Multilayered Films Fabricated from an Oligoarginine-Conjugated Protein Promote Efficient Surface-Mediated Protein Transduction

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The conjugation of cationic protein transduction domains to proteins results in an increase in the extent to which proteins are internalized by cells. This investigation sought to determine whether the conjugation of a protein transduction domain to a functional protein could be used to facilitate the incorporation of the protein into multilayered polyelectrolyte films and, subsequently, whether these films could be used to promote surface-mediated protein transduction. We demonstrate that it is possible to fabricate multilayered assemblies 80 nm thick using sodium polystyrene sulfonate (SPS) and bovine pancreatic ribonuclease (RNase A) conjugated to the cationic protein transduction domain nonaarginine (R_9) using an entirely aqueous layer-by-layer process. We demonstrate further that the conjugation of R_9 to RNase A permits the assembly of multilayered films under conditions that do not allow for the incorporation of the unmodified protein. This result suggests that R_9 functions as a cationic anchor and serves to increase the strength of electrostatic interactions with SPS and facilitate layer-by-layer assembly. We also demonstrate that RNase A−R_9/SPS films dissolve rapidly in physiologically relevant media and that macroscopic objects coated with these materials can be used to mediate high levels of protein transduction in mammalian cells. These results suggest the basis of general methods that could contribute to the design of materials that permit spatial and temporal control over the delivery of therapeutic proteins to cells and tissues.

Introduction

The alternating, layer-by-layer adsorption of positively and negatively charged polymers on surfaces is a convenient and versatile method for the fabrication of well-defined, nanostructured thin films.[1−4] The stepwise nature of this process permits precise control over the compositions, thicknesses, and surface properties of multilayered assemblies fabricated from a wide variety of water-soluble polymers. The ability to incorporate biologically active species such as peptides,[5,6] proteins,[7−26] and DNA[27−32] into these assemblies without loss of biological function has made possible the development of catalytically and biologically active thin films,[5−8,10,19−21,30] membranes,[4,18,20,23,26,33] and microcapsules with potential applications in many areas of biology, biotechnology, and medicine.[34,35]

The development of new tools and methods that provide control over the incorporation of proteins into multilayered films would be useful in the contexts of both basic biomedical research and the development of new catalytic and therapeutic applications of these materials. Here, we report an approach to the fabrication of ultrathin, protein-containing assemblies that makes use of a cationic protein transduction domain conjugated to a functional protein. These assemblies permit the efficient and spatially localized delivery of functional proteins to cells and could prove useful for the localized release of therapeutic proteins from the surfaces of objects coated with multilayered polyelectrolyte assemblies.

Past work describing the incorporation of proteins into multilayered polyelectrolyte assemblies has focused largely on naturally occurring (that is, wild-type) proteins.[7−27] Numerous studies have demonstrated that manipulating the pH or ionic strength of polyelectrolyte, protein, or polypeptide solutions used during fabrication can influence the growth and structures of these films as well as the structure and function of incorporated proteins.[16,22,24,25,36−40] One general limitation of this approach, however, is that assembly conditions and film properties are often dependent upon the magnitude and sign of the net charge, isoelectric point, and other physical properties of the native proteins or polyelectrolytes that are used. Of particular relevance to the work reported here is a recent report by Li and Haynie demonstrating that model peptides rationally designed to contain high densities of cationic residues (e.g., lysine) or anionic residues (e.g., glutamic acid) can be used to facilitate the assembly of multilayered films using layer-by-layer procedures.[38] On the basis of this report, we hypothesized that appending a highly charged domain to a protein would facilitate its incorporation into multilayered assemblies under conditions that do not otherwise allow for the incorporation of the unmodified protein. We report here that the conjugation of a cationic protein transduction domain to the enzyme bovine pancreatic ribonuclease (RNase A) increases dramatically the extent to which this enzyme can be incorporated into multilayered polyelectrolyte films.

The work reported here is based on past studies by us[41,42] and others[43−50] describing the conjugation of protein transduction domains (PTDs) to functional proteins. These studies have demonstrated that appending short, cationic peptides or non-

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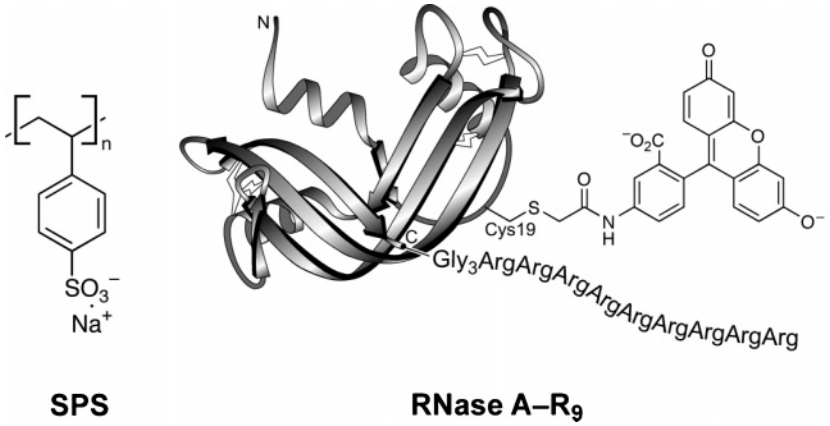

