Layer-by-Layer Assembly of DNA- and Protein-Containing Films on Microneedles for Drug Delivery to the Skin

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Microneedle patches contain micrometer-scale needles coated with bioactive agents for minimally invasive drug delivery to the skin. In this study, we introduce layer-by-layer approaches to the fabrication of ultrathin DNA- and protein-containing polyelectrolyte films (or "polyelectrolyte multilayers", PEMs) on the surfaces of stainless steel microneedles. DNA-containing PEMs were fabricated on microneedles by the alternating deposition of plasmid DNA and a hydrolytically degradable poly(β-amino ester). Protein-containing PEMs were fabricated using sodium poly(styrene sulfonate) (SPS) and bovine pancreatic ribonuclease A (RNase A) conjugated to a synthetic protein transduction domain. Layer-by-layer assembly resulted in ultrathin, uniform, and defect-free coatings on the surfaces of the microneedles, as characterized by fluorescence microscopy. These films eroded and thereby released DNA or protein when incubated in saline or when inserted into porcine cadaver skin and deposited DNA or protein along the edges of microneedle tracks to depths of ~500 to 600 μm. We conclude that PEM-coated microneedles offer a novel and useful approach to the transdermal delivery of DNA- and protein-based therapeutics and could also prove useful in other applications.

Introduction

Methods for the delivery of therapeutic or cosmetic agents into skin are of interest in a broad range of fundamental and applied contexts. Although many different approaches have been developed for topical administration of small-molecule agents that can diffuse more readily across the skin, the outer layer of skin (i.e., stratum corneum) presents a formidable barrier to passive transport of larger, macromolecular therapeutics (e.g., peptides, proteins, and nucleic acid-based agents, such as DNA and siRNA).[1,2] Approaches to transdermal delivery of these larger molecules have therefore been based, in large measure, on methods that temporarily or reversibly disrupt the stratum corneum and allow transport to underlying dermal tissue.[3,4]

One approach to reversibly disrupt stratum corneum is to use micrometer-scale needles (or "microneedles") that are long enough to penetrate the stratum corneum, but not long enough to cause pain or significant tissue damage. This approach has been developed extensively for the pain-free administration of small- and large-molecule therapeutics and vaccines using arrays of solid or hollow microneedles fabricated from a broad range of materials.[5−7] Key to the continued development of approaches based on the use of solid microneedles, in particular, is the development of robust methods to immobilize and subsequently release therapeutic agents from the surfaces of microneedle arrays.

Several past studies by our group and others have demonstrated that methods developed for the encapsulation of proteins, oligonucleotides, viruses, DNA, and other agents in thin films of bulk polymer can be used to coat the surfaces of microneedle arrays and promote transdermal delivery in vitro and in vivo.[8−12] However, the continued development of this approach would benefit from the design of polymers and thin films with behaviors that can be tuned or tailored to take into account the specific needs of each of these different biotech-based drugs. For example, the doses, release kinetics, and presence of auxiliary delivery agents required for the transdermal delivery of DNA could vary significantly from those that are necessary for the efficient delivery of a protein or peptide construct. Moreover, an optimal microneedle coating method should (i) incorporate a controlled dose of drug into the coating, (ii) make use of aqueous or otherwise gentle processes to protect sensitive biotherapeutics, (iii) provide drug release kinetics that can be tuned or controlled with precision, and (iv) provide a uniform, adherent coating that maximizes drug loading and minimizes excipient content on the limited surface area available on microneedles. The work reported here takes a significant step toward addressing several of these broader issues by demonstrating approaches to the layer-by-layer fabrication of DNA- and protein-containing films that can be used to localize the release of these agents from the surfaces of microneedles.

The approach reported here builds upon past reports demonstrating methods for the layer-by-layer assembly of multilayered polyelectrolyte thin films (or "polyelectrolyte multilayers", PEMs) on surfaces.[13,14] These methods can be used to fabricate nanostructured films using a broad range of natural and synthetic polyelectrolytes, including therapeutically relevant macromolecules such as DNA and protein.[15−24] Numerous past

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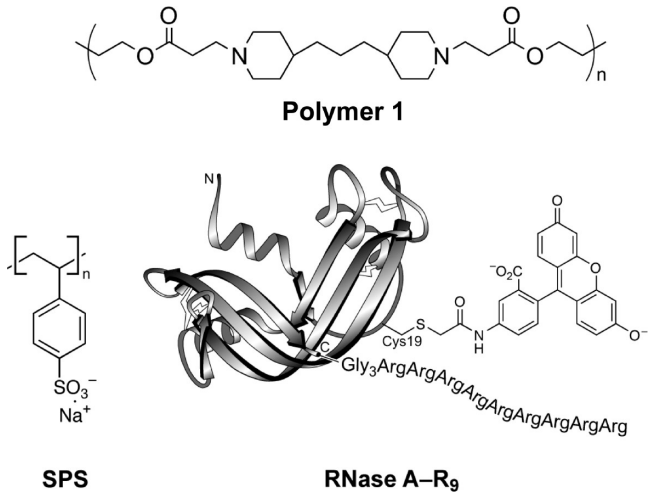

Figure 1. Structures of polymer **1**, sodium poly(styrene sulfonate) (SPS), and fluorescein-labeled RNase A-R_9 used in this study (N = amino terminus, C = carboxyl terminus, Cys = cysteine, Gly = glycine, Arg = arginine).

studies by a broad range of different research groups have demonstrated that, provided that the components of these assemblies are designed appropriately, PEMs can serve as thin-film platforms for the controlled, localized, and/or surface-mediated release of DNA, proteins, and other macromolecular or small-molecule agents.[15−24]

This current study sought to (i) develop approaches to fabricate ultrathin DNA- and protein-containing PEMs on the surfaces of arrays of stainless steel microneedles and (ii) characterize the ability of film-coated needles to promote delivery of DNA and protein into skin. In the context of these broader goals, we investigated two different approaches to the design of PEMs for microneedle-mediated delivery. The first approach is based on the fabrication of PEMs using a degradable cationic polymer (polymer **1**, see Figure 1) to promote controlled film disruption and surface-mediated release of incorporated DNA. We demonstrated previously that this approach can be used to fabricate thin films (e.g., ∼100 nm thick) that erode gradually and promote surface-mediated cell transfection in vitro[25−27] and that this approach can be used to provide tunable control over film disassembly and the release of DNA (or other agents) from surfaces.[28−30] The second approach is based on the fabrication of PEMs using the model protein bovine pancreatic ribonuclease (RNase A) covalently modified with a synthetic cationic protein transduction domain (Figure 1).[31] Our past studies demonstrate that it is possible to fabricate films that promote rapid release of this model cationic protein and enable surface-mediated transduction of RNase A into cells when these films are incubated in physiologically relevant media (e.g., phosphate-buffered saline or cell culture media).[31] When combined, these two approaches provide attractive platforms for the localized and surface-mediated delivery of proteins and DNA from coated microneedle arrays.

