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Assembly of Erodible, DNA-Containing Thin Films on the Surfaces of Polymer Microparticles: Toward a Layer-by-Layer Approach to the Delivery of DNA to Antigen-Presenting Cells

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Abstract

We report a layer-by-layer approach to the assembly of ultrathin and erodible DNA-containing films on the surfaces of polymer microparticles. DNA-containing multilayered films were fabricated layer-by-layer on the surfaces of polystyrene microspheres (~6 μm) by iterative and alternating cycles of particle suspension, centrifugation, and resuspension in solutions of plasmid DNA and a hydrolytically degradable polyamine. Film growth occurred in a stepwise manner, as demonstrated by characterization of the zeta potentials and fluorescence intensities of film-coated particles during film assembly. Characterization of film-coated particles by confocal fluorescence microscopy and scanning electron microscopy (SEM) revealed the multilayered particle coatings to be smooth, uniform, and free of large-scale physical defects. Film-coated microparticles sustained the release of transcriptionally active DNA into solution for approximately three days when incubated in physiologically relevant media. Past studies have demonstrated that the adsorption of DNA onto the surfaces of cationic microparticles can be used to target the delivery of DNA to antigen-presenting cells. As a first step toward the application of this layer-by-layer approach to the development of methods for the delivery of DNA to antigen-presenting cells, we demonstrated that film-coated microparticles could be used to transport DNA into macrophage cells *in vitro* using a model mouse macrophage cell line. Our results suggest the basis of a general approach that could, with further development, prove useful for the delivery of DNA-encoded antigens to macrophages, or other antigen-presenting cells, and provide new materials-based methods for the formulation and delivery of DNA vaccines.

Introduction

Approaches to vaccination based upon the delivery of DNA that encodes a protein-based antigen (so-called ‘DNA vaccines’) have the potential to provide protective immunity against diseases for which conventional vaccines are either ineffective or dangerous [1–3]. Relative to conventional vaccines, which generally require the production, formulation, and storage of live attenuated viruses, bacteria, or protein-based antigens, approaches based on the delivery of DNA have the potential to be both (i) less expensive to manufacture and (ii) more stable over the range of conditions typically encountered upon the shipment and long-term storage of vaccines [4]. DNA vaccines encoding model and clinically relevant antigens have also been demonstrated to generate long-lasting humoral and cellular immune responses *in vivo* [1–7].

One key challenge associated with the design of DNA vaccines is the development of new materials that permit the efficient and targeted delivery of DNA to macrophages and other

antigen-presenting cells (APCs) of the immune system [7–10]. Whereas traditional vaccines simply require methods for the administration of protein-based antigens to APCs, DNA vaccines require methods that promote efficient cell transfection – that is, both the successful delivery of DNA to APCs and the subsequent processing of the DNA to produce sufficient intracellular levels of encoded antigen. The development of materials-based delivery platforms that promote the efficient targeting, delivery, and processing of DNA by APCs is thus critical to improving the effectiveness of DNA vaccines.

Polymer-based approaches to the development of DNA vaccines have focused, in large measure, on the design and formulation of polymer microparticles as DNA delivery vehicles [6,8,11]. Polymer microparticles ranging from 1–10 micrometers in diameter present attractive platforms for targeted delivery to APCs because particles of this size are internalized preferentially by macrophages and dendritic cells, as opposed to other cell types [12]. Past studies have demonstrated that conventional methods for the encapsulation of proteins in degradable polymers such as poly(lactide-co-glycolide) (PLG) can be used to formulate DNA-containing microspheres that generate immune responses in animals [6–8,13]. Potential limitations of this approach, however, are that the relatively slow degradation of PLG can result in incomplete intracellular release of DNA [14] or the release of DNA at rates that may not be optimal for generating an immune response [15]. In addition, the hydrolysis of PLG has been demonstrated to generate low pH environments that could degrade encapsulated DNA [16]. These potential limitations have been addressed, at least in part, by the encapsulation of DNA using other polymers, such as poly(orthoester)s [17] or poly(β -amino ester)/PLG mixtures [15], that can release DNA more rapidly. These latter approaches have been demonstrated to result in improved immune responses *in vivo* [15,17].

An additional potential limitation of the approaches described above is that conventional methods for the encapsulation of DNA in microspheres typically expose DNA to high shear stresses that can degrade DNA [18]. In general, this type of physical degradation can be avoided by adopting formulation strategies that involve the physical adsorption of DNA directly onto the surfaces of pre-formed polymer microparticles (rather than direct encapsulation within them during fabrication). O'Hagan and coworkers demonstrated that the adsorption of DNA onto the surfaces of surfactant-modified PLG microparticles significantly reduced DNA damage during formulation, and that the resulting DNA-coated particles generated high antibody levels and strong T cell responses in mice [19,20]. The adsorption of DNA onto the surfaces of these microparticles was also reported to confer protection against the enzymatic degradation of DNA *in vitro* [20]. The investigation reported here was motivated by this second approach to formulation and, more broadly, by our general interest [21–34] in the design of thin films and polymer-based coatings that permit the adsorption and subsequent controlled release of DNA and other polyelectrolytes from surfaces. In this paper, we report a materials-based approach to the fabrication of ultrathin and erodible plasmid DNA-containing films on the surfaces of polymer microparticles.

The work reported here makes use of methods developed for the layer-by-layer assembly of ultrathin multilayered polyelectrolyte films (or 'polyelectrolyte multilayers') on surfaces [35]. These methods are entirely aqueous and provide precise, nanometer-scale control over the fabrication of ultrathin films composed of alternating layers of positively and negatively charged polymers selected from a broad range of natural and synthetic materials, including DNA (an anionic polyelectrolyte) [36–48]. Numerous past studies have demonstrated that it is possible to use these methods to fabricate multilayered films on the surfaces of colloidal microparticles for a broad range of potential applications [41,43,45,48]. Applications of this approach to the fabrication of DNA-containing multilayers and the development of methods for surface-mediated or microparticle-based cell transfection have been reviewed recently [49]. Of particular relevance to the work reported here, Delair and coworkers used this approach

to adsorb a layer of cationic polymer and a layer of plasmid DNA onto the surfaces of poly (lactic acid) microparticles, and demonstrated that these coated particles could promote transgene expression using the HEK 293T cell line [50–52]. Riebetanz et al. also reported the layer-by-layer assembly of multilayered films containing either one or two layers of imbedded plasmid DNA on the surfaces of silica microparticles, and the use of these coated microparticles to transfect HEK 293T cells [53]. These past investigations represent important steps toward the development of polyelectrolyte multilayer-coated microparticles as general platforms for the delivery of DNA to cells.

