), a model anionic polymer, on planar silicon substrates.[46] In these previous experiments, rates of film erosion and release were also measured to be independent of film thickness or the number of bilayers of material deposited during fabrication.

Figure 4 shows SEM images of a stent coated with eight bilayers of polymer **1** and DNA that was incubated in PBS at 37 °C for 1.5 h and then dried prior to imaging. These images demonstrate that the multilayered film, which was initially smooth and continuous (e.g., Figures 1 and 2), underwent a transformation upon incubation in PBS that resulted in the formation of a topographically complex and textured surface. The morphology of this partially eroded film is consistent with the results of a previous study that characterized the surface structure and morphology of polymer **1**/DNA films fabricated on planar silicon substrates. This past study demonstrated that films fabricated on silicon undergo a transformation that results in the formation of nanoparticulate structures ranging in size from 50 to 400 nm when incubated in PBS.[28,44] The physical basis of this behavior is not yet understood, and the nature of this transformation is currently under active investigation in our group. However, we speculate on the basis of these observations that it could ultimately prove possible to design ultrathin polycation/DNA films that act to condense DNA at the surfaces of coated stents and, thus, present or release DNA in a physical form that contributes actively to the transfection of cells (as described below).[28]

In Vitro Cell Transfection. The experiments above demonstrate that stents coated with multilayered polymer **1**/DNA films release plasmid DNA into solution for up to four days when incubated in physiologically relevant media. Characterization of the DNA released from coated stents using agarose gel electrophoresis demonstrated that the plasmid was released in an open circular topology. These gel electrophoresis results were consistent with the results of our past studies characterizing DNA released from polymer **1**/DNA films fabricated on silicon and quartz substrates (data not shown).[27] We performed several additional cell-based transfection experiments to characterize the functional integrity of the DNA released from film-coated stents at various time points during the film erosion process. Figure 5a shows a representative fluorescence microscopy image of COS-7 cells incubated with a commercially available cationic lipid transfection agent (Lipofectamine 2000) and an aliquot of PBS solution collected during the incubation of a coated stent (see Materials and Methods for additional details; additional time points not shown).

The data shown in Figure 5a are consistent with our past studies using film-coated silicon substrates[27] and demonstrate that the DNA released from the surface of an expanded stainless steel stent is released into solution in a form that is capable of yielding EGFP expression in cells. However, the interpretation of this result with respect to the potential of these materials for stent-mediated delivery is limited by the use of an auxiliary

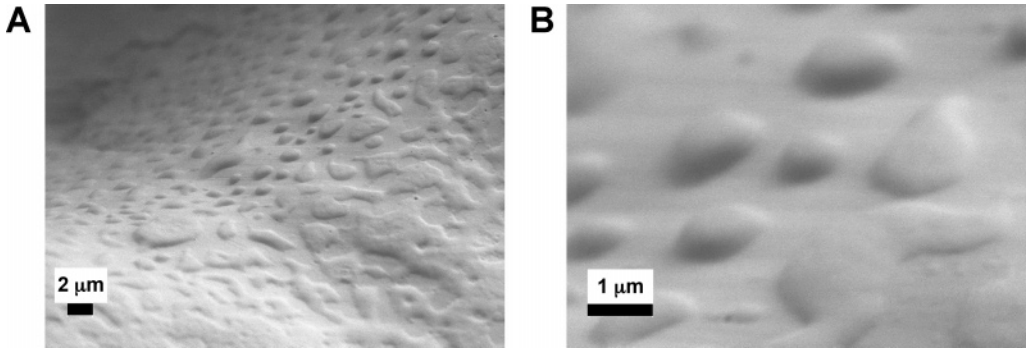


Figure 4. Scanning electron microscopy images of intravascular stents coated with eight bilayers of a multilayered polymer **1**/DNA film and incubated in PBS buffer at 37 °C for 1.5 h prior to imaging (see text).

