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## Degradable Polyelectrolyte Multilayers that Promote the Release of siRNA

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### Abstract

We report an approach to the design of degradable polyelectrolyte-based films for the controlled release of siRNA from surfaces. Our approach is based on stepwise, layer-by-layer assembly of multilayered polyelectrolyte films (or ‘polyelectrolyte multilayers’, PEMs) using siRNA and a hydrolytically degradable poly( $\beta$ -amino ester) (polymer **1**). Fabrication of films using siRNA sequences for green fluorescent protein (GFP) or firefly luciferase resulted in linear growth of ultrathin films (~50 nm thick) that promoted the surface-mediated release of siRNA upon incubation in physiologically relevant media. Physicochemical characterization of these siRNA-containing films revealed large differences in film growth profiles, physical erosion profiles, and siRNA release profiles as compared to PEMs fabricated using polymer **1** and larger plasmid DNA constructs. For example, whereas films fabricated using plasmid DNA erode gradually and release DNA over a period of ~48 hours, films fabricated using siRNA released ~65% of incorporated siRNA within the first hour of incubation, prior to the onset of any observed film erosion. This initial burst of release was followed by a second, slower phase of release (accompanied by gradual film erosion) over the next 23 hours. These differences in release profiles and other behaviors likely result, at least in part, from large differences in the sizes of siRNA and plasmid DNA. Finally, we demonstrate that the siRNA in these films is released in a form that remains intact, functional, and able to silence targeted protein expression upon administration to mammalian cells *in vitro*. The results of this investigation provide a platform for the design of thin films and coatings that could be used to localize the release of siRNA from surfaces in a variety of fundamental and applied contexts (e.g., for development of new research tools or approaches to delivery from film-coated implants and other devices).

### Introduction

Materials that provide control over the immobilization and release of nucleic acids from surfaces are of interest in a broad range of fundamental and applied contexts. Methods for the release of DNA from the surfaces of implantable materials and polymer-based scaffolds, for example, have contributed to the development of localized approaches to therapeutic gene delivery and platforms for the engineering of complex tissues.<sup>1-7</sup> The ability to immobilize and/or release DNA from surfaces is also critical for the development of tools for biomedical research.<sup>8-10</sup> Progress in each of these areas has been facilitated by general

**Supporting Information Available.** Additional plots expressing the data shown in Figure 2 as actual values of film thickness, amount of siRNA released, and amount of polymer released versus time. This material is available free of charge via the Internet at <http://pubs.acs.org>.

advances in polymer science and the design of materials and interfaces that can be used to encapsulate, bind to, or protect nucleic acid constructs while providing control over both the form and rates at which they are released (e.g., approaches that can be used to release DNA rapidly, slowly, or as an aggregate with other agents that promote the internalization and processing of DNA by cells).<sup>11-14</sup>

The work reported here was motivated by the recent explosion of interest in small interfering RNA (siRNA) constructs as research tools and as potential therapeutic agents. Numerous past studies have demonstrated that siRNA (small, double-stranded RNA constructs 20-25 base pairs long) can be used to silence or knock down the expression of targeted proteins in a potent and highly specific manner *in vitro* and *in vivo*.<sup>15-20</sup> The results of these and other past studies, when combined, highlight (i) the utility of siRNA as a tool for investigating protein function, (ii) the potential of siRNA to provide new approaches for the treatment of a broad range of different diseases, and (iii) the need for new materials and methods that can be used to deliver and control the release of siRNA to cells and tissues.

Many materials-based approaches to the delivery of siRNA are based upon approaches originally developed and optimized for the delivery of plasmid DNA. Because the backbones of DNA and siRNA are both negatively charged, many cationic lipids and cationic polymers developed for DNA delivery can also be adopted to formulate electrostatic complexes (or 'polyplexes') that promote the internalization of siRNA by cells.<sup>20-24</sup> It is important to note, however, that there are significant differences between plasmid DNA and siRNA (including size, hydrolytic stability, and mechanisms/locations of action), and that, as a result, materials that were developed or optimized for the delivery of DNA may be less effective for the delivery of siRNA.<sup>15,16,20,23,25-27</sup> In addition to the development of new cationic agents designed specifically for use with siRNA, recognition of these and other differences has also motivated development of new delivery platforms based on covalent modification of siRNA with polymers and other agents.<sup>20,28-34</sup>

To date, efforts to develop polymer-based siRNA delivery systems have focused largely on formulation of nanoscale aggregates of siRNA that can be administered *in vitro* or delivered systemically or locally *in vivo* (e.g., by direct injection).<sup>15-18,20</sup> In contrast, less attention has been focused on the design of thin films, scaffolds, or hydrogels that can be used to control or sustain the release of siRNA and/or achieve localized release of siRNA (e.g., from the surface of an implantable device or in the vicinity of a tissue engineering scaffold, etc.). Two recent reports have demonstrated the ability to release siRNA from hydrogels to knock down green fluorescent protein expression in cells embedded within the hydrogel *in vitro*<sup>35</sup> and to silence the expression of MMP2 in vascular cells *in vivo* using hydrogel-coated intravascular stents.<sup>36</sup> In a more recent study, Takahashi and co-workers also demonstrated the release of siRNA polyplexes from PEG-based, hydrogel-coated polymer implants and the knockdown of mTOR expression *in vitro*.<sup>37</sup> Additional studies have demonstrated approaches to the incorporation of polyplexes of siRNA and siRNA-loaded nanoparticles into or on top of thin polymer films for the *in vitro* delivery of siRNA (these studies are described in additional detail below).<sup>38-40</sup>

Here, we report an approach to the layer-by-layer assembly of polyelectrolyte-based thin films that can be used to promote the release of siRNA from film-coated surfaces. These methods exploit electrostatic interactions between oppositely charged polymers to fabricate thin multilayered films (or 'polyelectrolyte multilayers', PEMs) by the alternating deposition of cationic and anionic materials on surfaces.<sup>41,42</sup> This approach is entirely aqueous and can be used to fabricate thin films using a wide range of natural and synthetic building blocks, including charged biomacromolecules such as nucleic acids, proteins, and viruses.<sup>7,43-46</sup> Numerous past studies have demonstrated the ability to fabricate DNA-containing PEMs by

the alternating deposition of plasmid DNA with a range of different synthetic and natural cationic polymers; these approaches and other investigations of DNA-containing films as potential agents for the surface-mediated delivery of DNA have been reviewed comprehensively.<sup>7</sup>