We have demonstrated in a series of recent reports that hydrolytically degradable poly(β -amino ester)s, such as polymer **1**, can be used to fabricate polyelectrolyte multilayers that permit the gradual and controlled release of plasmid DNA and other anionic polymers from the surfaces of macroscopic objects (e.g., planar substrates or biomedical devices such as intravascular stents) [21–25,27–30,32]. Here, we report that this general approach can be used to fabricate erodible DNA-containing films on the surfaces of polymer microparticles in ways that could prove useful for the delivery of DNA to APCs. Characterization of microparticles coated with multilayers fabricated from polymer **1** and plasmid DNA using fluorescence microscopy and measurements of zeta potential demonstrate that film growth occurs in a stepwise manner, and that the DNA-containing films are deposited uniformly on the surfaces of the particles. We demonstrate further that DNA is released gradually from the surfaces of these film-coated particles (e.g., over a period of ~3 days) upon incubation in physiologically relevant media, and that it is released in a form that remains transcriptionally active when administered to cells. Finally, as a first step toward the potential application of this approach to the delivery of DNA to APCs, we demonstrate that microparticles coated with these DNA-containing films can be internalized by a model macrophage cell line. Our results suggest the basis of a general approach that could, with further development, prove useful for the delivery of DNA-encoded antigens to APCs for effective DNA vaccination.

Polymer 1

Experimental Section

Materials and General Considerations

Monodisperse amine-functionalized polystyrene microspheres (diameter = $5.844 \pm 0.453 \mu\text{m}$ or $6.303 \pm 0.224 \mu\text{m}$) and linear poly(ethylene imine) (LPEI, $M_w = 25,000$) were purchased from Polysciences, Inc. (Warrington, PA). Polymer **1** ($M_n \sim 10,000$ to $15,000$) was synthesized as previously described [54]. Plasmid DNA encoding enhanced green fluorescent protein (pEGFP-N1, >95% supercoiled) was purchased from Elim Biopharmaceuticals, Inc. (San Francisco, CA). Sodium acetate buffer was purchased from Sigma-Aldrich, Inc. (St. Louis, MO). HEPES and concentrated phosphate-buffered saline (PBS) were purchased from EMD Chemicals (Gibbstown, NJ). Hoechst 34580 and wheat germ agglutinin (WGA)-Alexa 488 fluorescent stains were purchased from Invitrogen (Carlsbad, CA). Deionized water (18 M Ω) was used to prepare all buffer and polymer solutions. Solutions of polymer **1** used for film fabrication (10 mM with respect to the molecular weight of the repeat unit) were prepared in sodium acetate buffer (100 mM, pH = 5) and filtered through a 0.2 μm membrane syringe filter prior to use. Solutions of LPEI (40 mM with respect to the molecular weight of the repeat unit) were prepared in deionized water with 8 mM HCl added to aid dissolution and filtered through a 0.2 μm membrane syringe filter prior to use. Solutions of plasmid DNA were prepared at 80 $\mu\text{g/mL}$ in filtered sodium acetate buffer and were not filtered prior to use. For experiments

requiring fluorescently labeled DNA, plasmid DNA was labeled with Cy3 using a Label-IT Cy3 Labeling Kit (Mirus Bio Corp., Madison, WI). Labeled DNA was purified by ethanol precipitation, and labeling densities (~1 label per 200 base pairs) were determined using a UV/vis spectrophotometer, as described by the manufacturer of the labeling kit. All UV/visible absorbance values were recorded using a Beckman Coulter DU520 UV/vis Spectrophotometer (Fullerton, CA). Zeta potential measurements were performed using a Zetasizer 3000 HSA instrument (Malvern Instruments, Westborough, MA). Fluorescence microscopy images used to evaluate the expression of enhanced green fluorescent protein (EGFP) in cell transfection experiments were recorded using an Olympus IX70 microscope and were analyzed using the Metavue version 7.1.2.0 software package (Molecular Devices; Toronto, Canada). Scanning electron microscopy was performed using an accelerating voltage of 3 kV on a LEO DSM 1530 scanning electron microscope. Samples were coated with a thin layer of gold using a sputterer (30 s at 45 mA, 50 mTorr) prior to analysis. Laser Scanning Confocal Microscopy (LSCM) was performed using a Bio-Rad Radiance 2100 MP Rainbow laser scanning confocal microscope equipped with a multiphoton laser. LSCM images were processed using the LaserSharp 2000 processing kit (Bio-Rad; Hercules, CA), ImageJ 1.38q (National Institutes of Health; Washington, D.C.), and Photoshop CS2 (Adobe Systems; San Jose, CA).

Fabrication of Multilayered Films on Microparticles

The layer-by-layer fabrication of polyelectrolyte multilayers on the surfaces of polystyrene microparticles was performed in the following general manner. An aqueous suspension of amine-functionalized microspheres (25 μ L of a 2.7 wt% stock solution) was added to 225 μ L of filtered sodium acetate buffer (100 mM, pH = 5) in a microcentrifuge tube. The microparticle suspension was then agitated gently using a mechanical vortexer and a solution of polymer **1** (250 μ L) was added (total volume = 500 μ L). The resulting suspension was then rotated gently end-over-end using a mechanical laboratory rotator (Barnstead Thermolyne, Dubuque, IA) for 10 minutes at ambient room temperature. The sample was then centrifuged for 10 minutes at 4,000 g using a Microfuge 18 Centrifuge (Beckman Coulter, Fullerton, CA), and the supernatant was removed by pipette. The remaining particles were then washed to remove weakly adsorbed polyelectrolytes using a resuspension/centrifugation washing process. Briefly, the pellet of particles was resuspended in sodium acetate buffer (500 μ L) using a mechanical vortexer, the resulting suspension was centrifuged for 10 minutes at 4,000 g, and the supernatant was removed by pipette. The resulting particles were then resuspended in sodium acetate buffer (250 μ L) in preparation for the adsorption of DNA. To ensure complete resuspension of the particles, and to disrupt large aggregates of multiple particles before the adsorption of DNA (see text), the microparticle suspension was passed 10 times through a 22G $\times$ 1" stainless steel needle (Becton, Dickinson and Co., Franklin Lakes, NJ) mounted on a 1 mL syringe (Terumo Medical Corp., Somerset, NJ). The microparticle suspension was then agitated using a mechanical vortexer, and a solution of plasmid DNA (250 μ L) was added (total volume = 500 μ L). This sample was then rotated for 10 minutes and particles were collected, rinsed, and resuspended using the methods described above. This general process was repeated to deposit alternating layers of polymer **1** and DNA until the desired number of layers was deposited. In experiments for which a final outer layer of LPEI was adsorbed (see text), microparticles were resuspended in deionized water prior to the adsorption of LPEI and rinsed in water following the adsorption of LPEI.

Characterization of Zeta Potentials

Characterization of the zeta potentials of microparticles after the deposition of polymer or DNA layers was conducted in the following general manner. Particle samples, prepared as described above, were suspended in sodium acetate buffer (100 mM, pH = 5), and 3 mL of this suspension was injected into the inlet of a Zetasizer 3000 HSA instrument (Malvern Instruments, Westborough, MA). Measurements were made at ambient temperature using an applied voltage

of 150 V. A minimum of five measurements was recorded for each sample, and the Henry equation was used to calculate zeta potentials from measurements of electrophoretic mobility using the Smoluchowski approximation [55]. For this calculation, we assumed the viscosity of the solution to be the same as that of water.