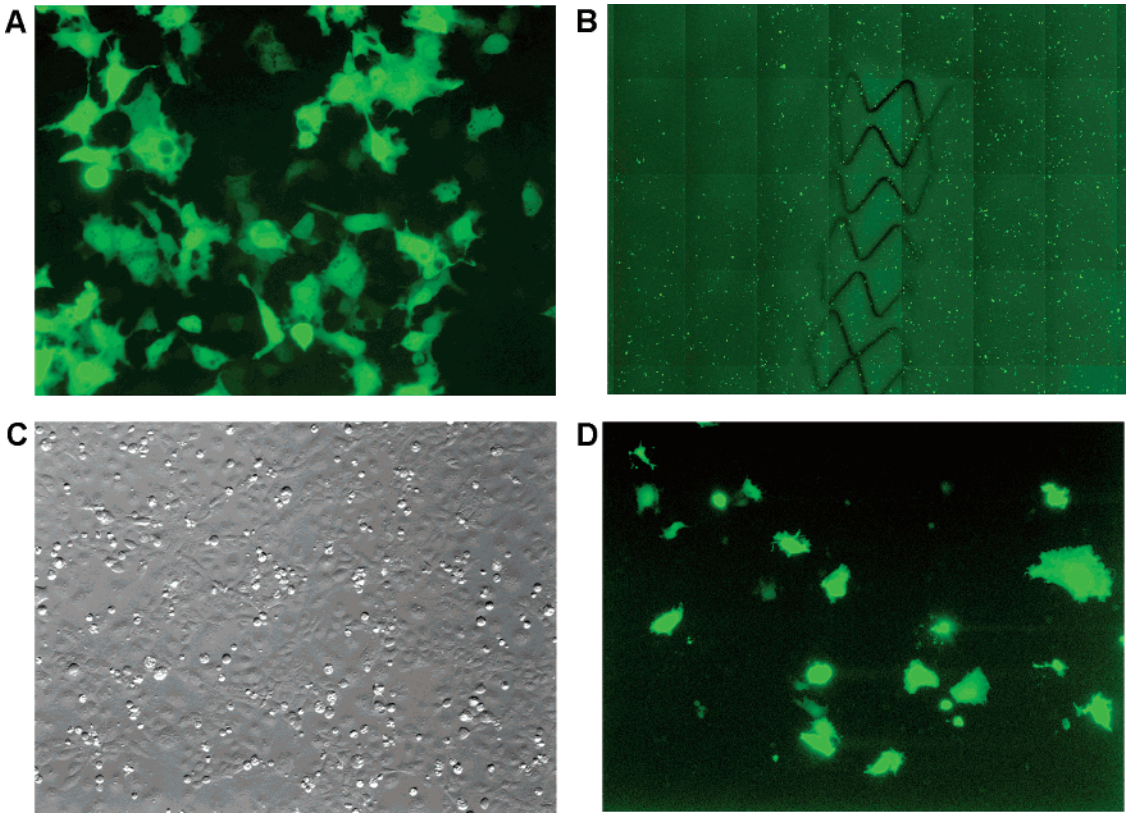


Figure 5. (A) Fluorescence microscopy image (20×) showing expression of EGFP in COS-7 cells transfected with DNA released from a multilayered polymer **1**/DNA film incubated in PBS at 37 °C. Transfection was conducted by combining released DNA with a commercially available cationic lipid (see text). (B) Series of adjacent low magnification (4×) fluorescence microscopy images showing expression of EGFP in a confluent population of COS-7 cells 48 h after the introduction of a stent coated with eight bilayers of polymer **1** and DNA (total area shown is approximately 1.8 mm^2; a portion of the expanded stent is shown for comparison). This experiment was conducted without the use of additional transfection agents (see text). Images C and D show representative higher magnification phase contrast and fluorescence microscopy images (10×) of cells shown in (B) used to quantify levels of transfection.

lipid-based transfection agent. We also sought to investigate the ability of stents coated with these multilayered films to mediate the transfection of cells self-sufficiently, e.g., in the absence of any additional transfection agents. As noted above, the DNA in these multilayered films is juxtaposed intimately with a cationic polymer, a class of materials used broadly to promote nonviral transfection.[15,16] Polymer **1** has been demonstrated to be an effective polymer-based gene delivery agent,[43] and we demonstrated recently that planar quartz slides coated with polymer **1**/DNA films are capable of mediating the localized and contact-mediated transfection of cells.[28] On the basis of these past studies, we conducted a second set of transfection experiments in which coated stents were expanded and placed directly into the wells of culture dishes containing cells growing in serum-containing culture medium. Figure 5b shows a series of adjacent low-magnification fluorescence microscopy images of a confluent population of COS-7 cells 48 h after the introduction of a stent coated with eight bilayers of polymer **1** and DNA (approximate area shown is 1.8 mm^2; a portion of the expanded stent is shown for comparison). Parts c and d of Figure 5 show representative, higher magnification phase contrast and fluorescence microscopy images of cells shown in Figure 5b. The average percentage of cells expressing EGFP mediated by film-coated stents was measured to be 4.5 ± 0.98% relative to the total number of cells in corresponding phase contrast images.