Figure 1. Structures of sodium poly(styrene sulfonate) (SPS) and fluorescein-labeled RNase A−R_9 used in this study (N = amino terminus, C = carboxyl terminus, Cys = cysteine, Gly = glycine, Arg = arginine).

natural, cationic oligomers to proteins can facilitate their uptake by cells. For example, we demonstrated recently that the conjugation of nonaarginine (R_9) to fluorescently labeled RNase A (Figure 1) dramatically increases the cellular internalization of RNase A without loss of ribonucleolytic activity.[41] In addition to the biological activity endowed by PTDs, the conjugation of R_9 to proteins also provides a general and straightforward method for conferring cationic charge without compromising protein function. For example, our previous studies also demonstrate that the conjugation of R_9 to RNase A increases its adsorption onto negatively charged glass and silica substrates.[41] This result suggests the basis of a general approach to facilitate incorporation of proteins into multilayered polyelectrolyte assemblies using anionic polyelectrolytes.

This investigation sought to determine whether the conjugation of nonaarginine to RNase A could be used to facilitate the incorporation of RNase A into multilayered assemblies and, subsequently, whether films fabricated using RNase A−R_9 conjugates could be used to localize the delivery of RNase A to cells. We demonstrate here that it is possible to fabricate multilayered films using RNase A−R_9 conjugates and a model anionic polymer [sodium poly(styrene sulfonate) (SPS)], and that R_9 functions as a cationic anchor that permits the incorporation of RNase A into films under conditions that do not allow for the incorporation of unmodified RNase A. We also demonstrate that macroscopic objects coated with these ultrathin assemblies can be used to provide spatial control over the delivery of RNase A−R_9 to mammalian cells. This work could lead to the development of new tools and methods for the localized, surface-mediated delivery of therapeutic proteins to cells.

Experimental Methods

Materials. Test grade n-type silicon wafers were obtained from Si-Tech (Topsfield, MA). Quartz microscope slides were purchased from Chemglass (Vineland, NJ). Linear poly(ethylene imine) (LPEI, MW = 25,000) was obtained from Polysciences (Warrington, PA). Poly(sodium 4-styrenesulfonate) (SPS, MW = 70000) was obtained from Aldrich (Milwaukee, WI). All commercial polyelectrolytes were used as received without further purification. Hoechst 34580 and wheat germ agglutinin−Alexa 594 fluorescent stains were purchased from Invitrogen (Carlsbad, CA). Deionized water (18 MΩ) was used for washing steps and to prepare all polymer and protein solutions. PBS buffer was prepared by diluting commercially available concentrate (EM Science, Gibbstown, NJ). Glass inset dishes used for laser scanning confocal microscopy (LSCM) were purchased from MatTek (Ashland, MA).

General Considerations. All buffers and polymer solutions were filtered through a 0.2 μm membrane syringe filter prior to use. Quartz and silicon substrates (3.5 cm × 0.5 cm) were cleaned with acetone, ethanol, methanol, and deionized water, dried under a stream of filtered air, and cleaned further by etching in an oxygen plasma (Plasma Etch, Carson City, NV) for 5 min prior to film deposition. UV−vis absorbance values used to quantify film deposition on quartz substrates were recorded using a DU 520 UV−vis spectrophotometer (Beckman Coulter, Fullerton, CA) at a wavelength of 226 nm (corresponding to the absorbance maximum of SPS) in at least four different locations on each sample. The optical thicknesses of films deposited on silicon substrates were determined in at least five locations 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 by assuming an average refractive index of 1.58 for the multilayered films. Fluorescence measurements of solutions used to erode multilayered films were made using a Fluoromax-3 fluorimeter (Jobin Yvon, Edison, NJ) at an excitation wavelength of 490 nm. The mean fluorescence emission intensity was determined from intensity values recorded from 514 to 520 nm. Laser scanning confocal microscopy was performed using a Bio-Rad Radiance 2100 MP Rainbow laser scanning confocal microscope equipped with a multiphoton laser. Images were processed using the Bio-Rad LaserSharp 2000 processing kit and Adobe Photoshop 8.0.