Of particular relevance to this current study are several recent reports demonstrating the ability to incorporate RNA-based constructs into the structures of PEMs. Recksiedler et al. reported on the assembly and electrochemical erosion of PEMs fabricated using commercially available, high molecular weight RNA and a cationic polymer.<sup>47</sup> In a different study, Dimitrova and co-workers reported on the incorporation of a layer of pre-formed polyplexes of siRNA targeted toward hepatitis C virus (HCV) replication into PEMs fabricated using enzymatically degradable polyelectrolytes and demonstrated that the resulting films inhibited replication of HCV in attached cells *in vitro*.<sup>38</sup> Mehrotra et al. demonstrated that siRNA-containing polyplexes can be patterned on top of pH-sensitive PEMs and that the resulting assemblies could be used to deliver the immobilized polyplexes to cells and silence the expression of RNA-dependent protein kinase (PKR) *in vitro*.<sup>39</sup> Finally, Zhang and co-workers demonstrated recently an approach to the fabrication of PEMs using nanoparticles made of calcium phosphate and shRNA (a single-stranded RNA sequence with a hairpin loop that functions similar to siRNA).<sup>40</sup> These films were demonstrated to knock down osteocalcin and osteopontin expression in human osteoblasts cultured on the films to varying extents depending on the loading of shRNA-calcium phosphate nanoparticles. In contrast to these approaches based on the incorporation of pre-formed complexes, the study reported here sought to investigate and characterize the behaviors of PEMs fabricated by direct, layer-by-layer incorporation of siRNA into degradable assemblies.

We and others have demonstrated in past studies that hydrolytically degradable poly( $\beta$ -amino ester)s, such as polymer **1**, can be used to design PEMs that erode gradually and release plasmid DNA<sup>48-57</sup> and other therapeutically relevant agents<sup>54,58-63</sup> from surfaces. We sought to determine whether polymer **1** could be used to fabricate PEMs using small siRNA constructs, and, subsequently, whether physicochemical differences between siRNA and plasmid DNA would result in differences in the properties and physical behaviors of the resulting films (e.g., differences in film stability, siRNA release rates, etc.). The results of this investigation demonstrate (i) that it is possible to fabricate ultrathin films (e.g., ~50 nm thick) layer-by-layer using polymer **1** and siRNA constructs targeted to the knockdown of either enhanced green fluorescent protein (GFP) or firefly luciferase, and (ii) that surfaces coated with these films promote the release of siRNA upon incubation in physiologically relevant media. We also demonstrate that film growth profiles and the release profiles of films fabricated using siRNA vary significantly compared to those of otherwise identical films fabricated using larger plasmid DNA. For example, whereas films fabricated using polymer **1** and DNA typically erode gradually and release DNA over a period of ~48 hours,<sup>49</sup> films fabricated using siRNA release ~65% of incorporated siRNA within the first hour of incubation. Finally, we demonstrate that siRNA is released from these materials in a form that remains functional and capable of silencing targeted protein expression upon administration to mammalian cells expressing EGFP. The results of this study suggest a platform for the design of thin films and coatings that could, with further development, be used to promote the localized and surface mediated release of siRNA in a range of therapeutic and biomedical research applications.

## Experimental Section

### Materials

Test grade n-type silicon wafers were purchased from Silicon Inc. (Boise, ID). 316 Stainless steel mesh was purchased from McMaster-Carr (Elmhurst, IL). Linear poly(ethylene imine) (LPEI, MW = 25,000) was purchased from Polysciences (Warrington, PA). Poly(sodium 4-styrenesulfonate) (SPS, MW = 70,000), acrylamide:N,N'-methylenebisacrylamide (29:1), TRIS borate-EDTA (TBE) buffer, ammonium persulfate, N,N,N',N'-tetramethylethylenediamine (TEMED), and 4,4'-trimethylenedipiperidine were obtained from Aldrich Chemical Co. (Milwaukee, WI). 1,4-Butanediol diacrylate was purchased from Alfa Aesar (Ward Hill, MA). All commercial polyelectrolytes were used as received without further purification. polymer **1** ( $M_n \approx 10,000$ ) was synthesized as previously described.<sup>64</sup> polymer **1<sub>FL</sub>** was synthesized as recently described<sup>65</sup> by conjugation of the small-molecule fluorophore tetramethylrhodamine cadaverine (TMR-Cad) to acrylate end-functionalized samples of polymer **1** prepared using a variation of a procedure reported previously for the design of amine end-labeled poly( $\beta$ -amino ester)s.<sup>66</sup> Plasmid DNA encoding enhanced green fluorescent protein [pEGFP-N1, >90% supercoiled] was purchased from Elim Biopharmaceuticals, Inc. (San Francisco, CA). siRNA complementary to green fluorescent protein (GFP) and firefly luciferase mRNA was obtained from Dharmacon. Deionized water (18 M $\Omega$ ) was used for washing steps during film fabrication and to prepare all polymer and siRNA solutions. Compressed air used to dry films and coated substrates was filtered through a 0.4  $\mu$ m membrane syringe filter. Phosphate-buffered saline (PBS) (EM Science, Gibbstown, NJ) and sodium acetate buffer (VWR, West Chester, PA) were prepared by diluting commercially available concentrates.

