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Characterization of Nanoscale Transformations in Polyelectrolyte Multilayers Fabricated from Plasmid DNA Using Laser Scanning Confocal Microscopy in Combination with Atomic Force Microscopy

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Abstract

Laser scanning confocal microscopy (LSCM) and atomic force microscopy (AFM) were used to characterize changes in nanoscale structure that occur when ultrathin polyelectrolyte multilayers (PEMs) are incubated in aqueous media. The PEMs investigated here were fabricated by the deposition of alternating layers of plasmid DNA and a hydrolytically degradable polyamine onto a precursor film composed of alternating layers of linear poly(ethylene imine) (LPEI) and sodium poly(styrene sulfonate) (SPS). Past studies of these materials in the context of gene delivery revealed transformations from a morphology that is smooth and uniform to one characterized by the formation of nanometer-scale particulate structures. We demonstrate that in-plane registration of LSCM and AFM images acquired from the same locations of films fabricated using fluorescently labeled polyelectrolytes allows the spatial distribution of individual polyelectrolyte species to be determined relative to the locations of topographic features that form during this transformation. Our results suggest that this physical transformation leads to a morphology consisting of a relatively less disturbed portion of film composed of polyamine and DNA juxtaposed over an array of particulate structures composed predominantly of LPEI and SPS. Characterization by scanning electron microscopy (SEM) and energy-dispersive X-ray (EDX) microanalysis provides additional support for this interpretation. The combination of these different microscopy techniques provides insight into the structures and dynamics of these multicomponent thin films that cannot be achieved using any one method alone, and that could prove useful for the further development of these assemblies as platforms for the surface-mediated delivery of DNA.

Keywords

Thin Films; Nanostructure; Polymers; Layer-by-Layer; DNA Delivery

Introduction

The layer-by-layer fabrication of multilayered polyelectrolyte assemblies (or ‘polyelectrolyte multilayers’, PEMs) has emerged as a versatile approach to the fabrication of nanostructured thin films (Ai et al., 2003; Bertrand et al., 2000; De Geest et al., 2007; Decher, 1997; Hammond, 2004; Peyratout and Dähne, 2004; Sukhishvili, 2005; Tang et al., 2006). These methods are entirely aqueous and provide control over both the compositions and physicochemical properties of thin films and coatings fabricated from a broad range of synthetic and natural polyelectrolytes. This approach is also particularly well suited for the incorporation or immobilization of therapeutically relevant polyelectrolytes, such as proteins and DNA, on surfaces. As a result, PEMs have been investigated in a broad range of fundamental and applied biomedical contexts (Ai et al., 2003; Boudou et al., 2009; De Geest et al., 2007; Jewell and Lynn, 2008a; ^b; Lynn, 2006; 2007; Peyratout and Dähne, 2004; Sukhishvili, 2005; Tang et al., 2006). In the context of potential applications in the area of drug delivery, numerous past studies have demonstrated that PEMs can serve as thin-film platforms for the controlled release of small molecules and macromolecular agents (Boudou et al., 2009; De Geest et al., 2007; Jewell and Lynn, 2008a; Peyratout and Dähne, 2004; Tang et al., 2006). One challenge associated with the continued development of these new materials-based approaches lies in developing methods for characterization of the molecular-level and nanoscale structures of these polyelectrolyte-based assemblies and, in particular, understanding the ways in which these structures can change when exposed to the physiologically relevant environments in which they will be used. In this paper, we describe the use of three complementary microscopy techniques to characterize dynamic, nanometer-scale processes that occur when PEMs fabricated from transcriptionally active plasmid DNA are incubated in physiologically relevant media.