Production and Purification of Fluorescent RNase A and RNase A−R_9 Conjugates. Fluorescein-labeled RNase A and fluorescein-labeled RNase A conjugated to R_9 were prepared as described previously.[41] Untagged variants of RNase A were produced in *Escherichia coli* and purified as described previously.[51] Variants of RNase A containing a C-terminal R_9 tag were prepared by growing BL21(DE3)-PlysS cells containing plasmid encoding the RNase A variant at 37 °C with shaking (250 rpm) in Terrific Broth containing ampicillin (200 μg/mL) and chloramphenicol (35 μg/mL) to an optical density of 1.6 at 600 nm. cDNA expression was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG; 1 mM). Cells were grown for an additional 4 h before harvesting. Cell pellets were resuspended in a lysis buffer of 10 mM Tris−HCl (pH 8.0) containing ethylenediaminetetraacetic acid (EDTA; 1.0 mM), NaCl (0.10 M), and phenylmethylsulfonyl fluoride (1.0 mM), and lysed by sonication. Inclusion bodies were isolated by centrifugation at 11000*g* for 45 min and solubilized in a denaturing solution of 20 mM Tris−HCl buffer (pH 8.0) containing guanidine hydrochloride (7.0 M) and EDTA (10 mM) for 4 h at room temperature. Solubilized inclusion bodies were diluted 10-fold with acetic acid (20 mM) and clarified by centrifugation. The supernatant was dialyzed overnight against the same buffer. The resulting protein was then folded overnight at 4 °C in a redox buffer of 0.1 M Tris−HCl (pH 8.0) containing EDTA (10 mM), L-arginine (0.5 M), reduced glutathione (1 mM), and oxidized glutathione (0.2 mM). Refolded

protein was purified by cation-exchange chromatography on a 5 mL column of HiTrap SP-sepharaose FF resin (Amersham Biosciences, Piscataway, NJ) in 50 mM sodium acetate buffer (pH 5.0) with a linear gradient (50 + 50 mL) of NaCl (0−1.5 M). The identity of each variant was verified by MALDI-TOF mass spectrometry. Ribonucleases were labeled with fluorescein at one specific residue in a surface loop by using variants in which Ala19 was replaced with a cysteine residue.[51] Film stability and release experiments described below relied on the fluorescence of the dianionic form of fluorescein. The second pK_a of fluorescein is 6.3,[64] which is much less than the pH of PBS and is likely to be even lower in the proximity of a highly cationic protein such as RNase A. A19C RNase A or A19C RNase A−R_9 (100 μM) were incubated in PBS containing a 20-fold molar excess of 5-iodoacetamidofluorescein (Molecular Probes, Eugene, OR) and a 3-fold molar excess of tris[2-carboxyethylphosphine] (TCEP) hydrochloride for 4 h at room temperature. The resulting solution was dialyzed overnight against 50 mM sodium acetate buffer (pH 5.0), and then purified by cation-exchange chromatography using a 5 mL HiTrap CM-Sepharose Fast Flow column with a linear gradient (50 + 50 mL) of NaCl (0−1.00 M for A19C RNase A; 0−2.00 M for A19C RNase A−R_9). Conjugation to the fluorophore was confirmed by MALDI-TOF mass spectrometry.

Preparation of Protein and Polyelectrolyte Solutions. Solutions of protein (7.0 μM with respect to concentration of RNase A) contained either fluorescein-labeled RNase A (RNase A) or fluorescein-labeled RNase A conjugated to nonaarginine (RNase A−R_9) and were prepared by diluting a concentrated stock with water. The concentrations of protein in stock solutions were determined by using $\epsilon = 9860\ M^{-1}\ cm^{-1}$ for RNase A at 277 nm and correcting for the absorbance of the fluorescein moiety with the equation: $A_{277nm}^{protein} = A_{277nm}^{observed} - (A_{494nm}^{observed}/5)$.[52] 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 water. LPEI solutions contained 5 mM HCl to aid polymer solubility. SPS solutions used for the deposition of protein/SPS layers (20 mM with respect to the polymer repeat unit) were prepared in water, and the pH was adjusted to 5.0 with HCl.

Fabrication of Multilayered Films. All protein/SPS films were deposited on quartz or silicon substrates precoated with 10 bilayers of linear poly(ethylene imine) and poly(styrene sulfonate) fabricated using a previously optimized procedure.[31,53] These precursor layers were deposited manually or by using an automated dipping robot (Riegler & Kirstein GmbH, Potsdam, Germany). Multilayered films fabricated using RNase A and SPS were fabricated on these foundation layers manually using the following general protocol: (1) substrates were submerged in a solution of protein (RNase A or RNase A−R_9) for 5 min, (2) substrates were removed and immersed in a wash bath of deionized water for 1 min followed by a second wash bath for 1 min, (3) substrates were submerged in a solution of SPS for 5 min, and (4) substrates were rinsed in the manner described above. This cycle was repeated until the desired number of protein and SPS layers (typically eight each) had been deposited. To produce substrates coated with multilayered films on only one side, commercially available rubber cement was applied to one face of the substrate and allowed to dry prior to dipping. Removal of the rubber cement by peeling after film fabrication yielded substrates coated with protein/SPS films on a single side.