The study below is presented in two parts. In the first part, we demonstrate that layer-by-layer assembly can be used to fabricate DNA- and protein-containing films on the shafts of arrays of stainless steel microneedles ∼750 μm in length. The growth and uniformity of films fabricated using fluorescently labeled DNA and protein were characterized using fluorescence microscopy. Additional in vitro experiments demonstrated that film-coated microneedles released protein and transcriptionally active DNA into solution when incubated in physiologically relevant media. In the second part of this study, we demonstrate that film-coated microneedle arrays can be used to promote delivery of DNA and protein into porcine cadaver skin. Histological characterization of skin after removal of needle arrays demonstrated that DNA and protein were delivered to depths of ∼500 to 600 μm, demonstrating that these ultrathin films are mechanically robust and able to withstand forces associated with insertion of the needles. The results of this investigation suggest the basis of a versatile materials-based approach to the immobilization and release of macromolecular agents from the surfaces of microneedles (or other medical devices) with potential utility in the context of transdermal drug and vaccine delivery or a range of other biomedical applications.

Materials and Methods

Materials and General Considerations. Linear poly(ethylene imine) (LPEI, MW = 25000) was purchased from Polysciences, Inc. (Warrington, PA). Sodium poly(4-styrenesulfonate) (SPS, MW = 70000) and concentrated sodium acetate buffer were purchased from Aldrich Chemical Co. (Milwaukee, WI). All commercial polyelectrolytes were used as received without further purification. Polymer **1** (M_n = 8000−16000) was synthesized as previously described.[32] Plasmid DNA encoding enhanced green fluorescent protein [pEGFP-N1 (4.7 kb), >95% supercoiled] was purchased from Elim Biopharmaceuticals, Inc. (San Francisco, CA). Bovine pancreatic ribonuclease (RNase A) labeled with a single fluorescein molecule and conjugated at the C-terminus to a nonarginine sequence (referred to hereafter as RNase A-R_9) was a kind gift from Prof. Ronald T. Raines (U. Wisconsin) and was prepared as described previously.[33] For experiments requiring fluorescently labeled plasmid, Cy3 and TM-Rhodamine Label-IT nucleic acid labeling kits were purchased from Mirus Bio Corporation (Madison, WI) and used according to the manufacturer's instructions (labeling density = 1 label/200 bp). Fluorescence microscopy images were acquired using an Olympus IX70 microscope and the Metavue version 7.1.2.0 software package (Molecular Devices, Toronto, Canada). Images were analyzed using Adobe Photoshop and ImageJ software. Deionized water (18 MΩ) was used to prepare all buffers and polymer solutions, unless otherwise noted. Phosphate-buffered saline (PBS) was prepared by diluting commercially available concentrate (EMD Chemicals, Gibbstown, NJ). All buffers and polymer solutions (with the exception of DNA and RNase A solutions) were filtered through a 0.2 μm membrane syringe filter prior to use unless otherwise noted.

Microneedle Preparation and Cleaning. Microneedles were fabricated as previously described.[9] Briefly, the microneedle geometry was designed using a computer drafting software package (Autocad, Autodesk, Inc., San Rafael, CA), and microneedles were cut from stainless steel metal sheets (75 μm thick, Trinity Brand Industries, SS 304, McMaster-Carr, Atlanta, GA) using an infrared laser (Resonetics Maestro, Nashua, NH). Next, the microneedles were washed and cleaned to remove any loose burrs left during laser fabrication. To further sharpen the microneedles, the arrays were placed in a solution of glycerin, phosphoric acid, and water (Fisher Chemicals, Fair Lawn, NJ) and electropolished at a rate of 1.8 mA/mm^2 for 15 min.[9] Finally, the needles were washed with deionized water, dried using compressed air, and stored covered at room temperature prior to use.

To prepare the surfaces of microneedle arrays for the fabrication of multilayered films, the arrays were rinsed with acetone, ethanol, methanol, and water and dried under a stream of filtered air. The five needles on each array were pierced through a sheet of commercially available Parafilm (Pechiney Plastic Packaging, Chicago, IL) to restrict the deposition of multilayered films to the shafts of the needles themselves and avoid deposition on the base of the array (see text). The Parafilm was then folded back over the base of the microneedle row and sealed by squeezing the edges together with moderate pressure using forceps.

Preparation of Polyelectrolyte and Protein Solutions. Solutions of LPEI and SPS used for the fabrication of LPEI/SPS precursor layers

(20 mM with respect to the molecular weight of the polymer repeat unit) were prepared using a 26 mM NaCl solution. LPEI solutions contained 5 mM HCl to aid polymer solubility. Solutions of polymer **1** (5 mM with respect to the molecular weight of the polymer repeat unit) were prepared using 100 mM sodium acetate buffer (pH = 4.9). Solutions of plasmid DNA were prepared at 1 mg/mL in 100 mM sodium acetate buffer (pH = 4.9) and were prepared by mixing unlabeled plasmid with 20−50% (w/w) of a plasmid labeled with Cy3 (at a labeling density of 1 label per 200 base pairs). Solutions of RNase A-R_9 (7.0 μM with respect to concentration of protein) were prepared by diluting a concentrated stock with deionized water. The concentrations of protein in stock solutions were determined by UV absorbance (DU520 UV/vis spectrophotometer, Beckman-Coulter, Fullerton, CA) using $\varepsilon = 9860\ M^{-1}\ cm^{-1}$ for RNase A-R_9 at 277 nm and correcting for the absorbance of the fluorescein moiety with the equation:

$$A_{277\ nm}^{protein} = A_{277\ nm}^{observed} - (A_{494\ nm}^{observed}/5)$$

where A is absorbance.[34] SPS solutions used for the deposition of protein/SPS layers (20 mM with respect to the polymer repeat unit) were prepared in deionized water and the pH was adjusted to 5.0 with HCl.