### General Considerations

All buffers and polymer solutions were filtered through a 0.2  $\mu$ m membrane syringe filter prior to use. Silicon, quartz, and mesh substrates ( $\sim 3.5$  cm  $\times$  0.5 cm) were cleaned with acetone, ethanol, methanol, and deionized water, and dried under a stream of filtered air. The silicon and quartz substrates were cleaned further by etching in oxygen plasma (Plasma Etch, Carson City, NV) for 5 min prior to film deposition. The optical thicknesses of the films deposited on silicon substrates were determined in at least five locations using a Gaertner LSE Stokes ellipsometer (632.8 nm, incident angle = 70 $^\circ$ ). Data were processed using the Gaertner Ellipsometer Measurement Program software package. Relative thicknesses were calculated by assuming an average refractive index of 1.58 for the multilayered films. UV-vis absorbance values used to quantify the amount of siRNA released from the multilayered films during incubation in PBS were recorded using a DU 520 UV-vis spectrophotometer (Beckman Coulter, Fullerton, CA) at a wavelength of 260 nm (corresponding to the absorbance maximum of siRNA). Fluorescence measurements of solutions used to characterize the stability and erosion of films fabricated from polymer **1<sub>FL</sub>** were made using a Fluoromax-3 fluorometer (Jobin Yvon, Edison, NJ) at an excitation wavelength of 543 nm. The fluorescence emission intensity was collected at 580 nm (corresponding to the emission maximum of polymer **1<sub>FL</sub>**). COS-7 cells used for the qualitative analysis of GFP knockdown by siRNA were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Fluorescence micrographs used to characterize the expression of enhanced green fluorescent protein (EGFP) and siRNA knockdown experiments were recorded using an Olympus IX70 microscope. Images were processed using Metavue version 7.1.2.0 software package (Molecular Devices, Toronto, Canada), Adobe Photoshop, and ImageJ (NIH). Flow cytometry analyses were performed using a BD FACSCalibur flow cytometer (BD Bioscience, San Jose, CA), and data were analyzed using the WinMDI version 2.9 software package.

## Preparation of Polyelectrolyte and Nucleic Acid Solutions

Solutions of siRNA (900 µg/mL – 200 µg/mL) were prepared by diluting concentrated aqueous stock solutions into sodium acetate buffer to yield a final sodium acetate concentration of 100 mM (pH = 4.9). The concentration of the siRNA stock was determined by measuring the solution absorbance at 260 nm and then applying the following formula:  $A_{260} \times 40 = [\text{siRNA}, \mu\text{g/ml}]$ . Solutions of LPEI and SPS used for the fabrication of LPEI/SPS base layers (20 mM with respect to the molecular weight of the repeat unit) were prepared using a 26 mM NaCl solution in deionized water (18 MΩ). LPEI solutions contained 5 mM HCl to aid polymer solubility. polymer **1** solutions (5 mM with respect to the repeat unit) were prepared in sodium acetate buffer (100 mM, pH = 4.9). polymer **1<sub>FL</sub>** solutions were prepared using a 50/50 (w/w) mixture of polymer **1** mixed with polymer **1<sub>FL</sub>** at a total concentration of 5 mM (with respect to the repeat unit) in 100 mM sodium acetate buffer (pH = 4.9).

## Fabrication of Multilayered Films

Films were deposited on planar silicon and stainless steel meshes pre-coated with 10 bilayers of LPEI/SPS (~20 nm thick, terminated with a topmost layer of SPS) to ensure a suitably charged surface for the adsorption of polymer **1**, as described in our past studies on the fabrication of PEMs using polymer **1** and DNA.<sup>49</sup> Multilayered films were fabricated using an alternate dipping method in the following general manner: (1) substrates were submerged in a solution of polymer **1** or polymer **1<sub>FL</sub>** for 5 min, (2) substrates were removed and immersed in an initial sodium acetate buffer bath (100 mM) for 1 min followed by a second sodium acetate buffer bath for 1 min, (3) substrates were submerged in a solution of siRNA for 5 min, and (4) substrates were rinsed again in the manner described above. This cycle was repeated until the desired number of polymer and siRNA layer pairs (referred to hereafter as ‘bilayers’; typically eight) were deposited. After fabrication, the polymer **1**/siRNA films were dried under a stream of 0.4 µm filtered air and were either used immediately or stored covered at ambient temperature prior to use.

## Characterization of Film Stability and Evaluation of siRNA Release Profiles

Experiments designed to evaluate film stability and characterize the release of siRNA from polymer **1**/siRNA films were performed in the following general manner. Film-coated substrates were submerged in PBS (1 mL, pH = 7.4, 137 mM NaCl) in plastic UV transparent cuvettes. These samples were incubated at 37 °C and removed at predetermined intervals for characterization by ellipsometry, UV/vis spectrophotometry, and fluorometry. For films fabricated on silicon substrates, optical film thickness measurements were made using ellipsometry in at least five different predetermined locations on each substrate. The concentration of siRNA released into solution over time was characterized by measuring the UV absorbance of the buffer solutions (at a wavelength of 260 nm). For films fabricated using polymer **1<sub>FL</sub>**, the amount of polymer released into solution was measured by fluorometry using an excitation of 543 nm and collecting the fluorescence emission at 580 nm (corresponding to the maximum fluorescence emission of polymer **1<sub>FL</sub>**). After each measurement, the film-coated substrates were either placed in a new cuvette with a fresh aliquot of PBS (for experiments used to characterize erosion profiles) or placed back into the same PBS-filled cuvette (for experiments used to characterize GFP knockdown) and returned to the incubator at 37 °C.

## Characterization of Film Morphology

Experiments designed to investigate changes in film morphology during erosion were performed in the following general manner. Film-coated substrates were placed in a plastic UV-transparent cuvette containing 1.0 mL of PBS and incubated at 37 °C. At predetermined



intervals the films were removed from the cuvettes for characterization by atomic force microscopy (AFM). Films were rinsed under deionized water and dried under a stream of filtered compressed air prior to measurement. The films were then imaged over a scan area of  $25\ \mu\text{m} \times 25\ \mu\text{m}$  using the tapping mode on a Nanoscope Multimode atomic force microscope (Digital Instruments, Santa Barbara, CA).

### **Polyacrylamide Gel Electrophoresis Assays**

Samples of siRNA released into solution during film erosion experiments were evaluated by loading 20  $\mu\text{L}$  of solution into a 20% polyacrylamide gel (TBE buffer, 50 V, 5 hr). Samples were loaded into the gel with a loading buffer consisting of 50:50 (v/v) glycerol water mixture. siRNA bands were visualized by ethidium bromide staining.