The data in Figure 5b and d demonstrate that stents coated with films fabricated from polymer **1** and plasmid DNA are capable of mediating the transfection of cells (i) in the absence of additional transfection agents and (ii) in a serum-containing

cell culture environment. We note, however, that the levels of transfection observed here are lower than those observed in our previous study (e.g., from ca. 5 to 40%), in which planar quartz slides coated with polymer **1**/DNA films were placed in direct contact with cells.[28] In addition, we note that the transfection observed in Figure 5b and d was not localized to cells growing in contact with or adjacent to coated stent struts, as observed in our past study.[28] Rather, we observed cells expressing EGFP to be distributed equally in all regions of the exposed cell population (Figure 5b). This lack of localized transfection is likely due to the small percentage of the cell population contacted directly by stent struts and, in addition, by the fact that the stent was not immobilized during incubation. We note that in potential applications of these coated stents in vivo, films on the surfaces of expanded stents would be pressed into direct contact with vascular tissues. Such intimate contact could result in levels of transfection that are more localized and, potentially, higher than those shown in Figure 5. Additional investigations will be required to evaluate the suitability of these new materials for the localized delivery of DNA to vascular tissues in vivo.

The ability of stents coated with polymer **1** and DNA to transfect cells in the absence of additional transfection agents provides support for the view that polymer **1** could act to promote the internalization of DNA by cells (as described above). To examine this possibility more closely, we conducted an additional control experiment in which a population of cells identical to that used in Figure 5b−d was treated with a bolus of 15 μg of "naked" plasmid DNA (i.e., in the absence of polymer or any other DNA delivery agent). The amount of DNA used in this experiment corresponds to 2.1 times the estimated amount of DNA released from a stent coated with eight bilayers of polymer **1** and DNA (e.g., in Figure 3 and Figure 5b−d). We observed no significant expression of EGFP in cells in this control experiment after 48 h (data not shown). When combined with the results of this control experiment, the observation of the stent-mediated transfection in Figure 5b and d suggests that the DNA released from polymer **1**/DNA films may be released in a form that is at least partially condensed by polymer **1** and, therefore, capable of being internalized more effectively by cells.

Preliminary characterization using dynamic light scattering suggests the presence of polymer/DNA aggregates in solutions used to erode polymer **1**/DNA films. For example, intensity autocorrelation functions measured at 90° suggested the formation of aggregates with sizes ranging from 100 to 600 nm after 6 h of incubation in PBS. We caution, however, that additional experiments and measurements of scattering at multiple angles will be required to unambiguously assign the sizes (and to determine the compositions) of these aggregates. The results of this present study, however, do suggest the presence of aggregates in solutions of partially degraded films. These observations thus suggest future opportunities for the design and fabrication of multilayered films using other polyamines that either condense DNA more effectively or transfect cells more efficiently than polymer **1**.

Conclusions

We have reported an approach to the fabrication of ultrathin, multilayered polyelectrolyte films that permit both the immobilization and controlled release of plasmid DNA from the surfaces of stainless steel intravascular stents. Our approach makes use of an entirely aqueous-based, layer-by-layer method for the assembly of ultrathin films constructed from alternating layers of plasmid DNA and a hydrolytically degradable polyamine. We demonstrated that films ca. 100−120 nm thick could be fabricated conformally onto the topologically complex surfaces of stents and that these ultrathin coatings did not crack, peel, or delaminate from the surface after exposure to mechanical challenges (e.g., expansion) representative of those encountered during stent deployment. Stents coated with either eight or 16 bilayers of polymer **1** and a plasmid encoding enhanced green fluorescent protein sustained the release of DNA into solution for up to four days, and coated stents were capable of mediating the expression of EGFP in a model mammalian cell line without the aid of additional transfection agents.