Fabrication of Multilayered Films. Prior to the fabrication of DNA- or protein-containing films, the microneedles were precoated with 10 bilayers of a multilayered film composed of LPEI and SPS (~20 nm thick, terminated with a topmost layer of SPS), as previously described for the fabrication of polymer **1**/DNA or RNase A-R_9/SPS films.[25,31] Polymer **1**/DNA or RNase A-R_9/SPS layers were then deposited as follows: (1) substrates were submerged in a solution of a positively charged species (e.g., either polymer **1** or RNase A-R_9) for 5 min; (2) substrates were removed and immersed in a wash bath of 100 mM sodium acetate buffer (for films fabricated using polymer **1**) or deionized water (for films fabricated from RNase A-R_9) for 1 min followed by a second wash bath for 1 min; (3) substrates were submerged in a solution of a negatively charged species (e.g., either DNA or SPS) for 5 min; and (4) substrates were rinsed in the manner described above. This cycle was repeated until the desired number of bilayers (typically 16) had been deposited.

Following the final rinse step, the substrates were dried under a stream of filtered air. After drying, the Parafilm used to prevent film deposition on the base of the microneedle arrays (see above) was carefully removed. Microneedles coated using this procedure were either used immediately or stored in a dark location prior to use. All films were fabricated and stored at ambient room temperature. The presence and condition of multilayered films on the surfaces of the microneedles was characterized visually using fluorescence microscopy.

Characterization of Film Erosion upon Incubation of Coated Microneedles in PBS. Experiments designed to characterize the erosion profiles of films fabricated on microneedles were performed in the following manner. A coated microneedle array was submerged in PBS (pH = 7.2, 137 mM NaCl) in a plastic UV-transparent cuvette, incubated at 37 °C, and removed at predetermined intervals to permit characterization of the incubation solution. For films fabricated from polymer **1** and DNA, a Fluoromax-3 fluorimeter (Jobin Yvon, Edison, NJ) was used to measure the fluorescence emission of Cy3-labeled DNA in solution. The solution was excited at 550 nm, and the fluorescence emission intensity was recorded at 570 nm. For films fabricated from RNase A-R_9 and SPS, erosion solutions were excited at 490 nm and the fluorescence emission intensity was recorded at 514−520 nm (corresponding to the maximum fluorescence emission range of fluorescein). After each measurement, the substrates were placed in a new cuvette containing fresh PBS buffer. The presence or absence of film and gross changes in film morphology during and after these experiments were characterized visually using fluorescence microscopy.

Cell Transfection Assays. COS-7 cells (American Type Culture Collection, Manassas, VA) were seeded into 96-well plates at 15000 cells/well in 200 μL of Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. After 24 h, the medium was aspirated and replaced with fresh medium, and 50 μL of a Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and plasmid DNA mixture was added to each well. The Lipofectamine 2000/plasmid DNA mixture was prepared by mixing 25 μL of the plasmid solution collected at each time point during release experiments (arbitrary concentrations but constant volumes) with 25 μL of diluted Lipofectamine 2000 (24 μL of diluted into 976 μL of Opti-MEM I Reduced Serum Medium). Fluorescence microscopy images used to characterize expression of EGFP were acquired after 48 h.

Characterization of Release from Coated Microneedles in Porcine Cadaver Skin. Experiments designed to characterize the ability of film-coated microneedles to deliver DNA and protein into the skin were performed in the following manner. Porcine cadaver skin obtained with approval from the Georgia Tech Institutional Animal Care and Use Committee (IACUC) was thawed, shaved, and dried prior to microneedle insertion. Prior to insertion, the microneedles were imaged by brightfield and fluorescence microscopy using a stereomicroscope (Olympus SZX9, Japan). The arrays of coated microneedles were then manually inserted into the skin. After 2 h, the needles were removed and imaged again by stereomicroscopy to characterize the amount of film remaining on the surfaces of the microneedles. Histology was used to characterize DNA and protein delivery profiles in the skin. Skin samples were flash frozen using liquid nitrogen and histology sections (8 μm) were collected using a microcryostat (MICROM HM560, Waldorf Germany). The resulting sections were examined by fluorescence microscopy (Nikon E600W, Japan) to characterize the extent and distribution of DNA and protein film delivered from the microneedles. These sections were also stained using hematoxylin and eosin and imaged by stereomicroscopy to further characterize the depth of microneedle insertion.

Results

Layer-by-Layer Assembly of DNA- and Protein-Containing Films on Microneedle Arrays. Polymer **1**/DNA and RNase A-R_9/SPS films were fabricated on the surfaces of stainless steel microneedle arrays using alternate dipping protocols developed and optimized previously for the fabrication of these films on planar silicon and glass substrates.[25,31] For these initial characterization experiments, we used a plasmid DNA construct (pEGFP) encoding enhanced green fluorescent protein (EGFP) that was labeled with red-fluorescent Cy3 to permit characterization of the growth and uniformity of films using fluorescence microscopy (we note that the RNase A-R_9 construct used here is also labeled with fluorescein; see Figure 1). For the work described here, in-plane row microneedle arrays containing five microneedles each were cut from a thin sheet of 304 stainless steel (Figure 2A; see Materials and Methods for additional details related to processing and finishing). The finished needles were approximately 750 μm long, 150 μm wide, and 50 μm thick.

To limit the deposition of PEMs to the shafts of the needles on each array, the bases of the arrays were masked using Parafilm prior to use in film fabrication experiments (see Materials and Methods). To provide surfaces suitable for the adsorption of DNA- and protein-containing films, microneedles were precoated with a thin PEM composed of 10 layer pairs (or "bilayers") of linear poly(ethylene imine) (LPEI) and SPS prior to the deposition of either 16 bilayers of polymer **1** and DNA or 16 bilayers of RNase A-R_9 and SPS. Based on our past studies characterizing the growth of these assemblies using ellipsometry and UV/visible absorbance, we estimate the thicknesses of the 16-bilayer polymer **1**/DNA and RNase A-R_9/SPS films deposited on the surfaces of the microneedles used