Characterization of Film Stability and Protein Release Experiments. Experiments designed to evaluate film stability and characterize the release of protein from multilayered protein/SPS films were performed in the following general manner: film-coated substrates were placed in a plastic UV-transparent cuvette, and phosphate-buffered saline (PBS, pH 7.4, 137 mM NaCl) was added in an amount sufficient to cover the substrate. The samples were incubated at 37 °C and removed at predetermined intervals for analysis by ellipsometry (for silicon substrates) or UV−vis spectrophotometry (for quartz substrates). Optical thickness and absorbance measurements were made in at least four different predetermined locations on each substrate. For experiments designed to monitor the concentrations of protein released into the buffer solution, fluorescence readings at 514−520 nm (corresponding to the maximum fluorescence emission range of fluorescein) were made directly on the buffer solution. After each measurement, substrates were placed in a fresh aliquot of PBS and returned to the incubator at 37 °C. Measurement of the pH of the buffer at each time point indicated that pH did not change during the course of these experiments. Arbitrary fluorescence units arising from these experiments were converted to micrograms of protein released using a standard curve prepared using known concentrations of RNase A−R_9.

In Vitro Protein Transduction Experiments. COS-7 cells were grown in glass inset confocal microscopy dishes at initial seeding densities of 7.5×10^4 cells/mL in 3.0 mL of growth medium [90% (v/v) Dulbecco's modified Eagle's medium, 10% (v/v) fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin]. Cells were allowed to grow overnight to approximately 90% confluence, and growth medium was replaced with 3.0 mL of serum-free culture medium (OptiMEM). Quartz slides coated with multilayered films on one or both sides were placed manually into dishes on top of cells. In experiments involving slides coated on a single face, the substrate was placed such that the film-coated face was in direct contact with the cells. In both cases, cells were incubated for 3 h at 37 °C and analyzed directly (without removal of the quartz slide) using a Bio-Rad Radiance 2100 MP Rainbow LSCM. Immediately prior to imaging, cells were stained using wheat germ agglutinin (WGA)−Alexa 594 membrane stain and Hoechst nuclear stain according to the manufacturer's protocols. LSCM images were acquired using a 60×/1.40 NA oil-immersion objective. Images were recorded for populations of cells growing either directly under the film-coated substrates or in random remote locations of the culture well up to 2 mm away from the film-coated substrates. Fluorescein, Hoechst, and WGA−Alexa 594 probes were excited sequentially using laser lines at 488, 543, and 800 nm (multiphoton laser), respectively. Fluorescence emission signals were collected for three individual channels using direct scanning mode ($N = 1$, scan speed = 50 lps) and merged to create three-color images.

Results and Discussion

Past studies demonstrate that proteins can be incorporated into ultrathin, multilayered polyelectrolyte assemblies using layer-by-layer fabrication procedures.[7−26] The work reported here is based on our recent discovery that the conjugation of nonaarginine (R_9) to RNase A (Figure 1) leads to an increased affinity of this enzyme for glass and silica substrates.[41] We hypothesized that the conjugation of nine additional cationic arginine groups to RNase A might also (1) increase the strength of electrostatic interactions between RNase A and negatively charged polyelectrolytes, and (2) provide a mechanism for the incorporation of RNase A into films under conditions that do not allow for incorporation of the unmodified protein. In addition, numerous past studies have shown that the conjugation of short cationic peptide sequences such as R_9 to proteins increases their uptake by cells.[41−50] Therefore, we also sought to determine whether macroscopic objects coated with films fabricated using RNase A−R_9 could be used to localize the delivery of RNase A to mammalian cells.