We have reported in a series of past publications that PEMs fabricated from plasmid DNA and hydrolytically degradable polyamines (such as polymer **1**) can be designed to erode gradually and promote the surface-mediated delivery of DNA to cells (Fredin et al., 2005; 2007; Jewell and Lynn, 2008a; ^b; Jewell et al., 2005; Lynn, 2006; ²⁰⁰⁷; Saurer et al., 2009; Vazquez et al., 2002; Zhang et al., 2004; Zhang et al., 2007). These past studies have focused largely on films having the general structure (LPEI/SPS)₁₀(**1**/DNA)₈, fabricated by the deposition of eight alternating layers of polymer **1** and plasmid DNA directly onto thin precursor films fabricated from ten alternating layers of linear poly(ethylene imine) (LPEI) and sodium poly(styrene sulfonate) (SPS). During experiments to characterize the release of DNA and levels of cell transfection mediated by these materials (Jewell et al., 2005; Jewell et al., 2006; Zhang et al., 2004), we also identified physical transformations and unexpected changes in the morphologies of these materials that occur when they are incubated in physiologically relevant media (Fredin et al., 2005; 2007). For example, characterization of the surfaces of these films using atomic force microscopy (AFM) and scanning electron microscopy (SEM) demonstrated that these materials transform gradually from an initial state that is flat, smooth, and devoid of significant micrometer- and nanometer-scale defects to a state characterized by nanometer-scale particulate structures over a period of approximately five hours when incubated in phosphate-buffered saline (PBS) (Fredin et al., 2005; 2007). The results of these past studies also demonstrated that it is possible to promote or prevent these changes in morphology and, to varying extents, exert spatial and/or temporal control over these changes by varying polymer structure, film composition, or the environmental conditions (e.g., changes in ionic strength, pH, etc.) to which these materials are exposed (Fredin et al., 2007).

Polymer 1

The results of our past studies provide a basis for understanding the nature of these physical changes in film morphology at the nanometer scale. Unfortunately, however, methods such as AFM and SEM provide little insight into changes in film composition or changes in the relative distributions of the four different polyelectrolyte species in these materials that could occur as a result of these transformations. For example, characterization by AFM or SEM alone cannot be used to determine whether changes in film morphology occur as a result of the large-scale transport of all components (or layers) of these multicomponent films, or whether redistributions of mass occur predominantly in layers composed of polymer **1** and DNA or layers composed of LPEI and SPS. In addition, we note that films composed of polymer **1** erode gradually (e.g., on a time scale of several days) and release DNA into solution upon incubation in aqueous media (Jewell et al., 2006; Zhang et al., 2004). Our past studies demonstrate that the most significant changes in film morphology described above occur, in general, more rapidly than these processes of film erosion (e.g., over a time scale of several hours) (Fredin et al., 2005; 2007). However, important questions remain with respect to (i) the roles that polymer **1** and DNA could play in mediating this transformation, (ii) the physical locations of these polyelectrolytes once the transformation has occurred, and (iii) the extents to which they remain as components of these films after the transformation has occurred. A more complete understanding of changes in the distribution of the individual polyelectrolyte components of these films would contribute to a broader physical and molecular-level understanding of the dynamics of these new materials in aqueous media. In a broader context, additional information about the physical locations and dynamics of DNA and polymer **1** in these assemblies could also provide insight that would be useful in interpreting the results of past studies describing the DNA delivery behavior of these materials or in guiding the design of films fabricated to transfect cells more effectively.