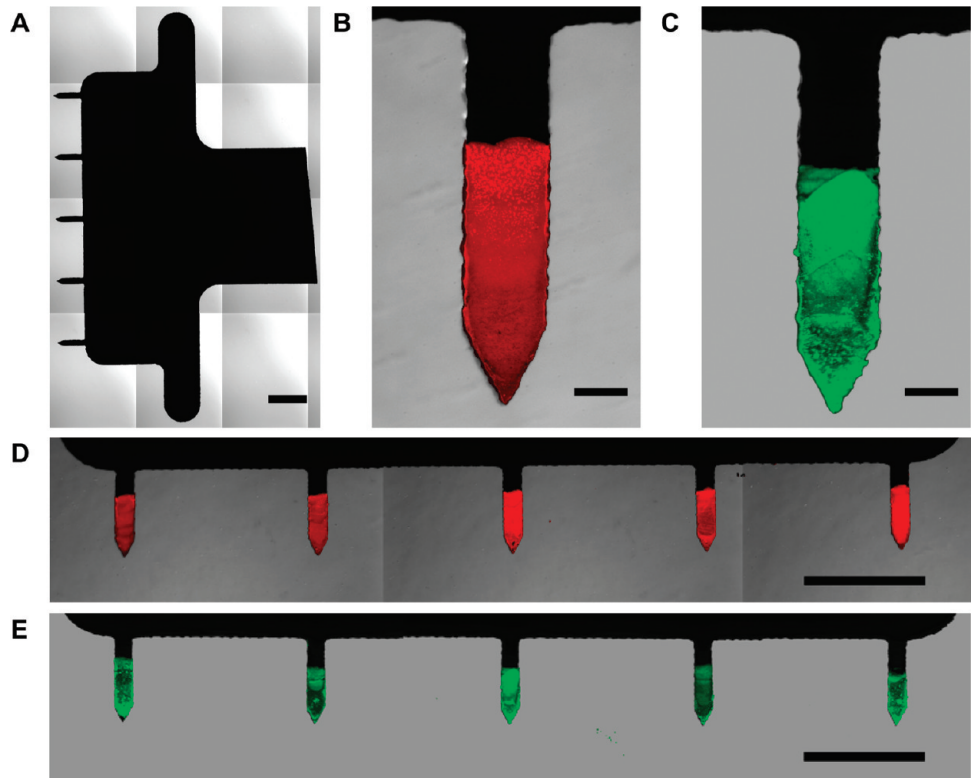

Figure 2. Microneedles used in this study. (A) Uncoated in-plane microneedle array. (B−E) Fluorescence microscopy images of stainless steel microneedle arrays coated with multilayered films fabricated from fluorescently labeled DNA (B,D) or protein (C,E). (B) High-magnification image of a microneedle coated with a film having the structure (polymer **1**/pEGFP-Cy3)$_{16}$. (C) High-magnification image of a microneedle coated with a film having the structure (RNase A-R$_9$/SPS)$_{16}$. Images in D and E show low-magnification views of coated five-needle arrays. Scale bars = 100 μm (B,C) or 1 mm (A,D,E).

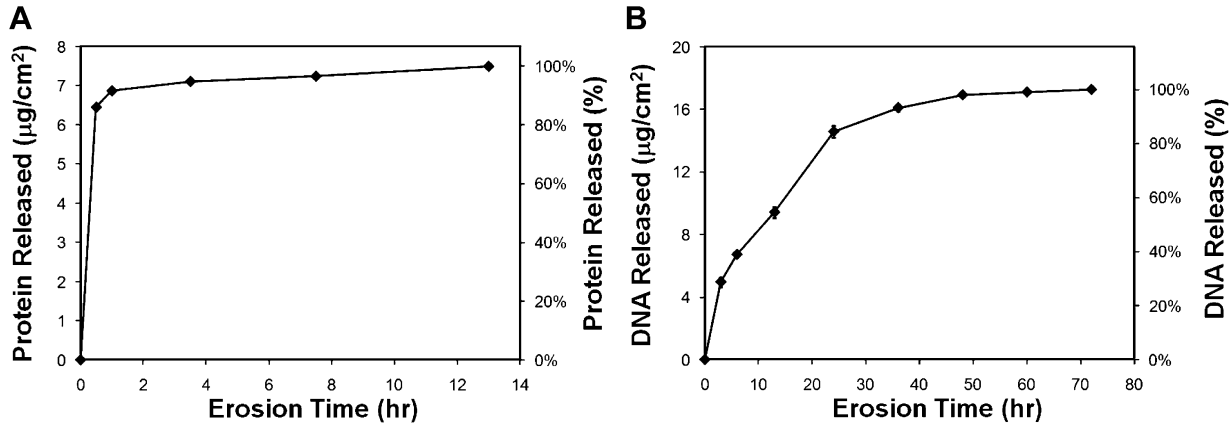

Figure 3. Cumulative release of (A) RNase A-R$_9$ or (B) DNA into PBS solution at 37 °C from the surface of stainless steel microneedles coated with either (RNase A-R$_9$/SPS)$_{16}$ or (polymer **1**/pEGFP-Cy3)$_{16}$.

in this current study to be approximately 200 and 160 nm, respectively.[25,31]

The images in Figure 2B−E show representative high- and low-magnification images of microneedle arrays coated with PEMs 16 bilayers thick produced by overlaying a series of fluorescence microscopy images onto phase contrast images of the same arrays. Inspection of these images reveals red and green fluorescence that is consistent with the presence of uniform and conformal DNA- and protein-containing films, respectively, on the shafts of each microneedle. These images also demonstrate that the application of Parafilm was sufficient to prevent the deposition of film on the bases of these arrays during assembly and that the removal of these masks post-fabrication can be achieved without damaging or tearing the films deposited on the needles (as determined by inspection of fluorescence microscopy images of the arrays before and after removal of the Parafilm).

Characterization of the Release of DNA and RNase A-R$_9$ from Film-Coated Microneedle Arrays. To characterize the loading and release profiles of the films fabricated above, we incubated film-coated microneedles in PBS at 37 °C. Figure 3A shows a representative RNase A-R$_9$ release profile obtained using a single five-needle array coated with a RNase A-R$_9$/SPS film 16 bilayers thick. This release profile was obtained by measuring the fluorescence emission of fluorescein-labeled RNase A-R$_9$ released into solution. We observed RNase A-R$_9$ to be released rapidly from the surfaces of the microneedles, with over 90% of the protein being released during the first hour of incubation in PBS. This release profile is generally consistent with the RNase A-R$_9$ release profiles observed in our

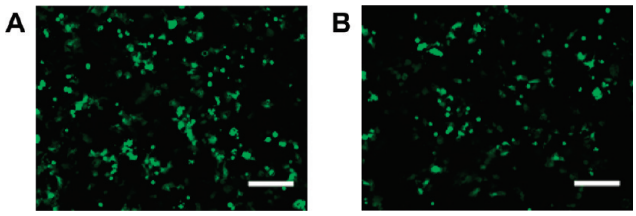

Figure 4. Representative fluorescence microscopy images showing EGFP expression in COS-7 cells transfected with DNA released from coated microneedles into PBS solution over (A) 0−3 h and (B) 36−47 h using Lipofectamine 2000 (see text). Scale bars = 200 μm.

past studies of RNase A-R_9/SPS films fabricated on larger silicon and glass substrates.[31]

Figure 3B shows a representative profile for the release of DNA from a microneedle array coated with a polymer **1**/DNA film 16 bilayers thick, determined by measuring the fluorescence emission of Cy3-labeled DNA released into solution over time. Inspection of these data reveals the release of DNA to be sustained for ~2 days, with over 80% of the DNA released in the first 24 h. These results are again generally consistent with the results of our past studies on the erosion and release of plasmid DNA from the surfaces of other macroscopic objects coated with polymer **1**/DNA films.[25,27] We note that, in contrast to experiments using the protein-containing films described above, the relatively low levels of solution fluorescence observed in experiments using films fabricated using Cy3-labeled DNA made it necessary to perform these experiments using needle arrays with films coating both the shafts of the needles and a larger, defined area of the base of the needle array (e.g., fabricated by reducing the total area of the bases of these arrays covered by the Parafilm masks prior to coating; see Figure S1 of the Supporting Information for a schematic illustration). We return to a consideration of these results again in the discussion below.