Characterization of Film-Coated Particles Using Fluorescence Microscopy and Laser Scanning Confocal Microscopy (LSCM)

Experiments designed to characterize changes in the fluorescence intensities of particle coatings during the layer-by-layer fabrication of multilayers using polymer **1** and fluorescently labeled DNA were conducted using an Olympus IX70 fluorescence microscope. Aliquots (5 μ L) of particle suspensions, prepared as described above, were transferred via pipette onto a glass microscope slide and covered using a glass cover slip. The average grayscale fluorescence intensity of individual particles was determined from fluorescence microscopy images using ImageJ. Values of fluorescence intensity are reported as the average (with standard deviation) based upon measurements of at least 10 individual particles. To characterize the uniformity of multilayered films deposited on the surfaces of microparticles using LSCM, the slide/cover slip assembly was inverted on the microscope stage of a Bio-Rad Radiance 2100 MP Rainbow laser scanning confocal microscope. LSCM images were acquired using a 60x/1.40 NA oil-immersion objective. Multilayered films were fabricated using Cy3-labeled DNA and excited using the 543 nm laser line. The fluorescence emission signal was collected using direct scanning mode (N=1, scan speed = 50 lps).

Characterization of DNA Release Profiles

Experiments designed to characterize the release of DNA from film-coated particles were conducted in the following general manner. A microcentrifuge tube containing film-coated particles suspended in PBS was mounted on a mechanical laboratory rotator and incubated at 37 °C. At predetermined times, the suspension was centrifuged for 10 minutes at 4,000 g and the supernatant was removed via pipette and transferred to a clean centrifuge tube. The pelleted particles were resuspended in fresh PBS and placed back in the incubator. The supernatant was centrifuged once more at 9,000 g to remove any remaining particles, and the concentration of DNA in the supernatant was measured by recording the absorbance of the supernatant at 260 nm, the absorbance maximum of DNA.

Agarose Gel Electrophoresis

Samples of plasmid DNA released from coated particles (25 μ L) were mixed with a loading buffer [5 μ L of a 50:50 (v/v) glycerol:water mixture] and analyzed on a 1% agarose gel (20 mM HEPES, pH = 7.2, 108V, 45 min). DNA bands were visualized by ethidium bromide staining.

Cell Transfection Assays

COS-7 cells (American Type Culture Collection; Manassas, VA) were seeded into 96-well plates at 15,000 cells/well in 200 μ L of Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 100 units/mL of penicillin and 100 μ g/mL of streptomycin. After 24 hours, the medium was aspirated and replaced with fresh medium, and 50 μ L 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 μ L of the plasmid solution collected at each time point during release experiments (arbitrary concentrations but constant volumes) with 25 μ L of diluted Lipofectamine 2000 (24 μ L diluted into 976 μ L Opti-MEM I Reduced Serum Medium). Fluorescence microscopy images used to characterize expression of EGFP were acquired after 48 hours using an Olympus IX70 fluorescent microscope and the MetaVue version 7.1.2.0 software package.

Particle Internalization Assays

Experiments designed to investigate the ability of coated particles to be internalized by phagocytic cells were carried out in the following general manner. Murine macrophage-like cells (p388d1 cell line; American Type Culture Collection; Manassas, VA) were seeded into glass inset cell culture dishes (MatTek; Ashland, MA) at a density of 500,000 cells per dish in 2 mL of RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 µg/mL streptomycin. After 24 hours, the media was aspirated and replaced with fresh RPMI-1640. Coated particles were suspended in PBS buffer, and 500 µL of this suspension was added to the confocal microscopy dish. After 5 hours, the cells were stained with Hoechst 34580 and wheat germ agglutinin (WGA)-Alexa 488 using the following procedure. Hoechst staining solution was prepared at 2 µg/mL in PBS. WGA-Alexa 488 staining solution was prepared at 6 µg/mL in PBS. The media was aspirated from each confocal dish, replaced with 1.5 mL Hoechst staining solution, and incubated at 37 °C for 10 minutes. Following this incubation, 1.5 mL WGA-Alexa 488 staining solution was added to each confocal dish, and each sample was incubated at 37 °C for 5 minutes. The staining solution was aspirated and replaced with 1.5 mL PBS, which was aspirated and replaced with 1.5 mL of Opti-MEM. LSCM images were acquired using a 100x/1.40 NA oil-immersion objective. WGA-Alexa 488, Cy3-labeled DNA, and Hoechst were excited using laser lines at 488, 543 nm, and 750 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

In the context of developing new methods for the microparticle-based delivery of DNA, layer-by-layer fabrication offers several potential practical advantages relative to methods used for the encapsulation of DNA in bulk polymer microspheres [49]. First, these methods are entirely aqueous and do not subject DNA to mechanical challenges (such as high shear) that could cause damage to DNA. In addition, because DNA is an anionic polyelectrolyte, it can be incorporated directly into the structure of a polyelectrolyte multilayer (e.g., as an individual 'layer') during fabrication. Because the layer-by-layer procedure is an iterative process (i.e., film fabrication proceeds by the alternating and iterative adsorption of many different layers of positively and negatively charged polymers) this modular method of assembly also provides a straightforward means of controlling the amount, or loading, of DNA incorporated into a thin film coating (for example, simply by increasing or decreasing the number of polymer/DNA layers deposited during fabrication). Finally, the presence of layers of cationic polymers in these materials provides opportunities to incorporate additional functionality that could potentially be used to address important extracellular and/or intracellular barriers to cell transfection. Provided that films can be constructed in a manner that also permits the eventual disruption and release of DNA [33], these methods present a platform for the delivery of DNA in a variety of different fundamental and applied contexts [49].

The results of our past studies demonstrate that hydrolytically degradable polyamines such as polymer **1** can be used to fabricate polyelectrolyte multilayers on the surfaces of macroscopic objects, and that these ultrathin films erode and release DNA gradually when incubated in physiologically relevant media [22–24,28,30,32]. This current investigation sought to determine whether this general approach could be used to fabricate erodible, DNA-containing thin films on the surfaces of polymer microparticles in ways that could provide opportunities to deliver DNA to macrophages or other APCs. The study below is described in three parts. First, we describe the fabrication and physical characterization of DNA-containing multilayers fabricated using polymer **1** and plasmid DNA on the surfaces of 6 µm polymer microspheres. We then characterize the film erosion and DNA release profiles of these film-coated

microspheres upon incubation in physiologically relevant media and demonstrate that DNA is released in a form that is transcriptionally active when administered to mammalian cells *in vitro*. Finally, we describe a series of experiments designed to investigate the cellular internalization of these film-coated microparticles using a model mouse macrophage cell line.