This present study sought to use laser-scanning confocal microscopy (LSCM) in combination with AFM to (i) characterize changes in the spatial distribution of polyelectrolytes in films fabricated using polymer **1** and DNA and (ii) correlate the locations of each polyelectrolyte with changes in nanometer-scale topography that occur when these materials are incubated in physiologically relevant media. While AFM has been widely used to characterize the micrometer- and nanometer-scale surface features of PEMs (Kotov et al., 1998; Lobo et al., 1999; McAloney et al., 2001; Mendelsohn et al., 2000; Picart et al., 2001; Saremi et al., 1995; Schoeler et al., 2002), the use of LSCM to characterize the location or behavior of individual polyelectrolyte components in PEMs has been more limited. Past studies have demonstrated that LSCM can be used to characterize PEMs fabricated from fluorescently labeled polyelectrolytes deposited onto colloidal particles or formed as hollow capsules (Garza et al., 2004; Ji et al., 2006; Jourdainne et al., 2007; Lavallo et al., 2004; Peyratout and Dähne, 2004; Picart et al., 2002; Porcel et al., 2007; Richert et al., 2004; Sun et al., 2007; Zhang et al., 2005). In addition, several groups have demonstrated that LSCM can be used to image optical cross-sections of thick (e.g., micrometer-scale) PEMs fabricated from fluorescently labeled polyelectrolytes and characterize the diffusion of polyelectrolytes that can occur within these materials during fabrication (Garza et al., 2004; Ji et al., 2006; Jourdainne et al., 2007; Lavallo et al., 2004; Picart et al., 2002; Porcel et al., 2007; Richert et al., 2004; Sun et al., 2007; Zhang et al., 2005). Picart and coworkers have also demonstrated that LSCM can be used to characterize the *in vitro* and *in vivo* enzymatic degradation of micrometer-thick PEMs fabricated from polysaccharides (Etienne et al., 2005; Picart et al., 2005b; Schneider et al., 2007), and to investigate the lateral diffusion of polyelectrolytes within PEMs by characterizing

fluorescence recovery in photobleached films (Picart et al., 2005a). These past studies demonstrate that LSCM can be used to visualize and provide spatial information (e.g., in both the x-y and x-z planes) about fluorescently labeled components incorporated into PEMs. However, to our knowledge, LSCM has not previously been used in combination with AFM to relate spatial differences in the locations or concentrations of the individual components of PEMs with changes in film morphology and topography that occur at the micrometer- and nanometer-scale.

Here, we focus on the combined use of AFM and LSCM to characterize morphological transformations that occur in ultrathin films (e.g., ~100 nm thick) having the structure (LPEI/SPS)₁₀(1/DNA)₈ during the first five hours of incubation in PBS. Our past studies demonstrate that the transformations that occur over in this time frame are largely complete prior to the onset of the release of significant amounts of DNA or SPS (Fredin et al. 2007). We demonstrate that LSCM can be used to provide nanometer-scale spatial information about changes in the relative locations and concentrations of all four polyelectrolyte components of these materials. We demonstrate that films fabricated using plasmid DNA, polymer **1**, LPEI, and SPS that are each independently fluorescently labeled can be used to characterize nanometer-scale changes in the spatial distribution of each polyelectrolyte species when these films are incubated in PBS. We demonstrate further that careful in-plane registration of LSCM images with images of the same films acquired by AFM permits correlation of the locations of individual polyelectrolyte species with changes in the topographies and morphologies of these films observed in past studies. The results of these experiments demonstrate that both LPEI and SPS are located predominantly in the nanometer-scale particulate features that arise during film decomposition. In contrast, both DNA and polymer **1** are found to be present in these particulate features and in the regions of the films that exist between these particulate features. These results are consistent with a physical picture that involves the large-scale transport and accumulation of the bottommost foundation layers fabricated from LPEI and SPS in punctate structures, and suggest that the topmost layers of these films (comprised predominantly of polymer **1** and DNA) are less disturbed by this transformation. This physical picture is supported further by the results of additional SEM and energy dispersive X-ray (EDX) microanalysis of scratched films. The results of this investigation provide additional insight into the nature of the physical transformations that take place when these ionically crosslinked assemblies are incubated in aqueous media and could prove useful for the further development of these DNA-containing assemblies as platforms for the surface-mediated delivery of DNA.