Fabrication of Films Using RNase A, RNase A−R_9, and Sodium Poly(styrene sulfonate). Multilayered films were fabricated on planar quartz and silicon substrates to facilitate characterization of film growth and thickness by UV−vis absorbance and ellipsometry, respectively. For all experiments, substrates were precoated with a thin multilayered film composed of LPEI and SPS (approximately 30 nm thick, with a topmost layer of SPS) to provide a charged surface suitable for subsequent adsorption of RNase A or RNase A−R_9. In all

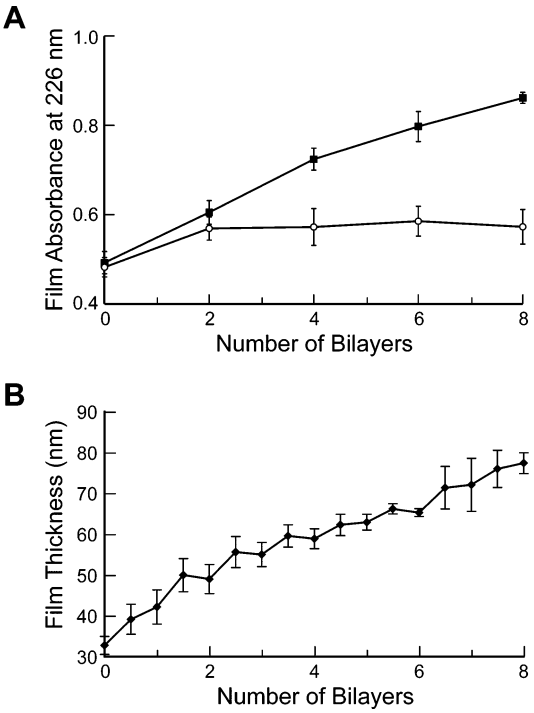

Figure 2. (A) Plot of absorbance (at 226 nm) versus the number of protein/SPS bilayers deposited onto quartz substrates. Data shown are for films fabricated using unmodified RNase A (○) or RNase A−R_9 (■). (B) Plot of ellipsometric film thickness versus the number of RNase A−R_9/SPS bilayers deposited onto a silicon substrate. In both cases, substrates were precoated with 10 bilayers of an LPEI/SPS film prior to fabrication of the protein/SPS films (see text).

experiments, RNase A and RNase A−R_9 conjugates were labeled with fluorescein at residue 19 to facilitate the visualization and tracking of RNase A in subsequent cellular internalization studies (described below).[41]

Fabrication of multilayered films was performed using an alternate dipping procedure. The iterative dipping of quartz substrates into RNase A−R_9 (7.0 μM in water; pH = 5.0) and SPS (20 mM in water; pH = 5.0) resulted in the growth of multilayered RNase A−R_9/SPS films. Figure 2a shows the increase in UV absorbance (at 226 nm, the absorbance maximum of SPS) for a representative RNase A−R_9/SPS film as a function of the number of protein/SPS layer pairs (referred to hereafter as "bilayers") deposited. These data demonstrate that film growth occurred in a linear manner, consistent with the growth of multilayered films fabricated from other conventional proteins.[8,11,54,55] This linear growth profile provides convenient and predictable control over the amount of RNase A−R_9 immobilized at the surface of a coated substrate by control over the number of RNase A−R_9/SPS bilayers deposited. In contrast to films fabricated using RNase A−R_9, we did not observe significant film growth when solutions of RNase A not tagged with R_9 were used for film fabrication (at concentrations and pH values identical to those used above). As shown in Figure 2a, the absorbance of substrates used to fabricate films using RNase A not tagged with R_9 remained essentially constant after the deposition of the first two bilayers.

We also fabricated RNase A−R_9/SPS and RNase A/SPS films on silicon substrates to characterize film growth and thickness using ellipsometry. As shown in Figure 2b, films fabricated using RNase A−R_9 increased in optical thickness to yield films up to 80 nm thick after the deposition of eight bilayers. Although the overall growth profile for these films is linear, closer inspection of these data reveals that the majority of the increase

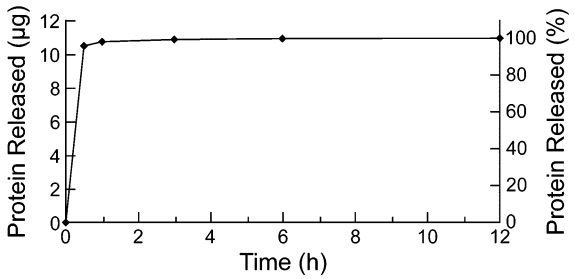

Figure 3. Plot of protein released versus time for an eight bilayer RNase A-R_9/SPS film incubated in PBS buffer at 37 °C.

in the thicknesses of these films resulted from the deposition of the protein-containing layers. On the basis of these ellipsometry data, we calculate the average thickness of each protein/SPS layer in these materials to be ~5.5 nm/bilayer. In contrast to films fabricated using RNase A−R_9, the optical thicknesses of films fabricated using solutions of RNase A that was not tagged with R_9 did not increase significantly for up to eight deposition cycles under otherwise identical fabrication conditions (data not shown). These differences in film growth profiles are consistent with the large differences in film growth observed by UV absorbance (Figure 2a). Taken together, these experiments demonstrate (1) that RNase A conjugated to R_9 can be incorporated effectively into multilayered films using SPS as an anionic film component, and (2) that RNase A−R_9 can be incorporated into films under conditions (e.g., 7 μM in water; pH 5.0) for which film growth *does not* occur using RNase A not tagged with R_9. We interpret these data to suggest that the conjugation of R_9 facilitates the growth of multilayered films under these conditions, presumably by increasing the strength of the electrostatic interactions between the more cationic protein and the anionic SPS. Nonaarginine may thus be viewed as a cationic "anchor", the conjugation of which permits the assembly of films under conditions that are not suitable for the assembly of films using native, unmodified RNase A.