Materials that permit control over the release of DNA from the surfaces of intravascular stents could contribute to the development of new approaches for the localized and nonviral delivery of DNA to vascular tissues. In this context, the results of this work suggest several opportunities for future study. For example, the layer-by-layer procedure used here provides a straightforward mechanism for the fabrication of films constructed from multiple different layers of multiple different DNA constructs[28] and could therefore present opportunities to design films that permit control over the release of multiple different genes from stent surfaces. Further, the internal structures of these ultrathin materials, in which DNA is juxtaposed intimately with cationic polymer, provide a potential platform upon which to design films that act to promote the uptake and expression of DNA more effectively than those fabricated from polymer **1**. The results of recent studies suggest that it may also be possible to fabricate ultrathin erodible polyelectrolyte assemblies that sustain the release of DNA for longer periods than those demonstrated here simply by increasing the hydrophobicity of the degradable polyamine used to fabricate these films.[46,47] Finally, in comparison to conventional methods reported for the encapsulation of DNA in polymeric stent coatings, the methods introduced here do not require the use of organic solvents and they reduce dramatically the amount of polymer required to immobilize and release DNA at the surface. The ultrathin films reported here could, with further development, contribute to the development of localized gene-based approaches to the treatment of cardiovascular diseases and related conditions.

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Supporting Information Available. Scanning electron microscopy image of a portion of a multilayered film fabricated from eight bilayers of polymer **1** and plasmid DNA used to estimate film thickness. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Morice, M. C.; Serruys, P. W.; Sousa, J. E.; Fajadet, J.; Ban Hayashi, E.; Perin, M.; Colombo, A.; Schuler, G.; Barragan, P.; Guagliumi, G.; Molnar, F.; Falotico, R. *N. Engl. J. Med.* **2002,** *346,* 1773−1780.
(2) Moses, J. W.; Leon, M. B.; Popma, J. J.; Fitzgerald, P. J.; Holmes, D. R.; O'Shaughnessy, C.; Caputo, R. P.; Kereiakes, D. J.; Williams, D. O.; Teirstein, P. S.; Jaeger, J. L.; Kuntz, R. E. *N. Engl. J. Med.* **2003,** *349,* 1315−1323.
(3) Sousa, J. E.; Serruys, P. W.; Costa, M. A. *Circulation* **2003,** *107,* 2383−2389.
(4) Tanabe, K.; Regar, E.; Lee, C. H.; Hoye, A.; van der Giessen, W. J.; Serruys, P. W. *Curr. Pharm. Des.* **2004,** *10,* 357−367.
(5) Stone, G. W.; Ellis, S. G.; Cox, D. A.; Hermiller, J.; O'Shaughnessy, C.; Mann, J. T.; Turco, M.; Caputo, R.; Bergin, P.; Greenberg, J.; Popma, J. J.; Russell, M. E. *N. Engl. J. Med.* **2004,** *350,* 221−231.
(6) Fishbein, I.; Stachelek, S. J.; Connolly, J. M.; Wilensky, R. L.; Alferiev, I.; Levy, R. J. *J. Controlled Release* **2005,** *109,* 37−48.