Materials and Methods

Materials

Test grade n-type silicon wafers were purchased from Si-Tech, Inc. (Topsfield, MA). Linear poly(ethylene imine) (LPEI, MW = 25,000) was obtained from Polysciences, Inc. (Warrington, PA). Poly(sodium 4-styrenesulfonate) (SPS, MW = 70,000), sodium acetate buffer, 4-styrenesulfonic acid sodium salt hydrate (SS), 4,4'-trimethylenedipiperidine, reagent grade dimethylsulfoxide, and sodium chloride were purchased from Aldrich Chemical Company (Milwaukee, WI). Polymer **1** (MW ≈ 20,000) was synthesized as previously described (Lynn and Langer, 2000). 1,4-Butanediol diacrylate was purchased from Alfa Aesar (Ward Hill, MA). Potassium persulfate was purchased from Fluka Chemical (Milwaukee, WI). Tetramethylrhodamine (TMR) cadaverine and 5-(and-6)-carboxytetramethylrhodamine, succinimidyl ester (TAMRA, SE) were purchased from Invitrogen Corp. (Carlsbad, CA). Methacryloxyethyl thiocarbamoyl rhodamine B (MRho) was purchased from Polysciences, Inc. Plasmid DNA [pEGFP-N1 (4.7 kb), > 95% supercoiled] was purchased from Elim Biopharmaceuticals, Inc. (San Francisco, CA). Anhydrous tetrahydrofuran was purchased from Acros Organics (Morris Plains, NJ). Absolute ethanol was purchased from Aaper Alcohol

(Shelbyville, KY). Reagent grade methanol was purchased from EMD (Gibbstown, NJ). Reagent grade hexane was purchased from BDH Chemicals (Poole, UK). Deionized water (18 M Ω) was used for washing steps and to prepare all polymer solutions. PBS buffer was prepared by diluting commercially available concentrate (EM Science, Gibbstown, NJ) and adjusting the pH to 7.4 with 1.0 M NaOH. All commercial materials were used as received without further purification unless otherwise noted.

General Considerations

Silicon and glass substrates (typically $\sim 0.5 \times 4$ cm) were successively rinsed with acetone, ethanol, methanol, and water and then dried under a stream of compressed air passed through a 0.2 μ m filter. Substrates were then activated by etching in an oxygen plasma for 5 minutes (Plasma Etch, Carson City, NV). Ellipsometric thicknesses of films deposited on silicon substrates were determined using a Gaertner LSE Stokes Ellipsometer (632.8 nm, incident angle = 70°). Data were processed using the Gaertner Ellipsometer Measurement Program software package. Relative thicknesses were calculated assuming an average refractive index of 1.55 for the multilayered films. Thicknesses were determined in at least four different standardized locations on each substrate and are presented as an average (with standard deviation) for each substrate. All films were dried under a stream of filtered compressed air prior to measurement. Characterization of film topography by atomic force microscopy (AFM) was conducted in tapping mode using a Nanoscope Multimode atomic force microscope (Digital Instruments, Santa Barbara, CA), using scan rates of 10–20 μ m/s to obtain 256×256 pixel images, using silicon cantilevers with a spring constant of 40 N/m (model NSC15/AIBS, MikroMasch USA, Inc., Portland, OR). Height data were flattened using a 2nd-order fit. Prior to imaging, a razor blade was used to scratch a pair of perpendicular lines in each film in an arbitrarily chosen location to facilitate imaging of the same area of a given film by AFM, laser-scanning confocal microscopy (LSCM), and scanning electron microscopy (SEM) (see text). For the characterization of surface morphology by SEM, an accelerating voltage of 3 kV was used to acquire images on a LEO DSM 1530 scanning electron microscope. Samples were coated with a thin layer of gold using a Denton Vacuum Desk II sputterer (30 seconds at 45 mA, 50 mTorr) prior to analysis. For experiments in which energy-dispersive X-ray (EDX) spectroscopy was used to characterize films, samples were coated with a thinner layer of gold (10 seconds at 45 mA, 50 mTorr), and an accelerating voltage of 3.5 kV was used. X-ray spectra were collected by focusing on either the center of a particle or on an area situated between particles (see text) at a magnification of 25000X, and selecting the “spot” option on the microscope’s control software (i.e., focusing the beam on one location rather than scanning over an area). The NORAN System SIX software was used to quantify the elemental composition of the location represented by each spectrum. For each sample, particles and regions between particles ($n = 10$ to 15 each) were selected arbitrarily for analysis. To eliminate differences in composition owing to potential differences in the thickness of different areas of the film (e.g., particles vs. film between particles), the compositions were recalculated with contributions from gold and silicon omitted (see text). LSCM was performed using a Bio-Rad Radiance 2100 MP Rainbow laser scanning confocal microscope. Film-coated substrates were placed face-down either in a confocal dish containing PBS or sodium acetate buffer or on a microscope cover slip coated with several drops of water, and were imaged using a 100X oil immersion objective. Additional optical zoom up to 10X was used to scan areas as small as $12 \mu\text{m} \times 12 \mu\text{m}$. Samples were excited with a 543 nm laser and emitted light was collected between 560 nm and 625 nm. Images were processed using Adobe Photoshop, ImageJ (NIH), and Microsoft PowerPoint. Preparation of some composite overlaid LSCM and AFM images required small, uniform changes (e.g., < 10%) to the overall dimensions of the AFM images to achieve suitable registration of the two different images.