Incubation of RNase A/SPS Films and Release of RNase A. Past studies have investigated the stability of protein-containing films in aqueous environments with a view toward designing assemblies with properties tailored for specific applications.[7−26] For example, films and assemblies that are stable in aqueous environments have been used to design catalytically active membranes and microcapsules.[4,12,13,18,20,23,26,33,36] In contrast, films and assemblies that are unstable or that erode in physiologically relevant media could be useful for the controlled, sustained, or localized release of proteins. Several groups have reported that multilayered polyelectrolyte assemblies can be disrupted or eroded upon changes in environmental pH, ionic strength, or other factors that change the nature of physical interactions in these ionically crosslinked materials.[28,56−61] We sought to investigate the stability of RNase A−R_9/SPS films in physiologically relevant media and determine whether it was possible to use these materials for the sustained or localized delivery of RNase A from surfaces.

Quartz substrates coated with films fabricated using SPS and either RNase A−R_9 or unmodified RNase A were incubated in phosphate-buffered saline (PBS, pH 7.4, 137 mM NaCl) at 37 °C in UV-transparent cuvettes. Substrates were removed at predetermined intervals, and the fluorescence of the incubation buffer was recorded directly from 514 to 520 nm (i.e., the maximum fluorescence emission range of fluorescein; see Experimental Methods for complete details of incubation procedures) and used to calculate the amount of protein released. Figure 3 shows a plot of the amount of protein released versus

time for films fabricated from eight bilayers of RNase A−R_9/SPS. Inspection of these data reveals a rapid and large increase in protein release over the first 30 min (corresponding to ∼96% of the protein released) and that the cumulative amount of protein released over this 12 h period was ∼11 μg. On the basis of these data and the dimensions of the film-coated portions of the substrates used in these experiments, we conclude that RNase A−R_9/SPS films eight bilayers thick contained approximately 6.1 μg of RNase A−R_9 per cm^2. No significant increase in solution fluorescence was observed during the incubation of films fabricated using RNase A not modified with R_9 (data not shown), consistent with our earlier observations that unmodified RNase A is not incorporated effectively into multilayered assemblies under the conditions used in this current study (e.g., Figure 2).

We interpret the results in Figure 3 to suggest that films fabricated from SPS and RNase A−R_9 dissolve and release RNase A−R_9 into solution rapidly when incubated in PBS. Characterization of the thicknesses of RNase A−R_9/SPS films fabricated on silicon substrates using ellipsometry revealed large and rapid decreases in optical thickness from ∼80 to ∼35 nm after incubation in PBS for 30 min. This remaining thickness of 35 nm corresponds closely to the thickness of the LPEI/SPS foundation layers used to coat these silicon substrates prior to fabrication of the protein/SPS films. These ellipsometry data are thus consistent with the large and rapid increase in solution fluorescence shown in Figure 3 over the same time period and provide additional support for the view that these films dissolve or disintegrate rapidly upon incubation in PBS.

As described above, many past studies have demonstrated that is it possible to disrupt multilayered polyelectrolyte assemblies by changing environmental parameters such as pH or ionic strength that change the nature of the ionic interactions in these ionically crosslinked assemblies.[28,56−61] We note here that the RNase A−R_9/SPS films used in this study were fabricated using protein and polymer solutions prepared in water, but that release and stability experiments were conducted in phosphate buffered saline. Thus, while the cationic oligopeptide R_9 does facilitate film assembly in water, the electrostatic interactions in these assemblies may be effectively disrupted upon transfer to a medium of higher ionic strength. The thickness of an RNase A−R_9/SPS film incubated in water (as opposed to PBS) did not decrease significantly for up to 200 h when incubated at 37 °C. In combination with the results shown in Figure 3, this control experiment provides support for the view that the large changes in pH and ionic strength that are experienced upon transfer to PBS play a significant role in the rapid dissolution and release of RNase A−R_9 from these materials. Although the films described here do not provide temporal control over the release of RNase A−R_9 from film-coated surfaces, we demonstrate below that objects coated with these materials can be used to exert spatial control over the release of protein and the internalization of protein by cells.