Layer-by-Layer Assembly of DNA-Containing Films on the Surfaces of Polymer Microspheres

We performed a series of experiments to determine whether DNA-containing multilayered films could be fabricated on the surfaces of polymer microparticles using polymer **1**, a plasmid DNA construct (pEGFP-N1) encoding enhanced green fluorescent protein (EGFP), and commercially available, monodisperse amine-functionalized polystyrene microspheres. Nondegradable polystyrene microspheres (diameter = 6 μm) were selected for these proof-of-concept experiments (rather than microspheres fabricated from degradable materials such as PLG) to avoid the potential for premature degradation or changes in surface chemistry or structure that could occur during film fabrication. For these and all other experiments described below, multilayered films were fabricated on the surfaces of these microspheres by iterative and successive cycles of particle suspension, centrifugation, and resuspension in solutions of polymer **1** and DNA (e.g., Scheme 1; see Experimental Section for complete details), using procedures similar to those described in past studies [41,43,45,48].

Initial attempts to fabricate polymer **1**/DNA films on the surfaces of microparticles resulted in substantial particle aggregation. For example, large white flocs developed during the adsorption and rinsing steps required for the sequential adsorption of two polymer **1**/DNA layer pairs (referred to hereafter as 'bilayers') on the particles. These flocs settled rapidly upon standing and were revealed by optical microscopy to be aggregates ~30 to 80 μm in size comprised of clusters of ~10 to 50 particles (data not shown). We investigated several different methods to prevent or reduce large-scale particle aggregation during film assembly and increase the number of single, isolated film-coated particles. Mechanical vortexing and brief sonication of particle suspensions after each particle wash cycle (i.e., before the resuspension of particles in the next polyelectrolyte solution) resulted in an increase in the fraction of individual particles, but did not completely eliminate larger aggregates. Unfortunately, exposure of these particles to brief periods of sonication also resulted in substantial physical damage to DNA (data not shown). As an alternative to this procedure, we found that passing microparticles through a narrow gauge needle multiple times (e.g., 10 times) between the rinsing step and the adsorption of the next polyelectrolyte layer resulted in a significant decrease in the size and number of aggregates observed.

Figure 1A shows a representative laser-scanning confocal microscopy (LSCM) image of particles coated with 2.5 bilayers of a polymer/DNA film fabricated using fluorescently labeled plasmid DNA and the physical method of particle treatment described above. Inspection of this image reveals the presence of single, isolated, film-coated particles (fluorescently labeled DNA is false-colored red; small aggregates consisting of two or three particles are also shown for comparison). Further inspection reveals that these particles are coated uniformly across the surface of each of the particles (e.g., see inset), without bare patches or uncoated areas that could result from the physical disruption of particle aggregates during assembly. Characterization of film-coated particles by scanning electron microscopy (SEM; Figure 1B) confirmed the uniformity of these films and revealed the surfaces of these film-coated particles to be smooth, continuous, and devoid of significant micrometer-scale holes, pits, or cracks.

For the assembly of multilayered films on the surfaces of macroscopic objects, layer-by-layer film growth can be characterized readily using methods such as ellipsometry or atomic force microscopy to measure stepwise increases in film growth. These techniques cannot generally be used to characterize the growth of films assembled on the surfaces of colloidal microparticles

[56]. However, past studies have demonstrated that changes in the zeta potentials of particles (e.g., as should occur after the adsorption of a negatively charged polymer or a positively charged polymer to the surface of a microparticle) can be used to characterize film growth and assembly [43,56–59]. Figure 2A shows representative changes in the zeta potentials of particles during the fabrication of a polymer/DNA film 2.5 bilayers thick. Bare, uncoated particles were measured to have an average zeta potential of -20 mV. Following the exposure of these particles to a solution of polymer **1** (layer 1), the zeta potentials of the particles increased to approximately $+3$ mV, consistent with adsorption of polymer **1** on the surfaces of the particles. Further inspection of these data reveals the zeta potential of these particles to become negative again (e.g., to a value of approximately -40 mV) upon exposure of the particles to a solution of DNA (layer 2). This large change in zeta potential from slightly positive (or near-neutral) values to large negative values was observed to continue upon the iterative exposure of these particles to solutions of polymer **1** and DNA, respectively. This oscillation of zeta potential is consistent with zeta potential profiles observed in past studies investigating the layer-by-layer fabrication of multilayered films on the surfaces of microparticles using a broad range of different polyelectrolytes [43,57–59].

Although the zeta potential profile in Figure 2A is consistent with layer-by-layer film growth, we note that these results could also arise from the iterative displacement (or stripping) of adsorbed layers of polymer or DNA from particle surfaces by an oppositely charged species upon exposure to each subsequent polyelectrolyte solution [56]. To further confirm layer-by-layer film growth, we characterized increases in the fluorescence intensities of film-coated particles during the assembly of films fabricated using fluorescently labeled DNA. Figure 2B shows the average grayscale intensities of film-coated particles as a function of the number of polymer/DNA layers deposited, and reveals fluorescence intensity to increase with the number of DNA layers deposited (e.g., see data for layers 2 and 4, which correspond to fabrication cycles during which DNA layers were adsorbed). The decrease in fluorescence intensity observed after the addition of the last polymer layer (layer 5) suggests that DNA in the penultimate layer may have been partially displaced or stripped from the surface to some extent during this final polymer adsorption step. We note, however, that such a decrease in fluorescence was not observed during the fabrication of the first two polymer/DNA bilayers (e.g., layers 1–4) and that, in general, these results are consistent with (i) stepwise, layer-by-layer film growth and (ii) an increase in the amount of DNA on the surface of the microparticles upon the addition of each individual polymer/DNA bilayer.

Colloidal stability is determined by the relative magnitudes of attractive van der Waals forces and repulsive electrical double-layer forces acting between particles in a suspension [60]. As a result, particles that are not sufficiently charged tend to form aggregates. The observation of near-neutral (or only slightly positive) zeta potentials for particles coated with an outer layer of polymer **1** (Figure 2A) suggests a possible explanation for the tendency of these particles to aggregate during fabrication. We caution, however, that several additional factors could contribute to the large-scale aggregation observed in our initial studies. For example, Vinogradova et al. have suggested that particle aggregation observed during the layer-by-layer fabrication of films on microparticles using calf thymus DNA could arise from the ‘bridging’ of high molecular weight DNA between particles [61]. Schneider and Decher also reported recently that aggregation during the layer-by-layer coating of gold nanoparticles with synthetic polyelectrolytes could be reduced substantially when lower molecular weight polyelectrolytes were used to fabricate the films [62]. It will likely prove possible to optimize the molecular weight of DNA or other factors to reduce or eliminate completely the particle aggregation observed for our polymer **1**/plasmid DNA system. However, the process described above resulted in reductions in particle aggregation and samples of individual particles sufficient for characterization and all subsequent experiments. Below, we demonstrate that microparticles

coated with polymer **1**/DNA films release transcriptionally active DNA into solution over a period of ~72 hours when incubated in physiologically relevant media.