To characterize the functional integrity of the DNA released during these experiments, we performed a series of cell-based transfection experiments using samples of released DNA and a commercially available cationic lipid reagent (Lipofectamine 2000). Figure 4A shows representative images of cells treated with lipoplexes formed using a sample of DNA released early in a release experiment (during the first 3 h); Figure 4B shows cells treated with lipoplexes formed using a sample of DNA released between 36 and 47 h of incubation. The observation of high levels of expression of enhanced green fluorescent protein (EGFP) in these cells demonstrates that the DNA released from these film-coated microneedle arrays is released in a transcriptionally active form that is able to promote transgene expression in mammalian cells. These results are consistent with our previous reports demonstrating that polymer **1**/DNA films fabricated on a wide range of substrates release DNA intact and in a transcriptionally active form.[25−27,35]

Delivery of DNA and RNase A-R_9 to Porcine Cadaver Skin. To characterize the ability of PEM-coated microneedles to promote delivery of protein and DNA into skin, we inserted arrays of film-coated microneedles into porcine cadaver skin. These experiments were performed in vitro by the manual insertion of film-coated microneedles into samples of skin for 2 h. After removal of the microneedles, the skin samples were sectioned and characterized by fluorescence and optical microscopy. Figure 5 shows representative histology and fluorescence microscopy images of tissue sections at the sites of insertion of microneedles coated with polymer **1**/DNA (Figure 5A,B) films or RNase A-R_9/SPS films (Figure 5C,D) 16 bilayers thick. The images in Figure 5A,C reveal bright fluorescence along the edges of the microneedle tracks and demonstrate that DNA and protein were transported into the epidermal and dermal layers of the skin. Figure 6 shows low magnification fluorescence and bright-field microscopy images of film-coated microneedles before (Figure 6A,C) and after (Figure 6B,D) insertion in porcine cadaver skin for 2 h. A comparison of these images reveals that the fluorescently labeled protein and DNA in these films was released almost completely from the surfaces of these needles over this time period.

Discussion

In the context of controlled release, layer-by-layer methods of assembly offer several potential practical advantages relative to conventional, polymer-based methods for the surface-mediated release of DNA and proteins[15,18,22−24] in ways that address several of the needs for optimal microneedle coatings noted above. First, because these agents can be incorporated directly within a PEM (e.g., as a "layer") during assembly, layer-by-layer methods allow precise control over the loading of these agents by control over film thickness (or the number of layers of agent deposited during fabrication). Second, these methods can be carried out in aqueous media at room temperature, and thus do not require the use of organic solvents that could remain in these materials postfabrication. Third, this approach permits the fabrication of films that provide control over the release of defined amounts of multiple different agents (thus providing a potential platform for the development of more sophisticated, multiagent approaches to therapy).[22,30,36−38] The multicomponent and multilayered nature of these materials also affords unique opportunities to incorporate in a controlled manner auxiliary agents (e.g., cationic polymers, lipids, or other materials) that could promote the efficient delivery of agents such as DNA or proteins to cells. Finally, this approach is well suited for the fabrication of thin films and conformal coatings on the surfaces of objects having complex or irregular shapes and microscale dimensions[15−24] typical of many medical devices and implantable materials, including microneedles.

Our past studies demonstrate that it is possible to fabricate PEMs using plasmid DNA and a range of different hydrolytically degradable[25,29,30,39] or charge-shifting cationic polymers[38,40] and that the rates at which these assemblies erode and release DNA into solution can be varied (over periods ranging from several hours to several days, weeks, or months) by changing the structures of the polymers used to fabricate these materials. We selected hydrolytically degradable polymer **1** to fabricate the films investigated in this current study because the general behavior of PEMs fabricated using this model polymer has been well characterized in several past studies.[25−27,35,39,41] In addition, a recent report demonstrated transcutaneous vaccination using multilayered films composed of polymer **1** and ovalbumin that were placed in contact with disrupted skin.[42] In general, PEMs fabricated using polymer **1** and plasmid DNA (referred to hereafter as "polymer **1**/DNA films") erode and release DNA over a period of ~2 days when incubated in PBS at 37 °C.[25,27,35]

We also demonstrated recently a layer-by-layer approach to the incorporation and rapid release of proteins from PEMs.[31] In contrast to the approach described above, which is based on the use of hydrolytically degradable polyamines to promote gradual film erosion, this latter approach is based on the disruption of ionic interactions in assemblies fabricated using SPS and a fluorescently labeled model protein (RNase A) conjugated to the synthetic protein transduction domain nona-