### **Characterization of *in vitro* siRNA Knockdown Using Fluorescence Microscopy**

COS-7 cells used in transfection experiments were grown in clear polystyrene 96-well culture plates at an initial seeding density of 15,000 cells/well in 200  $\mu\text{L}$  of growth medium (90% Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 100 units/mL penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin). After 24 hours of incubation at 37  $^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  atmosphere, the media in each well was aspirated and replaced with fresh medium and 200  $\mu\text{L}$  of a Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and pEGFP mixture. The Lipofectamine 2000/DNA mixture was prepared according to the manufacturer's suggested protocol. After 4 hours, the cell culture media was aspirated and replaced with fresh media. A 50  $\mu\text{L}$  mixture of siRNA (taken from either a stock solution of siRNA or a solution of siRNA released from polymer 1/siRNA films) and Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA), prepared according to the manufacturer's protocol, was then added to the transiently transfected COS-7 cells and the cells were allowed to incubate for another 48 hours. The RNAiMAX/siRNA solution was prepared by mixing 60 ng of siRNA from the erosion solutions (diluted to a total volume of 25  $\mu\text{L}$  using OptiMEM) with 25  $\mu\text{L}$  of diluted RNAiMAX reagent (7.2  $\mu\text{L}$  stock diluted into 593  $\mu\text{L}$  of OptiMEM). Levels of EGFP expression were then characterized by acquiring fluorescence microscopy images of each well using an Olympus IX70 microscope.

### **Characterization of *in vitro* siRNA Knockdown Using Flow Cytometry**

Untransformed HeLa cells and HeLa cells stably transfected with green fluorescent protein (GFP) used in flow cytometry experiments were cultured in clear 12-well polystyrene plates at an initial seeding density of 100,000 cells/well in 1 mL of growth media (90% Minimum Essential Media, 10% fetal bovine serum, 100 units/mL penicillin, 100  $\text{mg}/\text{mL}$  streptomycin). Cell growth medium for the stably transfected HeLa cells was also supplemented with 600  $\mu\text{g}/\text{mL}$  geneticin (G418) to select for those cells expressing GFP. After incubation at 37  $^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  atmosphere, the media was aspirated and replaced with fresh media and 250  $\mu\text{L}$  of a Lipofectamine RNAiMAX and siRNA mixture formulated according to the manufacturer's protocol. The RNAiMAX/siRNA solution was prepared by mixing 18.44  $\mu\text{L}$  of the siRNA erosion solution collected during the release experiments (arbitrary concentrations but constant volumes) diluted in 375  $\mu\text{L}$  of OptiMEM with 6  $\mu\text{L}$  of RNAiMAX diluted in 375  $\mu\text{L}$  OptiMEM. After an additional 72 hours of incubation, the media was aspirated from the wells and replaced with 500  $\mu\text{L}$  of trypsin with EDTA to detach the cells from the plate. The trypsin-EDTA solution was incubated with the cells for 5 minutes, after which 500  $\mu\text{L}$  of refrigerated growth media was added to each well. The contents of each well were transferred into separate centrifuge tubes and centrifuged at 3500 RPM for 10 minutes. The supernatant was removed and the cell pellets were resuspended in 500  $\mu\text{L}$  of PBS containing 1  $\text{mg}/\text{mL}$  bovine serum albumin, and the tubes were centrifuged again as outlined above. The supernatant was again aspirated and the cell pellets were resuspended in 500  $\mu\text{L}$  of the bovine serum albumin containing PBS and transferred to

plastic flow cytometry tubes. The cells were kept on ice prior to analysis by flow cytometry. Green fluorescence intensity along with forward- and side-scatter data were collected for populations of 25,000 cells. Intact cells were identified using the forward- and side-scatter data. The cell populations were gated into two populations (+GFP and -GFP) based on the green fluorescence intensity data collected from the control samples (untransformed HeLa cells and HeLa cells stably expressing GFP) and are presented as the percentage of the cell population that is positive for GFP expression.

## Results and Discussion

### Fabrication and Characterization of polymer **1**/siRNA Films

All PEMs used in our initial studies were fabricated on planar silicon substrates to facilitate characterization of film growth and optical thickness via ellipsometry and permit comparisons of film fabrication, erosion, and siRNA release profiles to those of our past studies on films fabricated using polymer **1** and plasmid DNA.<sup>49-51</sup> Silicon substrates were first pre-coated with a thin PEM composed of LPEI and SPS (~20 nm thick, with a topmost layer of SPS), as described in our past studies on DNA-containing films,<sup>49</sup> to provide a sufficient charge on the surface for the deposition of polymer **1**. Attempts to fabricate films directly on bare silicon (i.e., in the absence of these pre-coated foundation layers) did not result in observable film growth. The iterative dipping of these substrates into solutions of polymer **1** (5 mM with respect to the repeat unit in 100 mM sodium acetate, pH = 4.9) and siRNA targeted against GFP mRNA (GFP-siRNA, ~400 ug/mL in 100 mM sodium acetate, pH = 4.9) resulted in the layer-by-layer growth of polymer **1**/siRNA films. Figure 1 shows a plot of optical film thickness versus the number of polymer **1**/siRNA layer pairs (referred to hereafter as “bilayers”) deposited, and reveals film thickness to increase in a manner that is relatively linear, at least for the first six bilayers, to a thickness of ~50 nm after the deposition of eight bilayers. On the basis of these data, we estimate the average thickness of the polymer **1**/siRNA bilayers in these films to be ~3.8 nm.

The stepwise nature of film growth observed in Figure 1 is generally consistent with growth profiles observed during assembly of films fabricated using polymer **1** and plasmid DNA.<sup>49</sup> We note, however, that films fabricated using siRNA are significantly thinner than otherwise identical films fabricated using plasmid DNA (for comparison, films fabricated from eight bilayers of polymer **1** and plasmid DNA are ~120 nm thick, with an average bilayer thickness of ~12.5 nm).<sup>49</sup> These differences in film thickness are not surprising in view of large differences in the sizes and topologies of the siRNA and DNA constructs used in these experiments (e.g., 21 base pair-long linear strands of siRNA versus supercoiled plasmid DNA 4.7 kilobase pairs long) and other physicochemical differences in the structures and stabilities of siRNA and DNA that could also influence the manner in which these constructs adsorb to surfaces during film fabrication. We return to a consideration of these and other differences in the physicochemical properties of siRNA and DNA again in the discussion below.