Synthesis of Fluorescently Labeled LPEI

Fluorescently labeled LPEI was synthesized using a modification of a previously described procedure (Breunig et al., 2005). LPEI (45.0 mg) was dissolved in DMSO (1 mL), and this solution was then added to a separate vial containing TAMRA, SE (5.5 mg). The resulting solution was stirred at room temperature for three days. HCl (1.5 eq) was added to the reaction mixture, and NaOH (1 M) was added to precipitate the polymer. The precipitate was washed liberally using deionized water to remove unreacted fluorophore. The final product was dried in a vacuum desiccator for two days and the absence of unreacted fluorophore in the final product was confirmed by gel electrophoresis.

Synthesis of Fluorescently Labeled Polymer 1

Fluorescently labeled polymer **1** was synthesized using a modification of the procedure reported for the synthesis of unlabeled polymer **1** (Lynn and Langer, 2000). In separate vials, 1,3-trimethylene dipiperidine (407.9 mg) and 1,4-butanediol diacrylate were dissolved in anhydrous THF (3.239 mL and 3.240 mL, respectively). TMR cadaverine (1 mg) was dissolved in DMSO (100 μ L). These three solutions were combined in a glass vial equipped with a magnetic stir bar. The vial was sealed with a Teflon-lined cap, placed in an oil bath at 50 °C, and the reaction mixture was stirred for 48 hours. The solvent was then removed by rotary evaporation and THF (1 mL) was added to redissolve the product. This solution was then precipitated into hexanes. This process of dissolution and precipitation was repeated once more, and the resulting pink solid product was dried in a vacuum desiccator. The dried product was dissolved in dichloromethane, and several washes with deionized water were performed to remove unreacted fluorophore. The washed product was dried by rotary evaporation followed by placement in a vacuum desiccator. Absence of unreacted fluorophore in final product was confirmed by gel electrophoresis.

Synthesis of Fluorescently Labeled SPS

Labeled SPS was synthesized by radical copolymerization of 4-styrenesulfonic acid sodium salt hydrate (SS) and methacryloxyethyl thiocarbamoyl rhodamine B (MRho) following a procedure adapted from Dähne *et al* (Dähne et al., 2001). In a typical reaction, 4-styrenesulfonic acid sodium salt hydrate (0.4970 g, 2.42 mmol) was weighed into a 25 mL round-bottomed flask and dissolved in 10 mL 20% aqueous methanol. MRho (0.0083 g, 0.0121 mmol) and potassium persulfate (0.0065 mg, 0.024 mmol) were each dissolved in 20% aqueous methanol (1 mL) and added to the round-bottomed flask. The solution was heated to 80 °C under a nitrogen atmosphere for four hours. The methanol was removed under reduced pressure, and the resulting pink solution was dialyzed against DI water (MWCO = 3500) for four days and lyophilized to yield a pink solid.