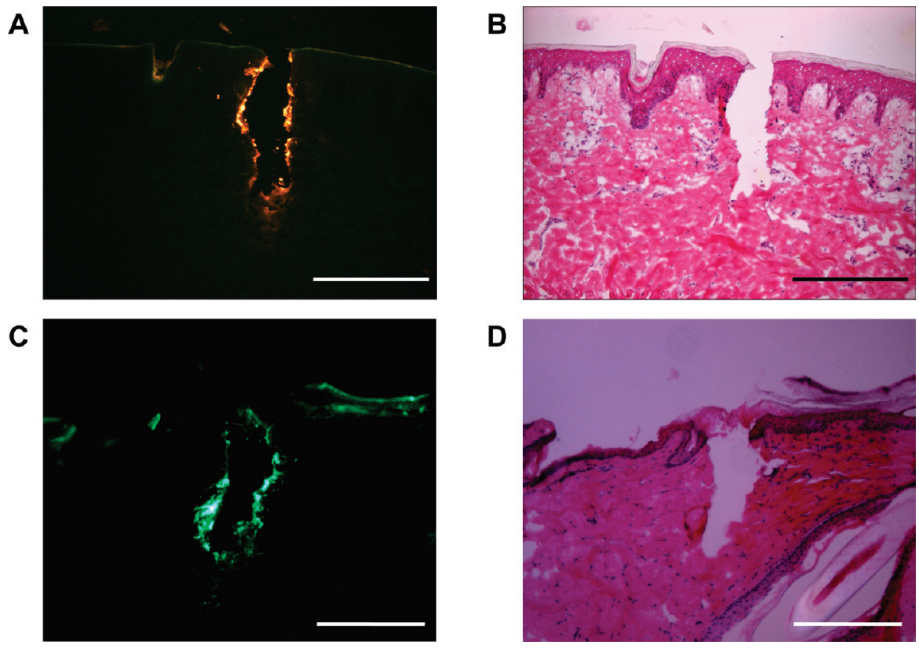

Figure 5. Representative fluorescence microscopy images (A,C) and companion histology images (B,D) of porcine cadaver skin after insertion and removal of film-coated microneedles. (A,B) Images of a needle track in skin pierced with a microneedle coated with a DNA-containing film having the structure (polymer **1**/pEGFP-Cy3)$_{16}$. (C,D) Images of a needle track in skin pierced with a microneedle coated with a protein-containing film having the structure (RNase A-R$_9$/SPS)$_{16}$. In both cases, needles were incubated in skin at 37 °C for 2 h before removal of the needle and imaging (see text). Each pair of images is taken from the same skin section but imaged using different optics. Scale bars = 400 μm.

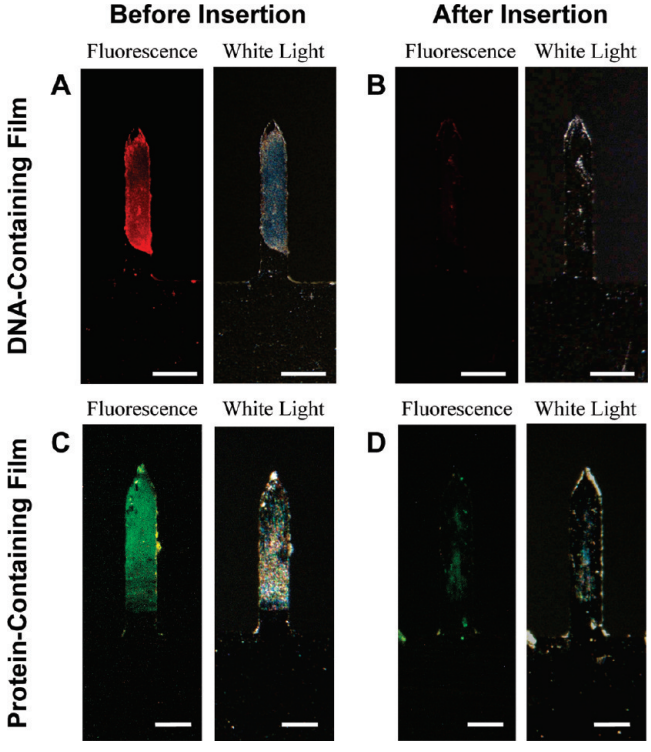

Figure 6. Representative fluorescence and bright field microscopy images of film-coated microneedles before insertion into porcine cadaver skin (A,C) and after removal (B,D) from porcine cadaver skin. In both cases, needles were left in skin at 37 °C for 2 h before removal of the needle and imaging (see text). (A,B) Before and after images of a microneedle coated with a DNA-containing film having the structure (polymer **1**/pEGFP-Cy3)$_{16}$. (C,D) Before and after images of a microneedle coated with a protein-containing film having the structure (RNase A-R$_9$/SPS)$_{16}$. Scale bars = 200 μm (A,B) and 150 μm (C,D).

arginine (RNase A-R$_9$).[31] RNase A-R$_9$/SPS films ~80 nm thick dissolve rapidly and release RNase A-R$_9$ into solution in ~30 min when incubated in PBS at 37 °C. Our past studies demonstrate that the presence of the cationic R$_9$ tag conjugated to RNase A plays an important role in facilitating film growth, and that this synthetic protein transduction domain promotes the localized transduction of RNase A into cells when film-coated substrates are placed in contact with mammalian cells in vitro.[31] These polymer **1**/DNA and RNase A-R$_9$/SPS systems thus provide two good model systems with which to explore the feasibility of microneedle-mediated transdermal delivery of DNA and proteins using PEMs.

To investigate the feasibility of using PEM-coated microneedles for the delivery of protein and DNA into skin, we selected in-plane arrays of five microneedles cut from sheets of stainless steel for several reasons: (i) this array geometry has been used in several past studies to fabricate polymer-coated microneedles,[9,12] (ii) the surfaces of these in-plane arrays can be characterized readily using bright-field and fluorescence microscopy, and (iii) past studies by our group and others demonstrate that layer-by-layer films can be fabricated readily on the surfaces of stainless steel substrates.[27,43,44]

The fluorescence microscopy images shown in Figure 2 demonstrate that layer-by-layer methods can be used to deposit thin and conformal DNA- and protein-containing films on the surfaces of these arrays. In this current study, we applied masks on the arrays prior to film fabrication (see Materials and Methods) such that only ~60−75% of the length of each microneedle was coated with PEMs. Microfluidic dipping cells similar to those reported in past studies for the conventional dip-coating of microneedles[9] could also be used to provide additional control over film deposition or to automate the layer-by-layer deposition process. In general, the layer-by-layer approach used here could also be used to coat three-dimensional arrays of microneedles.[8,10,11] However, the masking and manual dipping of these two-dimensional arrays was sufficient for the experiments described in this current study.

The results shown in Figure 3 demonstrate that RNase A-R$_9$ (Figure 3A) and plasmid DNA (Figure 3B) are released into

solution when PEM-coated microneedles are incubated in PBS at 37 °C. The relatively rapid release of RNase A-R_9 is generally consistent with the RNase A-R_9 release profiles observed in our past studies of RNase A-R_9/SPS films fabricated on larger substrates.[31] On the basis of these results, we estimate the loading of RNase A-R_9 on the these arrays coated with films 16 bilayers thick to be approximately 7.7 μg/cm^2 or approximately 10−20 ng of protein for each individual needle (considering a representative surface area of 0.22 mm^2 for each needle). We note again, in this context, that the layer-by-layer approach used to fabricate these films provides a means to increase (or decrease) the amount of protein incorporated into and released from these films (e.g., by control over the number of bilayers of polymer and protein deposited during assembly). Provided that the deposition of additional layers of polymer and protein also occurs in a linear manner, our data suggest that a coating 100−1000 bilayers thick could incorporate on the order of 0.1−1 μg of protein per microneedle. A microneedle patch containing, for example, 1000 microneedles could therefore be designed to contain up to 1 mg of drug.