(7) Sharif, F.; Daly, K.; Crowley, J.; O'Brien, T. *Cardiovasc. Res.* **2004,** *64*, 208−216.
(8) Klugherz, B. D.; Jones, P. L.; Cui, X.; Chen, W.; Meneveau, N. F.; DeFelice, S.; Connolly, J.; Wilensky, R. L.; Levy, R. J. *Nat. Biotechnol.* **2000,** *18*, 1181−1184.
(9) Klugherz, B. D.; Song, C.; DeFelice, S.; Cui, X.; Lu, Z.; Connolly, J.; Hinson, J. T.; Wilensky, R. L.; Levy, R. J. *Hum. Gene Ther.* **2002,** *13*, 443−454.
(10) Nakayama, Y.; Ji-Youn, K.; Nishi, S.; Ueno, H.; Matsuda, T. *J. Biomed. Mater. Res.* **2001,** *57*, 559−566.
(11) Perlstein, I.; Connolly, J. M.; Cui, X.; Song, C.; Li, Q.; Jones, P. L.; Lu, Z.; DeFelice, S.; Klugherz, B.; Wilensky, R.; Levy, R. J. *Gene Ther.* **2003,** *10*, 1420−1428.
(12) Takahashi, A.; Palmer-Opolski, M.; Smith, R. C.; Walsh, K. *Gene Ther.* **2003,** *10*, 1471−1478.
(13) Walter, D. H.; Cejna, M.; Diaz-Sandoval, L.; Willis, S.; Kirkwood, L.; Stratford, P. W.; Tietz, A. B.; Kirchmair, R.; Silver, M.; Curry, C.; Wecker, A.; Yoon, Y. S.; Heidenreich, R.; Hanley, A.; Kearney, M.; Tio, F. O.; Kuenzler, P.; Isner, J. M.; Losordo, D. W. *Circulation* **2004,** *110*, 36−45.
(14) Fishbein, I.; Alferiev, I. S.; Nyanguile, O.; Gaster, R.; Vohs, J. M.; Wong, G. S.; Felderman, H.; Chen, I. W.; Choi, H.; Wilensky, R. L.; Levy, R. J. *Proc. Natl. Acad. Sci. U.S.A.* **2006,** *103*, 159−164.
(15) Pack, D. W.; Hoffman, A. S.; Pun, S.; Stayton, P. S. *Nat. Rev. Drug Discovery* **2005,** *4*, 581−593.
(16) Luo, D.; Saltzman, W. M. *Nat. Biotechnol.* **2000,** *18*, 33−37.
(17) van der Giessen, W. J.; Lincoff, A. M.; Schwartz, R. S.; van Beusekom, H. M.; Serruys, P. W.; Holmes, D. R., Jr.; Ellis, S. G.; Topol, E. J. *Circulation* **1996,** *94*, 1690−1697.
(18) Decher, G. *Science* **1997,** *277*, 1232−1237.
(19) Bertrand, P.; Jonas, A.; Laschewsky, A.; Legras, R. *Macromol Rapid Commun.* **2000,** *21*, 319−348.
(20) Hammond, P. T. *Adv. Mater.* **2004,** *16*, 1271−1293.
(21) Lvov, Y.; Decher, G.; Sukhorukov, G. *Macromolecules* **1993,** *26*, 5396−5399.
(22) Schuler, C.; Caruso, F. *Biomacromolecules* **2001,** *2*, 921−926.
(23) Pei, R. J.; Cui, X. Q.; Yang, X. R.; Wang, E. K. *Biomacromolecules* **2001,** *2*, 463−468.
(24) Shi, X. Y.; Sanedrin, R. J.; Zhou, F. M. *J Phys Chem B* **2002,** *106*, 1173−1180.
(25) Serizawa, T.; Yamaguchi, M.; Akashi, M. *Angew. Chem., Int. Ed.* **2003,** *42*, 1115−1118.
(26) Ren, K. F.; Ji, J.; Shen, J. C. *Biomaterials* **2006,** *27*, 1152−1159.
(27) Zhang, J.; Chua, L. S.; Lynn, D. M. *Langmuir* **2004,** *20*, 8015−8021.
(28) Jewell, C. M.; Zhang, J.; Fredin, N. J.; Lynn, D. M. *J. Controlled Release* **2005,** *106*, 214−223.
(29) Segura, T.; Shea, L. D. *Bioconjugate Chem.* **2002,** *13*, 621−629.
(30) Segura, T.; Volk, M. J.; Shea, L. D. *J. Controlled Release* **2003,** *93*, 69−84.
(31) Segura, T.; Chung, P. H.; Shea, L. D. *Biomaterials* **2005,** *26*, 1575−1584.
(32) Shen, H.; Tan, J.; Saltzman, W. M. *Nat. Mater.* **2004,** *3*, 569−574.
(33) Farhat, T. R.; Schlenoff, J. B. *Electrochem Solid-State Lett.* **2002,** *5*, B13−B15.
(34) Tan, Q.; Ji, J.; Barbosa, M. A.; Fonseca, C.; Shen, J. *Biomaterials* **2003,** *24*, 4699−4705.