### Characterization of Film Stability and siRNA Release Profiles

The incorporation of hydrolyzable poly( $\beta$ -amino ester)s such as polymer **1** into the structure of a PEM provides a mechanism by which to promote the destabilization or erosion of a film when it is exposed to aqueous environments (e.g., aqueous buffer, cell culture media, etc.). Past studies have demonstrated that the esters in the backbone of polymer **1** play a critical role in promoting film erosion,<sup>67,68</sup> and that systematic changes in polymer structure and hydrophobicity can be used to change the rates of film erosion and the rates at which incorporated agents are released.<sup>51</sup> We performed a series of experiments to characterize the stability of our polymer **1**/siRNA films and characterize the release of siRNA from these



materials under physiologically relevant conditions. For these experiments, polymer **1**/siRNA films fabricated on planar silicon substrates were incubated in PBS at 37 °C in UV-transparent cuvettes. These film-coated substrates were removed at predetermined intervals to characterize changes in film thickness by ellipsometry (for films fabricated on silicon substrates) and to characterize the amount of siRNA released into the buffer solution as a function of time (by measurements of solution absorbance at 260 nm, the absorbance maximum of siRNA).

Figure 2 shows a plot of released siRNA as a function of time (open triangles) expressed as a percentage of total siRNA released over the duration of the experiment. Inspection of these data reveals that ~65% of the siRNA incorporated into the film was released within the first hour of incubation. Further inspection reveals that this rapid release phase was followed by a more gradual release of the remaining ~35% of the siRNA in the film over the next 23 hours. On the basis of solution absorbance measurements, we estimate the cumulative amount of siRNA released from polymer **1**/siRNA films eight bilayers thick to be ~1.8 μg, corresponding to a loading of ~0.9 μg of siRNA/cm<sup>2</sup> on the surface of a substrate (an additional plot showing the same data presented in Figure 2 expressed as the amount of siRNA released is included in Figure S1 of the Supporting Information). This siRNA release profile differs considerably from the release of plasmid DNA from polymer **1**/DNA films eight bilayers thick (which, as described above, release DNA gradually over a period of ~48 hours).<sup>49</sup> Our past studies also demonstrate that polymer **1**/DNA films release significantly more DNA (e.g., ~5.4 μg of DNA, corresponding to an average loading of ~2.7 μg/cm<sup>2</sup> of film) than these siRNA-containing films.<sup>49</sup> These large differences in siRNA and DNA loading correlate with the large differences in the thicknesses of these films, as discussed above and shown in Figure 1.

Figure 2 also shows a plot of changes in film thickness as a function of time (closed circles, expressed as a percentage of original film thickness) observed for the same films used to acquire the siRNA release profile described above (see also Figure S1 of the Supporting Information). These data demonstrate that film thickness decreases almost completely over a period of 24 hours, consistent with the disruption and time-dependent physical erosion of these films. We note, however, that this physical erosion profile does not correlate directly with the siRNA release profile (open triangles). For example, whereas the siRNA release profile reveals a rapid release of ~65% of the siRNA during the first hour of incubation, film thickness remained relatively constant (at or near the original thickness of ~50 nm) for the first three hours of incubation. These results suggest that large-scale physical erosion of these films is not required for the initial release of siRNA to occur. This behavior differs significantly from that of films fabricated using polymer **1** and plasmid DNA, for which the gradual release of DNA over ~48 hours is generally observed to occur with a simultaneous and gradual physical erosion of film thickness over the same time period.<sup>49</sup> We note, however, that film thickness does begin to decrease after ~3 hours and that the film erodes almost completely over the remaining 21 hours of the experiment (Figure 2). A comparison of these data with those shown in the siRNA release profile reveals this period of physical film erosion to coincide with the slower release of the remaining ~35% of siRNA that was not released during the initial rapid release phase.

When combined, the erosion and release results presented above suggest a physical picture for the behavior of films fabricated from siRNA that differs significantly from films fabricated using plasmid DNA. These differences in film behavior are likely to arise, at least partially, from the large differences in the sizes of these siRNA and plasmid DNA constructs. For example, because plasmid DNA constructs are large and are not likely to be able to diffuse readily through the ionically crosslinked structure of a PEM, the rates of release of DNA from a polymer **1**/DNA film are likely to depend critically upon the rate at

which the film itself is physically disrupted (e.g., as promoted by the gradual hydrolysis of polymer **1**). As a result, gradual release of DNA is observed to be concurrent with gradual decreases in film thickness.<sup>49</sup> In contrast, the observation of the rapid release of large amounts of siRNA from polymer **1**/siRNA films before the onset of significant decreases in film thickness suggests that these significantly smaller siRNA constructs may be able to diffuse through or be expelled rapidly from these films through a mechanism that does not involve loss of large amounts of polymer (e.g., promoted by changes in pH, ionic strength, or temperature or by other structural rearrangements of the film that could occur upon incubation in PBS). The slower release of the remaining ~35% of siRNA over a subsequent period of time (e.g., ranging from ~3 hrs to 24 hrs) that does correlate to decreases in film thickness suggests a second mechanism for release that depends, to some extent, on the rate at which the polymer **1**/siRNA film erodes physically (e.g., allowing entrapped siRNA that was not released or expelled initially to be released gradually).

To provide additional insight into the film erosion process and characterize the release of siRNA and polymer **1** simultaneously, we fabricated polymer **1**/siRNA films using a derivative of polymer **1** that was end-labeled with the small-molecule fluorophore tetramethylrhodamine (TMR) (referred to hereafter as polymer **1<sub>FL</sub>**).<sup>65</sup> Incorporation of polymer **1<sub>FL</sub>** into these films permitted characterization of the release of polymer from these films (via solution fluorometry measurements) during siRNA release as well as additional characterization of these films using fluorescence microscopy. Figure 2 shows a plot of the release of polymer **1<sub>FL</sub>** (closed squares; expressed as a percentage of total polymer released into solution over the duration of the experiment) as a function of time from a film incubated in PBS at 37 °C. Inspection of these data reveals that a small fraction of the polymer (~10%) was released from the film during the first hour of incubation, followed by a gradual linear release over the remaining 23 hours of incubation. In general, this release profile correlates inversely with the gradual decrease in film thickness (closed circles) observed over the same time period. These data provide additional support for the view that the rapid release of siRNA observed during the first hour of incubation (open triangles) is not dependent on physical film erosion (which would be accompanied by the release of polymer **1** into solution). These results are also consistent with the general view, as discussed above, that the second, slower phase of siRNA release is governed, to some extent, by the rate at which the polymer film erodes physically.