Surface-Mediated Delivery of RNase A−R_9 to Cells. Numerous past studies have demonstrated that the conjugation of protein transduction domains such as R_9 to proteins increases dramatically their transport into cells.[41−50] On the basis of these past reports and the results of our current study, we sought to determine whether ultrathin multilayered RNase A−R_9/SPS films could be used to promote the surface-mediated delivery of RNase A to cells.

To investigate the ability of these assemblies to mediate protein transduction, we fabricated RNase A−R_9/SPS films composed of eight bilayers on planar quartz substrates. Quartz substrates were used in these experiments to permit characterization of film growth using UV−vis spectrophotometry (e.g., Figure 2a) and to permit the tracking of fluorescently labeled RNase A−R_9 conjugates in cells using fluorescence microscopy.[41] Film-coated slides were placed in direct contact with COS-7 cells growing in a reduced serum cell-culture medium (e.g., Figure 4) and incubated in the presence of cells for 3 h. Cells were subsequently treated with fluorescent membrane and nuclear stains (WGA-Alexa 594 and Hoechst 34580) and imaged using LSCM without removal of the quartz slides.

Parts A and B of Figure 4 show representative 60×, three-color LSCM images of COS-7 cells incubated in the presence of a quartz slide coated with RNase A−R_9/SPS films on both sides (that is, on both the top and bottom faces of the slide). These images demonstrate that RNase A−R_9 is internalized efficiently by cells, as determined by the presence of numerous punctate green fluorescent spots in nearly all cells. The observation of punctate fluorescence is consistent with our previous observations that proteins and peptides conjugated to R_9 are trafficked to, and largely sequestered in, endosomes and lysosomes after internalization by cells.[41,62] We note here that while Figure 4A demonstrates the internalization of protein in cells growing directly beneath the film-coated slide, we also observed high levels of transduction in cells that were located in remote locations of the culture well (Figure 4B). This nonlocalized protein transduction likely resulted from the release of RNase A−R_9 from the top face of the coated slide (i.e., the face not placed in contact with cells), followed by the internalization of soluble protein by cells in adjacent areas of the culture well. These results are consistent with the results of our past studies using DNA-containing films, in which significant levels of nonlocalized cell transfection were observed when quartz slides coated on both sides were placed in direct contact with cells.[31]

We next prepared quartz slides coated on only a single face by obscuring one side of a slide with commercially available rubber cement prior to film fabrication.[31] Removal of the rubber cement after film deposition resulted in quartz slides coated with an RNase A−R_9/SPS film on only one side. Slides prepared in this manner contained ∼50% of the material immobilized on slides coated on both sides, as determined by UV−vis spectrophotometry (data not shown). When these slides were placed face down on cells, protein transduction was localized largely to cells growing directly beneath the films rather than cells growing in adjacent areas of the culture well (e.g., Figure 4C versus Figure 4D).

The results above demonstrate that films fabricated from RNase A−R_9 and SPS dissolve rapidly when incubated in physiologically relevant environments and that macroscopic objects coated with these materials can be used to promote the surface-mediated delivery of RNase A−R_9 to cells. However, several additional important points deserve comment. First, numerous past studies have demonstrated that proteins can be incorporated into multilayered films without changes in protein structure or loss of biological function.[5,6,9,10,14,15,20,23] We have conducted fluorescence-based enzyme activity assays using aliquots of PBS containing RNase A−R_9 released from RNase A−R_9/SPS films. These experiments demonstrated that a substantial fraction of the RNase A released from these materials remained catalytically active. We caution, however, that several synthetic polyanions are known to be potent inhibitors of RNase A.[63] Our initial experiments suggest that the presence of the SPS in these solutions can act to inhibit the activity of RNase A in these assays, and thus additional