Characterization of DNA Release Profiles and the Integrity of Released DNA

The incubation of film-coated microparticles in PBS at 37 °C resulted in the gradual release of plasmid DNA into solution. Figure 3 shows a representative DNA release profile for microspheres coated with 2.5 bilayers of a polymer **1**/DNA film (prepared as described above). This release profile was determined by characterizing the absorbance of the PBS solution (at 260 nm, the absorbance maximum of DNA) as a function of time, as described in our past studies of films fabricated on the surfaces of macroscopic objects (see Experimental Section for additional details) [22–24,28,30,32]. These data reveal that DNA is released gradually from the surfaces of these film-coated particles over a period of ~3 days. Further inspection reveals release to be more rapid during the initial stages of release (e.g., with ~80% of DNA being released during the first 12 hours). After the first 12 hours, the release of DNA was more gradual, with the remaining ~20% of DNA being released into solution over an additional period of ~60 hours. Additional experiments conducted using ethidium bromide to characterize the presence of DNA on the particles before and after erosion confirmed that, within the limits of visual inspection, the DNA was completely released from the surface of the particles after 72 hours (data not shown). The experiment shown in Figure 3 was performed with approximately 1.1×10^6 particles, as determined by counting the particles in three representative samples using a hemacytometer. On this basis, we estimate the DNA loading in our system to be ~52 µg DNA/mg of particles (or ~7 pg DNA/particle). It is important to note that this value is an estimate, as several factors, including the potential for the loss of particles during centrifugation and resuspension steps involved in this release experiment, could complicate determination of DNA loading.

Characterization of aliquots of PBS collected during the DNA release experiments described above using agarose gel electrophoresis confirmed qualitatively the general features of the release profile shown in Figure 3 and permitted characterization of the structure of the released DNA. Figure 4A shows a representative image of a gel used to characterize the DNA released into solution at six different time points (ranging from 3 hours to 72 hours) during incubation of the film-coated particles. Inspection of these data confirms that DNA was released gradually into solution for at least 14 hours, at which point the bands corresponding to the presence of DNA (as visualized by ethidium bromide staining) become faint and difficult to resolve. We note in this context that these release experiments were performed by centrifuging the microparticles at each time point to measure solution absorbance, followed by the resuspension of the particles in fresh PBS buffer (see Experimental Section for additional details). As a result, the amount of DNA measured at any given time point does not reflect the cumulative amount of DNA released, but rather the amount of DNA released into solution after the preceding measurement was made (and, as such, the intensity of the DNA bands in the gel is observed to decrease over time). Further inspection of the data in Figure 4A reveals that DNA was released in both supercoiled and open-circular topologies. This result is consistent with the results of our past studies on the characterization of DNA released from polymer **1**/plasmid DNA films fabricated on the surfaces of macroscopic silicon, stainless steel, and quartz substrates [22–24,28,30,32]. These results, when combined, suggest that the presence of open-circular DNA does not arise simply from the physical manipulation of the film-coated microspheres during fabrication.

We also performed a series of cell-based transfection experiments to characterize the functional integrity of released DNA. Figures 4B and 4C show representative fluorescence microscopy images of COS-7 cells incubated with lipoplexes formed from Lipofectamine 2000 (a commercially available cell transfection agent) and aliquots of the DNA-containing PBS

solutions used to generate the data shown in Figure 4A. These data are consistent with the results of our past studies using DNA released from macroscopic substrates [22–24, 28, 30, 32], and demonstrate that the DNA released from film-coated microparticles is released in a transcriptionally active form that is capable of promoting the expression of EGFP in cells (the images shown in Figures 4B and 4C correspond to DNA released into solution over 0–3 hours and over 24–48 hours, respectively; additional time points not shown).

Characterization of Internalization of Film-Coated Microparticles by Macrophages

As a first step toward the development of multilayered film-coated microparticles for the delivery of DNA to antigen-presenting cells, we conducted a series of experiments to investigate the internalization of film-coated particles using the p388d1 cell line, a murine macrophage cell line used as a model in past studies to evaluate new materials for the development of DNA vaccines [8,15]. Figure 5 shows representative confocal microscopy images of cells incubated with particles coated with films fabricated using Cy-3 labeled DNA [in these images, Cy3 fluorescence is false-colored red; blue and green fluorescence corresponds to Hoechst 34580 (a fluorescent nuclear stain) and wheat germ agglutinin-Alexa 488 (a fluorescent cell membrane stain), respectively]. These experiments were performed using microparticles coated with 2.5 bilayers of a polymer **1**/DNA film.

The image in Figure 5A shows a representative confocal microscopy image of a population of cells incubated in the presence of film-coated microparticles for five hours. The image in Figure 5B shows an enlarged view of the cell in Figure 5A that has internalized a film-coated particle. The results shown in these images are significant for two reasons. First, these data demonstrate that these film-coated microparticles can be internalized by macrophage cells over the course of the five-hour incubation period used in these experiments. Second, we note that the polymer **1**/DNA films on the surfaces of these microparticles begin to erode and release DNA immediately upon incubation in physiological media (e.g., see Figure 3). The red, particle-associated fluorescence observed in the images in Figures 5A and 5B demonstrates that these film-coated particles can be internalized rapidly enough that a significant amount of DNA remains on the surfaces of the particles and is transported into the cell. The percentage of cells found to have internalized film-coated particles was low in these experiments. On the basis of several different confocal microscopy experiments, we estimate that ~1% of cells internalized at least one film-coated microparticle under these conditions. A substantially smaller number of cells internalized multiple particles.

Past studies have demonstrated that macrophages internalize microparticles with positive zeta potentials more readily than microparticles with near-neutral zeta potentials [12]. The film-coated particles used in the experiments described above were coated with a film 2.5 bilayers thick (i.e., with a final layer of cationic polymer **1**). In an effort to increase the surface charge of these particles in a manner that could lead to increased levels of particle internalization, we also conducted a series of experiments using particles coated with polymer **1**/DNA films for which the final layer of polymer added was linear poly(ethylene imine) (LPEI) rather than polymer **1**. LPEI is a polyamine commonly used as a DNA delivery agent [63,64], and it contains a higher density of amine functionality than polymer **1**. The zeta potentials of particles fabricated to have an outer layer of LPEI were measured to be higher than those of particles fabricated to have an outer layer of polymer **1** (e.g., approximately +14 mV vs. +3 mV, as measured in sodium acetate buffer).