We also characterized the surfaces of polymer **1**/siRNA films before and after incubation in PBS using atomic force microscopy (AFM). Figure 3A shows a 25  $\mu\text{m}$   $\times$  25  $\mu\text{m}$  image of a polymer **1**/siRNA film just after fabrication and prior to incubation in PBS. This image reveals the surface of the film to be smooth and largely devoid of micrometer- and nanometer-scale defects or other topographic features. The topography of these films is similar in many respects to that of films fabricated using polymer **1** and plasmid DNA prior to incubation in PBS. Figures 3B-C show images of the same film after incubation in PBS at 37 °C for one hour and three hours, respectively. Inspection of these images reveals the film to undergo significant and large-scale changes (e.g., from a smooth film to a morphology characterized by a pattern of holes and cell-like structures) after one hour of incubation (Figure 3B). These structures were observed to decompose further upon incubation for an additional two hours to yield a surface morphology consisting of an array of nanoparticulate structures (Figure 3C; ranging from ~0.1-1.5  $\mu\text{m}$  in diameter and ~100-500 nm high as determined by AFM image analysis).

The large-scale structural rearrangements shown in Figure 3 occur on a time scale (e.g., within the first ~3 hours of incubation) that is similar to the time scale over which the first phase of rapid siRNA release is observed in the experiments described above (Figure 2). We note that these changes in morphology provide another point of departure from the behavior

of PEMs fabricated using polymer **1** and plasmid DNA. We demonstrated in a past study, for example, that films fabricated using plasmid DNA do also undergo nanometer-scale changes in film morphology upon incubation in PBS buffer.<sup>68</sup> However, changes in the morphology of these DNA-containing films (which also resulted in a progression from smooth films to arrays of nanoparticulate structures) were observed to take place over different time scales and through a mechanism that occurred *without* the concurrent release of DNA. These DNA-containing films were also observed to decompose initially into more complex morphologies that were significantly different than those observed in Figure 3B.<sup>68</sup> Although it is difficult to draw direct conclusions solely on the basis of these current experiments, these large differences in behavior are also likely to arise from differences in the sizes (and thus also the relative mobilities) of the siRNA and plasmid DNA constructs used to fabricate these films. Additional studies will be required to elucidate the physical factors that govern these changes in film morphology more completely. We note, however, that alternative explanations based on differences in the chemical stabilities and physical integrities of siRNA and DNA are not consistent with the results of additional siRNA characterization experiments described below.

### Characterization of Released siRNA

We performed additional experiments to characterize the physical and functional integrity of the siRNA released from polymer **1**/siRNA films. Figure 4 shows the results of polyacrylamide gel electrophoresis characterization of siRNA released after the erosion of a polymer **1**/siRNA film eight bilayers thick (a sample of siRNA taken from a stock solution is included for comparison and is shown in lane 1). These results demonstrate that siRNA released from these films (lanes 2 and 3) remains structurally intact and that it does not degrade or dissociate irreversibly during the processes of film fabrication or erosion.

The results of additional cell-based experiments demonstrated that siRNA released from these multilayered films was functional and able to promote the selective knockdown of gene expression in mammalian cells. For these cell-based experiments, we fabricated polymer **1**/siRNA films eight bilayers thick on stainless steel mesh substrates and incubated these film-coated substrates in PBS at 37 °C for 24 hours. Samples of siRNA released at different time points during these experiments were formulated using a commercially available lipid-based siRNA delivery agent (Lipofectamine RNAiMAX) and added to cultures of COS-7 cells transiently transfected using plasmid DNA encoding enhanced green fluorescent protein (EGFP).

The images in Figure 5 show representative, low-magnification fluorescence micrographs of transiently transfected cells imaged 72 hours after treatment (Figure 5B) or no treatment (Figure 5A) with samples of released siRNA. The reduced level of green fluorescence in the cells in the image in Figure 5B demonstrates that a significant amount of the siRNA that is released from polymer **1**/siRNA films is released in a form that remains functionally active and able to knock down the expression of EGFP. The levels of knockdown achieved using aliquots of released siRNA (Figure 5B) were qualitatively similar to those observed in control experiments using aliquots of control GFP-siRNA taken from an unused stock solution (Figure 5C). Additional control experiments conducted using RNAiMAX only (Figure 5D) or samples of RNAiMAX and siRNA released from a film fabricated using an siRNA construct targeted against luciferase mRNA (Luc-siRNA; Figure 5E) demonstrated that the silencing of EGFP expression was (i) sequence specific and (ii) not a result of lowered protein expression and/or toxicity exhibited by the lipid agent used in these experiments (Figure 5D) or the presence of polymer **1** (Figure 5E). Finally, the image shown in Figure 5F shows cells treated with aliquots of released siRNA in the absence of the lipid-based delivery agent, and demonstrates that the presence of polymer **1** alone in solutions of released siRNA is not sufficient to promote significant levels of EGFP knockdown under

these conditions. The results of these experiments, when combined, demonstrate that the siRNA released from these films was released in a form that remained able to promote the knockdown of EGFP in mammalian cells.

To characterize levels of gene silencing more quantitatively, we performed a second set cell-based experiments and measured differences in the levels of knockdown using flow cytometry. These experiments were conducted using lipoplexes formulated using aliquots of released siRNA and a second cell type (HeLa cells) stably transfected to express GFP constitutively. The results of these experiments are shown in Figure 6. Columns 1 and 2 of Figure 6 show relative levels of GFP expression in cells 72 hours after treatment (column 2) or no treatment (column 1) with lipoplexes of released siRNA. Column 4 shows levels of knockdown achieved using lipoplexes formulated using a sample of control siRNA, and column 3 shows levels of knockdown achieved in the absence of the cationic lipid reagent. These results confirm that released siRNA is able to silence GFP expression. We note here that the observed levels of knockdown (~80-90%) shown in columns 2 and 4 largely reflect the efficacy of the lipid reagent used. As a result, conclusions made on the basis of these experiments are limited to those regarding the functional integrity of the released siRNA (that is, the ability of released siRNA to mediate knockdown upon subsequent administration to cells). Finally, column 5 of Figure 6 shows the results of experiments performed using lipoplexes formulated using Luc-siRNA, and demonstrates again that knockdown induced by the GFP-siRNA construct is specific and not the result of delivery agent-induced toxicity.