(35) Ji, J.; Tan, Q.; Fan, D. Z.; Sun, F. Y.; Barbosa, M. A.; Shen, J. *Colloids Surf., B* **2004,** *34*, 185−190.
(36) Ai, H.; Jones, S. A.; Lvov, Y. M. *Cell Biochem. Biophys.* **2003,** *39*, 23−43.
(37) Etienne, O.; Gasnier, C.; Taddei, C.; Voegel, J. C.; Aunis, D.; Schaaf, P.; Metz-Boutigue, M. H.; Bolcato-Bellemin, A. L.; Egles, C. *Biomaterials* **2005,** *26*, 6704−6712.
(38) Etienne, O.; Schneider, A.; Taddei, C.; Richert, L.; Schaaf, P.; Voegel, J. C.; Egles, C.; Picart, C. *Biomacromolecules* **2005,** *6*, 726−733.
(39) Schultz, P.; Vautier, D.; Richert, L.; Jessel, N.; Haikel, Y.; Schaaf, P.; Voegel, J. C.; Ogier, J.; Debry, C. *Biomaterials* **2005,** *26*, 2621−2630.
(40) Pavoor, P. V.; Gearing, B. P.; Muratoglu, O.; Cohen, R. E.; Bellare, A. *Biomaterials* **2006,** *27*, 1527−1533.
(41) He, W.; Bellamkonda, R. V. *Biomaterials* **2005,** *26*, 2983−2990.
(42) Thierry, B.; Winnik, F. M.; Merhi, Y.; Silver, J.; Tabrizian, M. *Biomacromolecules* **2003,** *4*, 1564−1571.
(43) Lynn, D. M.; Langer, R. *J. Am. Chem. Soc.* **2000,** *122*, 10761−10768.
(44) Fredin, N. J.; Zhang, J.; Lynn, D. M. *Langmuir* **2005,** *21*, 5803−5811.
(45) Vazquez, E.; Dewitt, D. M.; Hammond, P. T.; Lynn, D. M. *J. Am. Chem. Soc.* **2002,** *124*, 13992−13993.
(46) Zhang, J.; Fredin, N. J.; Janz, J. F.; Sun, B.; Lynn, D. M. *Langmuir* **2006,** *22*, 239−245.
(47) Zhang, J.; Fredin, N. J.; Lynn, D. M. *J. Polym. Sci., Polym. Chem.* **2006,** *44*, 5161−5173.
(48) Lynn, D. M. *Soft Matter* **2006,** *2*, 269−273.
(49) Wood, K. C.; Boedicker, J. Q.; Lynn, D. M.; Hammond, P. T. *Langmuir* **2005,** *21*, 1603−1609.
(50) Peyratout, C. S.; Dahne, L. *Angew. Chem., Int. Ed.* **2004,** *43*, 3762−3783.
(51) Richert, L.; Engler, A. J.; Discher, D. E.; Picart, C. *Biomacromolecules* **2004,** *5*, 1908−1916.
(52) Nolte, A. J.; Rubner, M. F.; Cohen, R. E. *Macromolecules* **2005,** *38*, 5367−5370.
(53) Serizawa, T.; Yamaguchi, M.; Akashi, M. *Macromolecules* **2002,** *35*, 8656−8658.
(54) Picart, C.; Schneider, A.; Etienne, O.; Mutterer, J.; Schaaf, P.; Egles, C.; Jessel, N.; Voegel, J. C. *Adv. Funct. Mater.* **2005,** *15*, 1771−1780.
(55) Sukhishvili, S. A. *Curr. Opin. Colloid Interface Sci.* **2005,** *10*, 37−44.
(56) Sato, K.; Imoto, Y.; Sugama, J.; Seki, S.; Inoue, H.; Odagiri, T.; Hoshi, T.; Anzai, J. *Langmuir* **2005,** *21*, 797−799.
(57) Inoue, H.; Anzai, J. *Langmuir* **2005,** *21*, 8654−8359.
(58) Inoue, H.; Sato, K.; Anzai, J. *Biomacromolecules* **2005,** *6*, 27−29.
(59) Li, B. Y.; Haynie, D. T. *Biomacromolecules* **2004,** *5*, 1667−1670.
(60) Zelikin, A. N.; Quinn, J. F.; Caruso, F. *Biomacromolecules* **2006,** *7*, 27−30.
(61) Qiu, X. P.; Leporatti, S.; Donath, E.; Mohwald, H. *Langmuir* **2001,** *17*, 5375−5380.
(62) Dai, Z. F.; Heilig, A.; Zastrow, H.; Donath, E.; Mohwald, H. *Chem.—a Eur. J.* **2004,** *10*, 6369−6374.
(63) Zahr, A. S.; de Villiers, M.; Pishko, M. V. *Langmuir* **2005,** *21*, 403−410.
(64) Chung, A. J.; Rubner, M. F. *Langmuir* **2002,** *18*, 1176−1183.
(65) Berg, M. C.; Zhai, L.; Cohen, R. E.; Rubner, M. F. *Biomacromolecules* **2006,** *7*, 357−364.

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