Characterization of the internalization of particles coated with films having an outer layer of LPEI by confocal microscopy did not reveal any significant increases in the number of particles internalized by macrophages (data not shown). However, in contrast to our studies using particles coated with an outer layer of polymer **1**, we did observe diffuse red fluorescence located in the cytosol of cells that had internalized these LPEI-capped microparticles (Figures

5C and 5D show a low magnification image and an enlarged view of a representative example). We note again in this context that LPEI is used widely for the formulation of nanoscale polymer complexes for the delivery of DNA, at least in part as a result of its ability to promote the release of internalized DNA from intracellular vesicles such as endosomes [64]. One possible interpretation of the behavior described above is thus that the final layer of LPEI adsorbed to these film-coated particles could provide a means of promoting the disruption of phagosomes and the subsequent transport of released DNA into the cytosol. We caution, however, that additional experiments will be required to further evaluate this hypothesis and determine more completely the role that additional layers of LPEI (or other membrane-disruptive agents) could play in promoting the delivery of DNA into the cytosol under these conditions.

Finally, while the film-coated microparticles used in this study were fabricated using transcriptionally active plasmid DNA encoding EGFP, we did not observe significant levels of EGFP in macrophages in the particle internalization experiments described above (as determined by fluorescence microscopy). We note in this context that past reports have demonstrated that macrophages and other APCs can be difficult to transfect *in vitro* [65,66]. In addition, Denis-Mize et al. have reported that, despite levels of gene expression that were only observable using RT-PCR, bone marrow-derived dendritic cells were able to activate T cells *in vitro*, indicating that successful antigen presentation can occur even when transgene expression is relatively low [65]. The primary focus of this current study was directed toward physicochemical characterization of film-coated particles and the layer-by-layer fabrication procedure, as well as an initial evaluation of the potential of this approach to transport DNA into macrophage cells. Full characterization of this approach in the context of macrophage cell transfection will require the optimization of several additional significant variables – including DNA loading, particle sizes, the number of particles administered, plasmid DNA sequences and promoters, and other experimental conditions – that could have a substantial impact on levels of gene expression. Additional studies to optimize these variables, characterize levels of reporter protein expression, and, ultimately, assess levels of encoded antigen expression and presentation should provide a more complete understanding of the potential of this layer-by-layer approach to microparticle-based DNA delivery to promote effective transgene expression in APCs.

Summary and Conclusions

We have reported a layer-by-layer approach to the assembly of ultrathin and erodible DNA-containing films on the surfaces of polymer microparticles. The growth of multilayered films fabricated from plasmid DNA and hydrolytically degradable polymer **1** occurred in a stepwise manner, as demonstrated by monitoring the zeta potentials and fluorescence intensities of the particles during film fabrication, and the resulting film-coated microparticles sustained the release of transcriptionally active DNA into solution for ~3 days when incubated in physiologically relevant media. Finally, our results demonstrate that microparticles coated with polymer **1**/DNA films can be used to transport DNA into macrophage cells using a model mouse macrophage cell line.

Past studies have demonstrated that the adsorption of DNA onto the surfaces of cationic microparticles can be used to deliver DNA to antigen-presenting cells [19,20,65]. The results of this current study demonstrate a layer-by-layer approach to the assembly of erodible, DNA-containing films on the surfaces of polymer microparticles that could expand the versatility of this general approach and, with further development, provide new methods for the formulation of DNA vaccines. In addition to providing control over the amount of DNA immobilized at a surface, the modular nature of this method provides an approach to the incorporation of other functional polymers (or layers) and the tailoring of surface properties in ways that could be used to promote the targeting and internalization of film-coated particles, control rates at which

DNA release occurs, or increase levels of transgene expression. The focus of this current study was directed primarily toward characterization of the layer-by-layer assembly process and the physicochemical characterization of microparticles coated with erodible DNA-containing films, with a view toward providing a basis for the development of new microparticle-based methods for the delivery of DNA to phagocytotic cells of the immune system. We note, however, that the extension of this general approach to the fabrication of erodible films on the surfaces of smaller colloidal particles (e.g., $<1\ \mu\text{m}$) could also lead to new methods for the delivery of DNA to other cell types of interest in a broad range of fundamental and applied applications.

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References

1. Ulmer JB, et al. Heterologous Protection against Influenza by Injection of DNA Encoding a Viral Protein. *Science* 1993;259:1745–1749. [PubMed: 8456302]
2. Wang B, et al. Gene Inoculation Generates Immune-Responses against Human-Immunodeficiency-Virus Type-1. *Proc Natl Acad Sci USA* 1993;90:4156–4160. [PubMed: 8483929]
3. Fynan EF, Webster RG, Fuller DH, Haynes JR, Santoro JC, Robinson HL. DNA Vaccines-Protective Immunizations by Parenteral, Mucosal, and Gene-Gun Inoculations. *Proc Natl Acad Sci USA* 1993;90:11478–11482. [PubMed: 8265577]
4. Kowalczyk DW, Ertl HCJ. Immune responses to DNA vaccines. *Cell Mol Life Sci* 1999;55:751–770. [PubMed: 10379361]
5. Sato Y, et al. Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* 1996;273:352–354. [PubMed: 8662521]
6. Jones DH, Corris S, McDonald S, Clegg JCS, Farrar GH. Poly(DL-lactide-co-glycolide)-encapsulated plasmid DNA elicits systemic and mucosal antibody responses to encoded protein after oral administration. *Vaccine* 1997;15:814–817. [PubMed: 9234522]
7. McKeever U, et al. Protective immune responses elicited in mice by immunization with formulations of poly (lactide-co-glycolide) microparticles. *Vaccine* 2002;20:1524–1531. [PubMed: 11858858]
8. Hedley ML, Curley J, Urban R. Microspheres containing plasmid-encoded antigens elicit cytotoxic T-cell responses. *Nat Med* 1998;4:365–368. [PubMed: 9500615]
9. O'Hagan DT, Singh M, Ulmer JB. Microparticles for the delivery of DNA vaccines. *Immunol Rev* 2004;199:191–200. [PubMed: 15233735]
10. Donnelly JJ, Wahren B, Liu MA. DNA vaccines: Progress and challenges. *J Immunol* 2005;175:633–639. [PubMed: 16002657]
11. Putnam D. Polymers for gene delivery across length scales. *Nat Mater* 2006;5:439–451. [PubMed: 16738681]
12. Tabata Y, Ikada Y. Phagocytosis of Polymer Microspheres by Macrophages. *Adv Polym Sci* 1990;94:107–141.
13. Luby TM, Cole G, Baker L, Kornher JS, Ramstedt U, Hedley ML. Repeated immunization with plasmid DNA formulated in poly (lactide-co-glycolide) microparticles is well tolerated and stimulates durable T cell responses to the tumor-associated antigen cytochrome P4501B1. *Clin Immunol* 2004;112:45–53. [PubMed: 15207781]
14. Wang DQ, Robinson DR, Kwon GS, Samuel J. Encapsulation of plasmid DNA in biodegradable poly (D, L-lactic-co-glycolic acid) microspheres as a novel approach for immunogene delivery. *J Control Release* 1999;57:9–18. [PubMed: 9863034]