## Summary and Conclusions

The results of this study demonstrate that small, double-stranded siRNA constructs can be incorporated directly into and released from ultrathin polyelectrolyte multilayers fabricated using a hydrolytically degradable cationic polymer (polymer **1**). The siRNA that is released from these films is released in a form that remains physically intact and able to promote knockdown of gene expression upon subsequent administration to mammalian cells. Our results reveal several significant differences in the properties and behaviors of films fabricated using polymer **1** and siRNA as compared to films fabricated using polymer **1** and larger plasmid DNA constructs. When combined, the results of this investigation provide a platform for the design of thin films and surface coatings that could be used to control and localize the release of siRNA from surfaces in a variety of fundamental and applied contexts.

The layer-by-layer assembly process used here provides several potential practical advantages relative to other methods used for the encapsulation or immobilization of nucleic acid constructs, including (i) the ability to control with precision the amount of siRNA incorporated and released (e.g., by changing the number of polymer **1**/siRNA layers deposited), and (ii) the ability to fabricate thin conformal films on the complex surfaces typical of many implants and biomedical devices. In addition, these methods provide opportunities to incorporate additional layers of different polymers or other auxiliary agents that could also be used to change the rate at which siRNA is released or promote the more efficient internalization of siRNA by cells. Our current results demonstrate that the release of siRNA from polymer **1**/siRNA films occurs more rapidly than the release of plasmid DNA from polymer **1**/DNA films. The results of our past studies on DNA-containing films, however, suggest that it should prove possible to design films that release siRNA over longer time periods by incorporating different polymers. We note in this context that the observation of a mechanism for the release of siRNA that does not appear to be completely dependent on film erosion suggests that specific polymer structures and principles developed for the longer-term release of DNA may not provide a direct basis for the design of films that release siRNA more slowly. We note further, however, that the faster release profiles of

the polymer **1**/siRNA films investigated in this current study could also prove particularly advantageous in applications for which the rapid release of siRNA is required (e.g., for surgical procedures that require the insertion and rapid removal of interventional devices, etc.).

Finally, we note that we used a cationic lipid delivery agent to characterize the activity of siRNA and perform the knockdown experiments reported here. Our past studies demonstrate that films fabricated from polymer **1** and DNA can be used to promote surface-mediated cell transfection in the absence of additional agents,<sup>49-51</sup> suggesting the possibility that the cationic polymers in these PEMs could play a role in promoting the internalization of DNA (e.g., by formation of electrostatic complexes that could promote endocytosis). Although general barriers to the effective delivery of DNA and siRNA are different in several important ways, the juxtaposition of cationic polymers and siRNA in these PEMs creates opportunities to incorporate other polymers, polyplexes, or delivery agents<sup>38-40</sup> that promote the direct internalization and processing of siRNA by cells. Studies to this end are currently underway.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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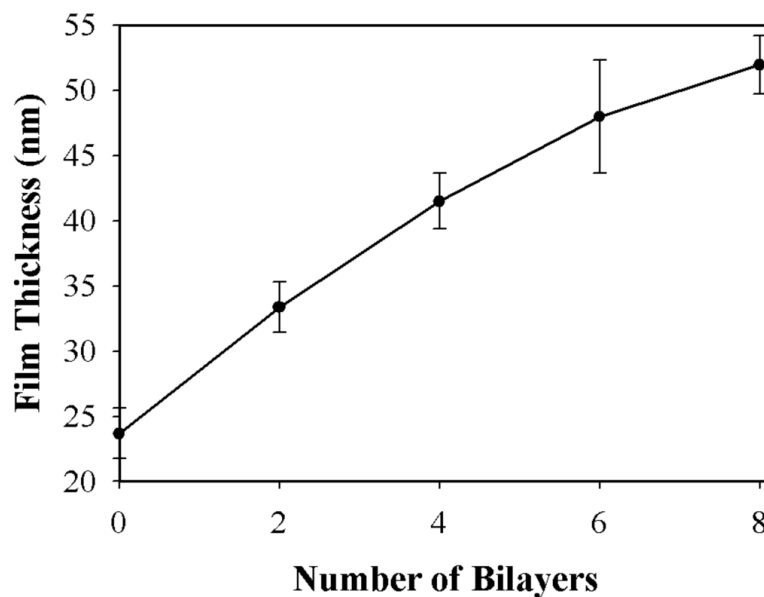
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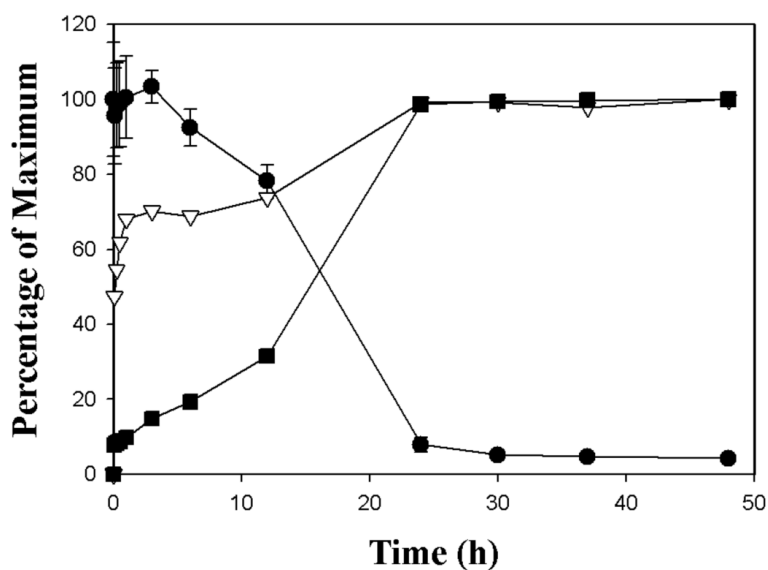
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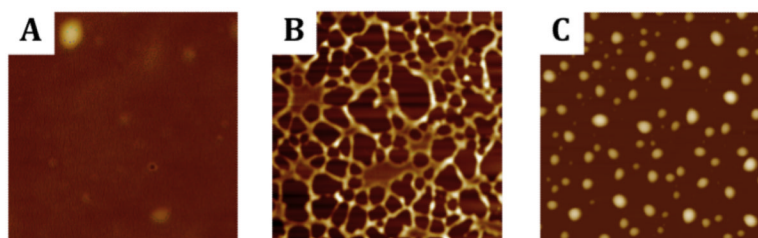
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**Figure 1.** Plot of ellipsometric film thickness versus the number of polymer 1/GFP-siRNA bilayers deposited onto a planar silicon substrate. Data points represent the average of multiple different film thickness values measured for two films fabricated on the same day under identical conditions. The silicon substrates were pre-coated with 10 bilayers of a LPEI/SPS film prior to the fabrication of the polymer 1/GFP-siRNA film (see text).