The release of DNA from film-coated microneedles over a period of ∼2 days (Figure 3B) is also generally consistent with the results of our past studies on the erosion and release of plasmid DNA from polymer **1**/DNA films fabricated on the surfaces of other macroscopic objects.[25,27] Based on the total amount of DNA released in these experiments, we estimate the loading of DNA in films 16 bilayers thick to be ∼17 μg/cm^2 of coated surface or ∼30−40 ng of DNA per needle. We conducted additional fabrication experiments to increase the loading of these DNA-containing arrays by fabricating polymer **1**/DNA films 50 bilayers thick on the shafts of each needle. Interestingly, while these thicker films released significantly more DNA (up to ∼900 ng/needle), these arrays also released DNA much more rapidly (e.g., over a period of ∼3 h; see Figure S2 of the Supporting Information) than those coated with films 16 bilayers thick (e.g., over ∼2 days, as shown in Figure 3B). We do not currently understand the basis for these faster release rates, and additional physical characterization will be required to characterize the film growth, structures and erosion profiles of these thicker films more completely (for example, our results suggest that the growth of these thicker films may not occur in the linear manner demonstrated for the fabrication of thinner, 16 bilayer films). In the context of this current study, however, our results do demonstrate that it is possible to increase the loading of DNA on the surface of a microneedle by increasing the number of bilayers of polymer and DNA deposited. In addition, we note that the more rapid release profiles exhibited by these thicker films could prove useful in microneedle-mediated delivery applications for which rapid insertion and removal of needle arrays may be desirable.

The results shown in Figures 5 and 6 demonstrate that our PEM-coated microneedles are able to transfer DNA and protein into porcine cadaver skin. The observation of continuous fluorescence at depths of up to ∼500−600 μm in these images demonstrates that the thin PEM films on the surfaces of these needles are sufficiently robust to withstand the mechanical challenges associated with insertion of the microneedles into skin (that is, these films do not appear to crack or peel off, but are carried into the skin by the needles when inserted manually through the stratum corneum). Observation of the complete release of RNase A-R_9 from the inserted needles over a period of 2 h is consistent with the relatively rapid release kinetics observed when RNase A-R_9/SPS films are incubated in PBS buffer (e.g., over 1 h, Figure 3A).

The relatively rapid release of DNA from these film-coated needles in skin over this 2 h period (shown in Figure 6B) is surprising, however, in view of the slower release of DNA into solution when these materials are incubated in PBS (e.g., ∼2 days, Figure 3B). We note, however, that there are several differences between incubation in aqueous buffer and placement in the complex intradermal environment of cadaver skin that could account for these differences. It is possible, for example, that differences in local pH, ionic strength, or levels of hydration in cadaver skin could change the rate of hydrolysis of polymer **1** and contribute to the more rapid erosion of these films. It also seems likely that substantial mechanical contact between skin and hydrated or partially eroded PEMs could promote faster or more efficient release of DNA (or facilitate the removal of remaining film) upon the removal of film-coated needles from skin.

The transdermal delivery experiments reported here were limited to the characterization of protein- and DNA-containing films on microneedle arrays inserted into porcine cadaver skin for 2 h. We note, however, that the range of time scales relevant for the microneedle-mediated delivery of therapeutic agents can vary considerably.[3] For example, whereas practical approaches to microneedle-mediated delivery of protein- or DNA-based vaccines are likely to benefit from protocols that involve rapid insertion and removal, other applications of microneedle-based delivery systems require longer residence times to achieve sustained release.

In this context, we note that past work by our group and others has demonstrated approaches that can be used to tune the properties of PEMs to achieve rapid, sustained, or, in some cases, multi-agent release of DNA or other agents by manipulating film structure or the structures of the polymers used to fabricate these films.[18,22−24] The work reported here thus provides a platform for the design of PEM-coated microneedles that could be used to design films of potential value in a range of different delivery applications. In this broader context, the observation of a more rapid release of film when polymer **1**/DNA films are inserted in skin (as opposed to incubated in aqueous buffer) is notable because (i) it demonstrates that the behaviors of these films could vary significantly when exposed to more complex tissue environments and (ii) it suggests that the manipulation of polymer and film structure may not always be required to achieve faster release profiles.

Finally, while our results demonstrate the ability of this approach to promote the delivery of transcriptionally active DNA from microneedles (Figure 4), the use of porcine cadaver skin prevented the characterization of subsequent transfection of dermal tissue with DNA administered from coated microneedles. We note, however, that the compositions and structures of the PEMs used here (i.e., multicomponent films composed of layers of plasmid DNA and a class of cationic polymer[45] known to facilitate the delivery of DNA to cells) provide a platform for the fabrication of film-coated microneedles that both facilitate the transdermal delivery of DNA and promote the subsequent transfection of cells residing in the dermis. Our approach to the delivery of a protein conjugated to a synthetic protein transduction domain (R_9) is also of potential value in this context, because this transduction domain is well documented to promote the efficient internalization of protein by cells.[33,46] Efforts to characterize the behavior of these materials in more complex tissue environments and explore the potential of this approach for the transdermal delivery of protein- and DNA-based therapeutics are currently underway.

Summary and Conclusions

We have demonstrated a layer-by-layer approach to the fabrication of DNA- or protein-containing PEMs on the surfaces of stainless steel microneedle arrays. Films fabricated using either (i) plasmid DNA and a hydrolytically degradable polyamine or (ii) SPS and a model protein conjugated to a synthetic, cationic protein transduction domain (RNase A-R_9) eroded and promoted the surface-mediated release of DNA or protein into solution when film-coated microneedles were incubated in physiological saline. Insertion of PEM-coated microneedles into porcine cadaver skin resulted in the transfer of DNA or protein along the edges of microneedle tracks to depths of ~500−600 μm. Our results demonstrate that PEM-coated microneedles provide a novel and useful platform for the delivery of proteins and DNA to skin and suggest opportunities for the development of PEM-based approaches to the delivery of these agents of interest in the contexts of transdermal drug and vaccine delivery or a range of other biomedical applications.