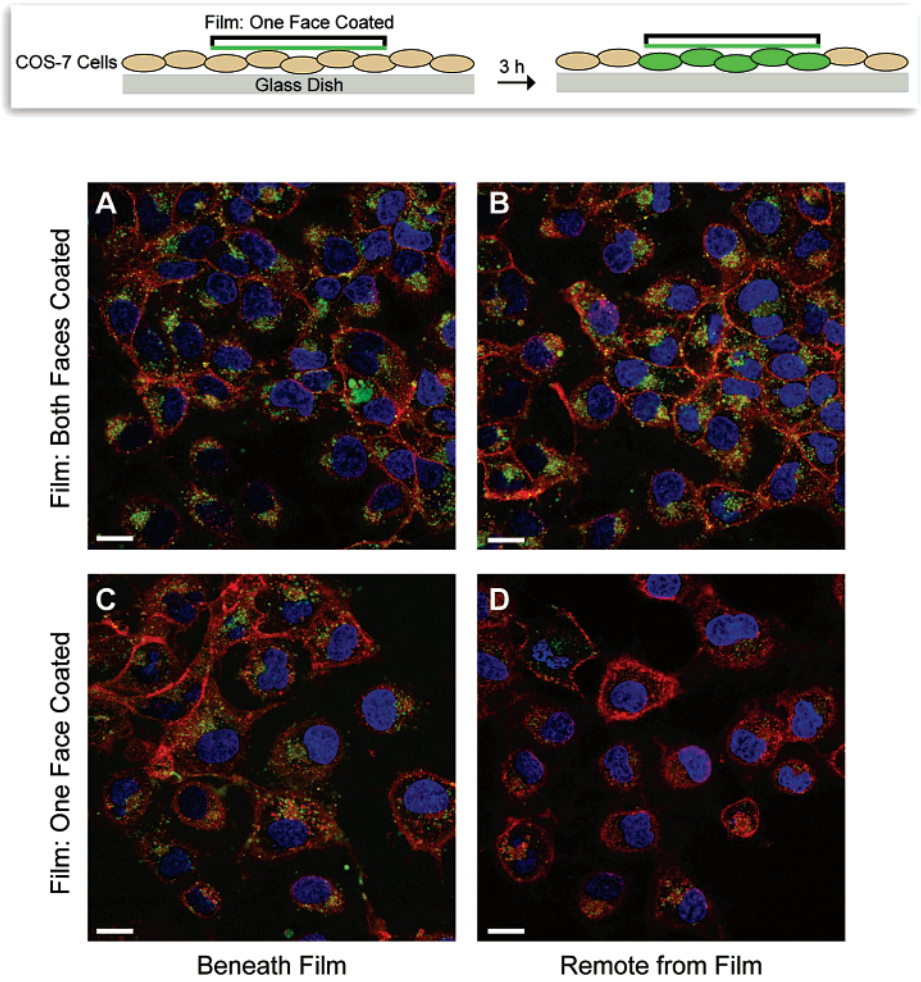

Figure 4. (Top) General scheme illustrating surface-mediated protein transduction in cells promoted by placing film-coated quartz slides in contact with cells. (Bottom) LSCM images of COS-7 cells incubated in the presence of film-coated quartz slides. (A) Cells growing directly beneath a slide coated on both faces. (B) Cells growing in a remote location in the culture well not in direct contact with the slide in panel A. (C) Cells growing directly beneath a slide coated on a single face. (D) Cells growing in a remote location in the culture well not in direct contact with the slide in panel C. The red, green, and blue channels in parts A−D correspond to WGA-Alexa 594, fluorescein, and Hoechst fluorescent probes, respectively (see Experimental Methods). Scale bar = 20 μm.

analytical experiments will be required to establish quantitatively the activity of the RNase A released from these materials. Second, although the RNase A−R_9/SPS films investigated here dissolve and release their contents rapidly, it may prove possible to incorporate new polymer structures or other design elements that permit gradual erosion and the sustained release of protein. Finally, we note that many past studies have demonstrated the conjugation of cationic protein transduction domains to proteins of therapeutic and biotechnological interest.[41−50] The work reported here thus suggests the basis of methods that could be used to fabricate ultrathin films that permit the localized delivery of therapeutic proteins to cells and tissues.

Summary and Conclusions

We have reported a general approach for the incorporation of proteins into multilayered polyelectrolyte assemblies that makes use of cationic protein transduction domains. Conjugation of the cationic protein transduction domain nonaarginine (R_9) to RNase A results in an increase in positive charge and, as a result, an increase in the extent to which RNase A is internalized by cells. One important result of this investigation is the demonstration that the conjugation of R_9 to RNase A permits the incorporation of RNase A into films under conditions that do not allow for incorporation of the unmodified protein. This result suggests that R_9 functions as a cationic "anchor" that increases the strength of electrostatic interactions with SPS and facilitates layer-by-layer assembly.

A second significant outcome of this investigation is the observation that films fabricated from RNase A−R_9 and SPS dissolve and release RNase A−R_9 into solution rapidly when incubated in physiologically relevant environments. These materials can thus be used to localize the release of RNase A−R_9 and the internalization of this protein by cells. We demonstrated that the placement of film-coated quartz slides in contact with COS-7 cells resulted in high levels of protein transduction in cells that were growing under or in contact with these materials. Many past studies have demonstrated the feasibility of conjugating R_9 or other synthetic or naturally occurring cationic protein transduction domains to proteins of therapeutic and biotechnological interest. The work reported here could thus contribute to the design of ultrathin films and coatings that permit the localized delivery of therapeutic proteins from the surfaces of implantable materials or provide spatial and temporal control over the release and internalization of engineered proteins in other biotechnological applications.

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