15. Little SR, et al. Poly-beta amino ester-containing microparticles enhance the activity of nonviral genetic vaccines. *Proc Natl Acad Sci USA* 2004;101:9534–9539. [PubMed: 15210954]
16. Fu K, Pack DW, Klivanov AM, Langer R. Visual evidence of acidic environment within degrading poly(lactic-co-glycolic acid) (PLGA) microspheres. *Pharm Res* 2000;17:100–106. [PubMed: 10714616]
17. Wang C, et al. Molecularly engineered poly(ortho ester) microspheres for enhanced delivery of DNA vaccines. *Nat Mater* 2004;3:190–196. [PubMed: 14991022]
18. Ando S, Putnam D, Pack DW, Langer R. PLGA microspheres containing plasmid DNA: Preservation of supercoiled DNA via cryopreparation and carbohydrate stabilization. *J Pharm Sci* 1999;88:126–130. [PubMed: 9874713]
19. Singh M, Briones M, Ott G, O'Hagan D. Cationic microparticles: A potent delivery system for DNA vaccines. *Proc Natl Acad Sci USA* 2000;97:811–816. [PubMed: 10639162]
20. Briones M, Singh M, Ugozzoli M, Kazzaz J, Klakamp S, Ott G, O'Hagan D. The preparation, characterization, and evaluation of cationic microparticles for DNA vaccine delivery. *Pharm Res* 2001;18:709–712. [PubMed: 11465430]
21. Vazquez E, Dewitt DM, Hammond PT, Lynn DM. Construction of hydrolytically-degradable thin films via layer-by-layer deposition of degradable polyelectrolytes. *J Am Chem Soc* 2002;124:13992–13993. [PubMed: 12440887]
22. Zhang JT, Chua LS, Lynn DM. Multilayered thin films that sustain the release of functional DNA under physiological conditions. *Langmuir* 2004;20:8015–8021. [PubMed: 15350066]
23. Fredin NJ, Zhang JT, Lynn DM. Surface analysis of erodible multilayered polyelectrolyte films: Nanometer-scale structure and erosion profiles. *Langmuir* 2005;21:5803–5811. [PubMed: 15952826]
24. Jewell CM, Zhang JT, Fredin NJ, Lynn DM. Multilayered polyelectrolyte films promote the direct and localized delivery of DNA to cells. *J Control Release* 2005;106:214–223. [PubMed: 15979188]
25. Zhang JT, Fredin NJ, Janz JF, Sun B, Lynn DM. Structure/property relationships in erodible multilayered films: Influence of polycation structure on erosion profiles and the release of anionic polyelectrolytes. *Langmuir* 2006;22:239–245. [PubMed: 16378427]
26. Lynn DM. Layers of opportunity: nanostructured polymer assemblies for the delivery of macromolecular therapeutics. *Soft Matter* 2006;2:269–273.
27. Zhang JT, Fredin NJ, Lynn DM. Erosion of multilayered films fabricated from degradable polyamines: Characterization and evidence in support of a mechanism that involves polymer hydrolysis. *J Polym Sci A: Polym Chem* 2006;44:5161–5173.
28. Jewell CM, Zhang JT, Fredin NJ, Wolff MR, Hacker TA, Lynn DM. Release of plasmid DNA from intravascular stents coated with ultrathin multilayered polyelectrolyte films. *Biomacromolecules* 2006;7:2483–2491. [PubMed: 16961308]
29. Zhang J, Lynn DM. Multilayered films fabricated from combinations of degradable polyamines: Tunable erosion and release of anionic polyelectrolytes. *Macromolecules* 2006;39:8928–8935. [PubMed: 19043614]
30. Fredin NJ, Zhang JT, Lynn DM. Nanometer-scale decomposition of ultrathin multilayered polyelectrolyte films. *Langmuir* 2007;23:2273–2276. [PubMed: 17309197]
31. Jewell CM, Fuchs SM, Flessner RM, Raines RT, Lynn DM. Multilayered films fabricated from an oligoarginine-conjugated protein promote efficient surface-mediated protein transduction. *Biomacromolecules* 2007;8:857–863. [PubMed: 17269822]
32. Zhang JT, Montanez SI, Jewell CM, Lynn DM. Multilayered films fabricated from plasmid DNA and a side-chain functionalized poly(beta-amino ester): Surface-type erosion and sequential release of multiple plasmid constructs-from surfaces. *Langmuir* 2007;23:11139–11146. [PubMed: 17887783]
33. Lynn DM. Peeling back the layers: Controlled erosion and triggered disassembly of multilayered polyelectrolyte thin films. *Adv Mater* 2007;19:4118–4130.
34. Zhang JT, Lynn DM. Ultrathin multilayered films assembled from “Charge-Shifting” cationic polymers: Extended, long-term release of plasmid DNA from surfaces. *Adv Mater* 2007;19:4218–4223.