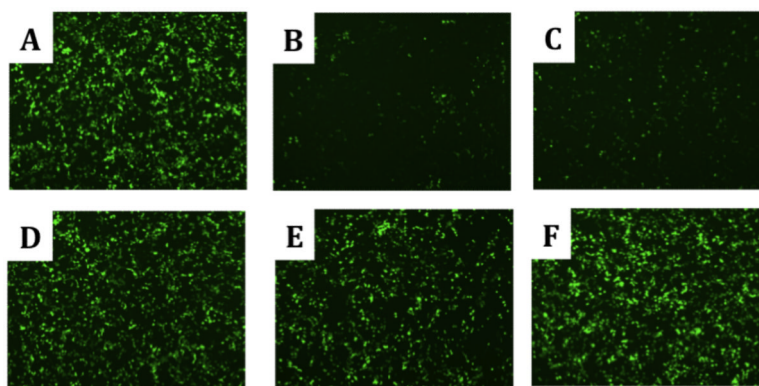
**Figure 2.** Plot of changes in film thickness (●), the release of siRNA (▽), and the release of polymer **1<sub>FL</sub>** (■) resulting from the incubation of a polymer **1<sub>FL</sub>**/GFP-siRNA film eight bilayers thick in PBS at 37 °C. Values are expressed as percentages of original film thickness or the total cumulative amounts of siRNA or polymer **1** released. Individual plots showing these data expressed as measured values of film thickness, siRNA released, and polymer released (rather than as relative percentages of each) are included as Supporting Information.



**Figure 3.** Representative  $25 \times 25 \mu\text{m}$  AFM images of a (polymer **1**/siRNA)<sub>8</sub> film (A) immediately after fabrication and after (B) 1 hour and (C) 3 hours of incubation in PBS at  $37^\circ\text{C}$  (see text). The z-scales for these images are 200 nm for the image in (A), 300 nm for the image in (B), and 1200 nm for the image in (C).

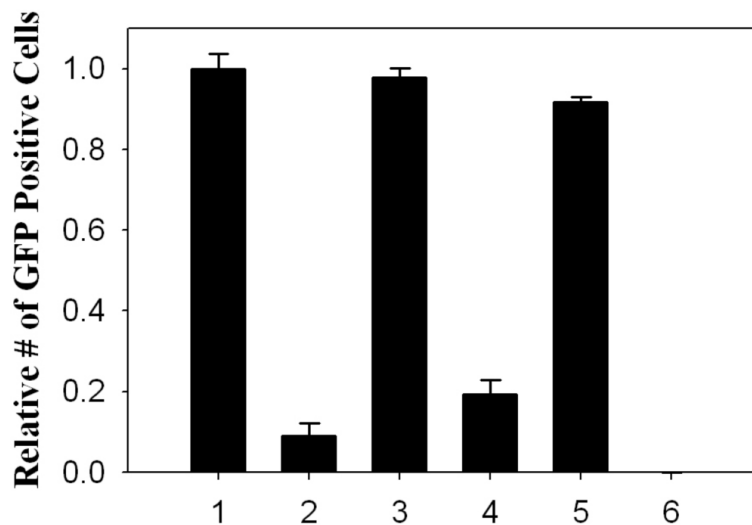


**Figure 4.** Polyacrylamide gel (stained with ethidium bromide) used to characterize GFP-siRNA released from a polymer 1/siRNA film eight bilayers thick (lanes 2 and 3). Lane 1 shows results for a control sample of GFP-siRNA for comparison.



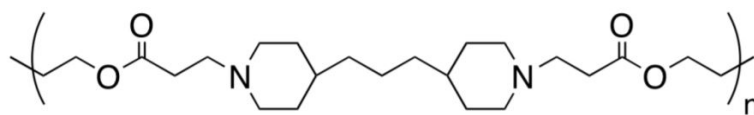
**Figure 5.** Representative low-magnification fluorescence microscopy images (4x) of transiently transfected COS-7 cells imaged 72 hours after treatment (B) or no treatment (A) with samples of siRNA released from a polymer **1**/GFP-siRNA film (formulated with RNAiMAX, see text). (C-F) Results of additional control experiments showing: (C) cells treated with unused GFP-siRNA stock + RNAiMAX, (D) cells treated with RNAiMAX only, (E) cells treated with Luc-siRNA erosion solution + RNAiMAX, and (F) cells treated with released GFP-siRNA in the absence of RNAiMAX. All images were acquired using the same exposure time.

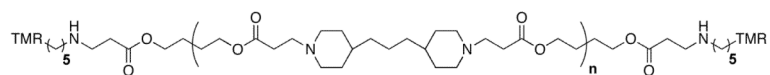




**Figure 6.**

Flow cytometry data for HeLa cells stably expressing GFP 72 hours after treatment (2) or no treatment (1) with samples of siRNA released from a polymer 1/GFP-siRNA film (formulated with RNAiMAX, see text). (3-6) Results of additional control experiments showing: (3) cells treated with released GFP-siRNA in the absence of RNAiMAX, (4) cells treated with unused GFP-siRNA stock + RNAiMAX, (5) cells treated with Luc-siRNA stock + RNAiMAX. Column (6) is a negative control representing a population of HeLa cells not expressing GFP. The data are averages of at least 3 replicates.

**Polymer 1.**



**Polymer 1FL.**