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Supporting Information Available. Schematic representation of the area of the microneedle array coated for DNA release experiments and DNA release curve for microneedles coated with a film fabricated from 50 bilayers of polymer **1** and pEGFP. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Barry, B. W. *Eur. J. Pharm. Sci.* **2001**, *14*, 101–114.
(2) Wertz, P. W.; Downing, D. T. In *Transdermal Drug Delivery: Developmental Issues and Research Initiatives*; Hadgraft, J., Guy, R. H., Eds.; Dekker, M.: New York, 1989; pp 1−17.
(3) Prausnitz, M. R.; Langer, R. *Nat. Biotechnol.* **2008**, *26*, 1261–1268.
(4) Arora, A.; Prausnitz, M. R.; Mitragotri, S. *Int. J. Pharm.* **2008**, *364*, 227–236.
(5) Coulman, S.; Allender, C.; Birchall, J. *Crit. Rev. Ther. Drug Carrier Syst.* **2006**, *23*, 205–258.
(6) Prausnitz, M. R.; Mikszta, J. A.; Cormier, M.; Andrianov, A. K. *Curr. Top. Microbiol. Immunol.* **2009**, *333*, 369–393.
(7) Donnelly, R. F.; Raj Singh, T. R.; Woolfson, A. D. *Drug Delivery* **2010**, *17*, 187–207.
(8) Cormier, M.; Johnson, B.; Ameri, M.; Nyam, K.; Libiran, L.; Zhang, D. D.; Daddona, P. *J. Controlled Release* **2004**, *97*, 503–511.
(9) Gill, H. S.; Prausnitz, M. R. *J. Controlled Release* **2007**, *117*, 227–237.
(10) Ameri, M.; Daddona, P. E.; Maa, Y. F. *Pharm. Res.* **2009**, *26*, 2454–2463.
(11) Andrianov, A. K.; DeCollibus, D. P.; Gillis, H. A.; Kha, H. H.; Marin, A.; Prausnitz, M. R.; Babiuk, L. A.; Townsend, H.; Mutwiri, G. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 18936–18941.
(12) Kim, Y. C.; Quan, F. S.; Compans, R. W.; Kang, S. M.; Prausnitz, M. R. *J. Controlled Release* **2010**, *142*, 187–195.
(13) Decher, G. *Science* **1997**, *277*, 1232–1237.
(14) Bertrand, P.; Jonas, A.; Laschewsky, A.; Legras, R. *Macromol. Rapid Commun.* **2000**, *21*, 319–348.
(15) Ai, H.; Jones, S.; Lvov, Y. *Cell Biochem. Biophys.* **2003**, *39*, 23–43.
(16) Peyratout, C. S.; Dähne, L. *Angew. Chem., Int. Ed.* **2004**, *43*, 3762–3783.
(17) Sukhishvili, S. A. *Curr. Opin. Colloid Interface Sci.* **2005**, *10*, 37–44.
(18) Tang, Z. Y.; Wang, Y.; Podsiadlo, P.; Kotov, N. A. *Adv. Mater.* **2006**, *18*, 3203–3224.
(19) Angelatos, A. S.; Katagiri, K.; Caruso, F. *Soft Matter* **2006**, *2*, 18–23.
(20) Johnston, A.; Cortez, C.; Angelatos, A.; Caruso, F. *Curr. Opin. Colloid Interface Sci.* **2006**, *11*, 203–209.
(21) De Geest, B. G.; Sanders, N. N.; Sukhorukov, G. B.; Demeester, J.; De Smedt, S. C. *Chem. Soc. Rev.* **2007**, *36*, 636–649.
(22) Lynn, D. M. *Adv. Mater.* **2007**, *19*, 4118–4130.
(23) Jewell, C. M.; Lynn, D. M. *Adv. Drug Delivery Rev.* **2008**, *60*, 979–999.
(24) Boudou, T.; Crouzier, T.; Ren, K. F.; Blin, G.; Picart, C. *Adv. Mater.* **2010**, *22*, 441–467.
(25) Zhang, J. T.; Chua, L. S.; Lynn, D. M. *Langmuir* **2004**, *20*, 8015–8021.
(26) Jewell, C. M.; Zhang, J. T.; Fredin, N. J.; Lynn, D. M. *J. Controlled Release* **2005**, *106*, 214–223.
(27) Jewell, C. M.; Zhang, J. T.; Fredin, N. J.; Wolff, M. R.; Hacker, T. A.; Lynn, D. M. *Biomacromolecules* **2006**, *7*, 2483–2491.
(28) Zhang, J.; Lynn, D. M. *Macromolecules* **2006**, *39*, 8928–8935.
(29) Zhang, J. T.; Fredin, N. J.; Janz, J. F.; Sun, B.; Lynn, D. M. *Langmuir* **2006**, *22*, 239–245.
(30) Zhang, J. T.; Montanez, S. I.; Jewell, C. M.; Lynn, D. M. *Langmuir* **2007**, *23*, 11139–11146.
(31) Jewell, C. M.; Fuchs, S. M.; Flessner, R. M.; Raines, R. T.; Lynn, D. M. *Biomacromolecules* **2007**, *8*, 857–863.
(32) Lynn, D. M.; Langer, R. *J. Am. Chem. Soc.* **2000**, *122*, 10761–10768.
(33) Fuchs, S. M.; Raines, R. T. *Protein Sci.* **2005**, *14*, 1538–1544.
(34) Abel, R. L.; Haigis, M. C.; Park, C.; Raines, R. T. *Anal. Biochem.* **2002**, *306*, 100–107.
(35) Saurer, E. M.; Jewell, C. M.; Kuchenreuther, J. M.; Lynn, D. M. *Acta Biomater.* **2009**, *5*, 913–924.
(36) Jessel, N.; Oulad-Abdeighani, M.; Meyer, F.; Lavalle, P.; Haikel, Y.; Schaaf, P.; Voegel, J. C. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 8618–8621.
(37) Wood, K. C.; Chuang, H. F.; Batten, R. D.; Lynn, D. M.; Hammond, P. T. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 10207–10212.
(38) Liu, X. H.; Zhang, J. T.; Lynn, D. M. *Adv. Mater.* **2008**, *20*, 4148–4153.
(39) Fredin, N. J.; Zhang, J. T.; Lynn, D. M. *Langmuir* **2007**, *23*, 2273–2276.
(40) Zhang, J. T.; Lynn, D. M. *Adv. Mater.* **2007**, *19*, 4218–4223.
(41) Fredin, N. J.; Zhang, J. T.; Lynn, D. M. *Langmuir* **2005**, *21*, 5803–5811.
(42) Su, X.; Kim, B. S.; Kim, S. R.; Hammond, P. T.; Irvine, D. J. *ACS Nano* **2009**, *3*, 3719–3729.
(43) Farhat, T. R.; Schlenoff, J. B. *Electrochem. Solid-State Lett.* **2002**, *5*, B13–B15.
(44) Ji, J. A.; Tan, Q. G.; Fan, D. Z.; Sun, F. Y.; Barbosa, M. A.; Shen, J. C. *Colloids Surf., B* **2004**, *34*, 185–190.
(45) Lynn, D. M.; Anderson, D. G.; Putnam, D.; Langer, R. *J. Am. Chem. Soc.* **2001**, *123*, 8155–8156.
(46) Fuchs, S. M.; Raines, R. T. *Cell. Mol. Life Sci.* **2006**, *63*, 1819–1822.

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