35. Decher G. Fuzzy nanoassemblies: Toward layered polymeric multicomposites. *Science* 1997;277:1232–1237.
36. Lvov Y, Decher G, Sukhorukov G. Assembly of Thin-Films by Means of Successive Deposition of Alternate Layers of DNA and Poly(Allylamine). *Macromolecules* 1993;26:5396–5399.
37. Bertrand P, Jonas A, Laschewsky A, Legras R. Ultrathin polymer coatings by complexation of polyelectrolytes at interfaces: suitable materials, structure and properties. *Macromol Rapid Commun* 2000;21:319–348.
38. Pei RJ, Cui XQ, Yang XR, Wang EK. Assembly of alternating polycation and DNA multilayer films by electrostatic layer-by-layer adsorption. *Biomacromolecules* 2001;2:463–468. [PubMed: 11749207]
39. Schuler C, Caruso F. Decomposable hollow biopolymer-based capsules. *Biomacromolecules* 2001;2:921–926. [PubMed: 11710050]
40. Shi XY, Sanedrin RJ, Zhou FM. Structural characterization of multilayered DNA and polylysine composite films: Influence of ionic strength of DNA solutions on the extent of DNA incorporation. *J Phys Chem B* 2002;106:1173–1180.
41. Ai H, Jones S, Lvov Y. Biomedical applications of electrostatic layer-by-layer nano-assembly of polymers, enzymes, and nanoparticles. *Cell Biochem Biophys* 2003;39:23–43. [PubMed: 12835527]
42. Serizawa T, Yamaguchi M, Akashi M. Time-controlled desorption of ultrathin polymer films triggered by enzymatic degradation. *Angew Chem-Int Ed* 2003;42:1115–1118.
43. Peyratout CS, Dahne L. Tailor-made polyelectrolyte microcapsules: From multilayers to smart containers. *Angew Chem-Int Ed* 2004;43:3762–3783.
44. Hammond PT. Form and function in multilayer assembly: New applications at the nanoscale. *Adv Mater* 2004;16:1271–1293.
45. Angelatos AS, Katagiri K, Caruso F. Bioinspired colloidal systems via layer-by-layer assembly. *Soft Matter* 2006;2:18–23.
46. Ren KF, Ji J, Shen JC. Construction and enzymatic degradation of multilayered poly-L-lysine/DNA films. *Biomaterials* 2006;27:1152–1159. [PubMed: 16102814]
47. Tang ZY, Wang Y, Podsiadlo P, Kotov NA. Biomedical applications of layer-by-layer assembly: From biomimetics to tissue engineering. *Adv Mater* 2006;18:3203–3224.
48. De Geest BG, Sanders NN, Sukhorukov GB, Demeester J, De Smedt SC. Release mechanisms for polyelectrolyte capsules. *Chem Soc Rev* 2007;36:636–649. [PubMed: 17387411]
49. Jewell CM, Lynn DM. Multilayered polyelectrolyte assemblies as platforms for the delivery of DNA and other nucleic acid-based therapeutics. *Adv Drug Del Rev* 2008;60:979–999.
50. Trimaille T, Pichot C, Delair T. Surface functionalization of poly(D,L-lactic acid) nanoparticles with poly(ethylenimine) and plasmid DNA by the layer-by-layer approach. *Colloids Surf A* 2003;221:39–48.
51. Messai I, Lamalle D, Munier S, Verrier B, Ataman-Onal Y, Delair T. Poly(D,L-lactic acid) and chitosan complexes: interactions with plasmid DNA. *Colloids Surf A* 2005;255:65–72.
52. Munier S, Messai I, Delair T, Verrier B, Ataman-Onal Y. Cationic PLA nanoparticles for DNA delivery: Comparison of three surface polycations for DNA binding, protection and transfection properties. *Colloids Surf B* 2005;43:163–173.
53. Reibetanz U, Claus C, Typlt E, Hofmann J, Donath E. Defoliation and plasmid delivery with layer-by-layer coated colloids. *Macromol Biosci* 2006;6:153–160. [PubMed: 16456874]
54. Lynn DM, Langer R. Degradable poly(beta-amino esters): Synthesis, characterization, and self-assembly with plasmid DNA. *J Am Chem Soc* 2000;122:10761–10768.
55. Hunter, RJ. *Zeta Potential in Colloid Science*. New York, NY: Academic Press; 1981.
56. Johnston APR, Zelikin AN, Lee L, Caruso F. Approaches to quantifying and visualizing polyelectrolyte multilayer film formation on particles. *Anal Chem* 2006;78:5913–5919. [PubMed: 16906740]
57. Donath E, Sukhorukov GB, Caruso F, Davis SA, Mohwald H. Novel hollow polymer shells by colloid-templated assembly of polyelectrolytes. *Angew Chem-Int Ed* 1998;37:2202–2205.
58. Sukhorukov GB, Donath E, Lichtenfeld H, Knippel E, Knippel M, Budde A, Mohwald H. Layer-by-layer self assembly of polyelectrolytes on colloidal particles. *Colloids Surf A* 1998;137:253–266.

59. Hiller S, Leporatti S, Schnackel A, Typlt E, Donath E. Protamine assembled in multilayers on colloidal particles can be exchanged and released. *Biomacromolecules* 2004;5:1580–1587. [PubMed: 15244481]
60. Israelachvili, J. *Intermolecular & Surface Forces*. San Diego, CA: Academic Press; 1991.
61. Vinogradova OI, Lebedeva OV, Vasilev K, Gong HF, Garcia-Turiel J, Kim BS. Multilayer DNA/poly(allylamine hydrochloride) microcapsules: Assembly and mechanical properties. *Biomacromolecules* 2005;6:1495–1502. [PubMed: 15877370]
62. Schneider G, Decher G. Functional core/shell nanoparticles via layer-by-layer assembly investigation of the experimental parameters for controlling particle aggregation and for enhancing dispersion stability. *Langmuir* 2008;24:1778–1789. [PubMed: 18225923]
63. Godbey WT, Wu KK, Mikos AG. Poly(ethylenimine) and its role in gene delivery. *J Control Release* 1999;60:149–160. [PubMed: 10425321]
64. Kircheis R, Wightman L, Wagner E. Design and gene delivery activity of modified polyethylenimines. *Adv Drug Del Rev* 2001;53:341–358.
65. Denis-Mize KS, et al. Plasmid DNA adsorbed onto cationic microparticles mediates target gene expression and antigen presentation by dendritic cells. *Gene Ther* 2000;7:2105–2112. [PubMed: 11223992]
66. Walter E, Merkle HP. Microparticle-mediated transfection of non-phagocytic cells in vitro. *J Drug Target* 2002;10:11–21. [PubMed: 11996082]

Figure 1.

A) Representative confocal microscopy image of particles coated with a film 2.5 bilayers thick fabricated from polymer **1** and Cy3-labeled DNA. Scale bar = 20 μm for image, 2 μm for inset.

B) SEM image of a particle coated with 2.5 bilayers of polymer **1** and DNA. Scale bar = 1 μm .

Figure 2.

A) Zeta potentials of particles as a function of the number of alternating polymer 1 and DNA layers adsorbed. Particles were suspended in sodium acetate buffer (100 mM; pH = 5) prior to measurement. B) Average grayscale intensities of coated particles as a function of the number of alternating polymer 1 and Cy3-labeled DNA layers adsorbed.

Figure 3.
Plot of DNA released into solution versus time for particles coated with a polymer 1/DNA film 2.5 bilayers thick incubated in PBS at 37 °C.

Figure 4.

A) Agarose gel electrophoresis data characterizing release of DNA from film-coated particles. Lane 1 shows control DNA at a concentration of 2 $\mu\text{g/mL}$. Lanes 2–7 are solutions from DNA release experiments (corresponding to solutions collected after 3, 6, 14, 24, 48, and 72 hours, respectively). B–C) Representative fluorescence microscopy images showing EGFP expression in COS-7 cells transfected with DNA released over 0–3 hours (B) and 24–48 hours (C) using Lipofectamine 2000 (see text). Scale bars = 100 μm .

Figure 5.

A) Representative confocal microscopy image of p388d1 cells treated with film-coated microparticles fabricated using polymer **1** and Cy3-labeled DNA. Scale bar = 10 μm . B) Higher magnification image of the cell in panel A that has internalized a coated particle. Scale bar = 5 μm . C) Representative image of p388d1 cells treated with microparticles coated with 2 bilayers of a polymer **1**/Cy3-labeled DNA film capped with an outer layer of LPEI. Scale bar = 10 μm . D) Enlarged image of the cell in panel C that has internalized a coated particle. Scale bar = 5 μm . The red, green and blue fluorescence in these images correspond to Cy3, WGA-Alexa 488, and Hoechst fluorescent probes, respectively.

Scheme 1.

Assembly of multilayered polyelectrolyte films on polymer microspheres is accomplished by successive cycles of centrifugation and resuspension in solutions of positively and negatively charged polymers. Passing the particles through a narrow-gauge syringe needle facilitates resuspension and reduces particle aggregation (see text).