Preparation of Polyelectrolyte Solutions

Solutions of polymer **1** used for dipping (5 mM with respect to the molecular weight of the polymer repeat unit) were prepared in sodium acetate buffer (100 mM, pH = 5.0). Solutions of LPEI, SPS, and fluorescently labeled LPEI 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 25 mM NaCl solution in 18 M Ω water. LPEI and labeled-LPEI solutions contained 5 mM HCl to aid polymer solubility. Solutions of MRho-SPS were also prepared at a concentration of 20 mM with respect to the SPS repeat unit but without added NaCl. Solutions of plasmid DNA were prepared at 1 mg/mL in sodium acetate buffer. DNA labeled with Cy3 was prepared using the Label-IT kit from Mirus Bio Corporation (Madison, WI) following the manufacturer's protocol. Labeling density was determined to be one label per 270 nucleotide bases by UV-vis absorbance. For experiments in which fluorescently labeled polymers were used to fabricate films, solutions used for film fabrication were prepared by mixing

commercially available polyelectrolyte with the labeled species [9:1 (w/w) in the cases of SPS and DNA, and 4:1 (w/w) in the cases of LPEI and polymer **1**].

Fabrication of Multilayered Films

Fabrication of multilayered films was conducted using an alternate dipping method according to the following general protocol: 1) Substrates were submerged in a solution of LPEI for 5 minutes, 2) substrates were removed and immersed in an initial water bath for 1 minute followed by a second water bath for 1 minute, 3) substrates were submerged in a solution of SPS for 5 minutes, and 4) substrates were rinsed in the manner described above. This cycle was repeated until ten alternating layers of LPEI and SPS (referred to hereafter as ten 'bilayers') had been deposited. Following the deposition process, films were dried under a stream of filtered compressed air. After characterization of these LPEI/SPS films (e.g., by ellipsometry, AFM, etc.), the alternate dipping process described above was repeated to deposit eight bilayers of polymer **1** and DNA on the surface of the LPEI/SPS films, as described in past publications (Fredin et al., 2005; 2007; Jewell et al., 2005; Zhang et al., 2004).

Characterization of Film Erosion and Changes in Film Morphology

Film-coated substrates were placed in a semi-micro cuvette containing PBS buffer (1 mL, sufficient to completely cover the film coated portion of the substrate) and incubated at 37 °C, as described in past publications (Jewell et al., 2006; Zhang et al., 2004). Samples were removed at predetermined intervals, rinsed in two water baths for one minute each, and dried under filtered compressed air for characterization by ellipsometry, AFM, SEM, LSCM, and EDX (additional details of these methods are described above under General Considerations).

Results and Discussion

Our past studies demonstrate that multilayered films of plasmid DNA and polymer **1** deposited on a thin precursor film fabricated from multiple layers of LPEI and SPS undergo significant changes in surface morphology when incubated in aqueous media (e.g., PBS) (Fredin et al., 2005; 2007; Jewell et al., 2005; Jewell et al., 2006). These changes occur as a transformation that proceeds from a morphology that is initially smooth to a morphology having a topography that consists of isolated particulate structures. Figures 1A and 1B show representative AFM images of a silicon substrate coated with a multilayered film approximately 170 nm thick having the structure (LPEI/SPS)₁₀(**1**/DNA)₈ both before (Figure 1A) and after (Figure 1B) incubation in PBS for six hours. These images illustrate that, prior to incubation, these films have a smooth appearance and lack significant defects or other surface features on the micrometer- or nanometer-scale (Figure 1A). However, after incubation (Figure 1B), film topography is characterized by an array of particulate structures with mean diameters of approximately 300 nm. These results are generally consistent with the results of our past work on the characterization of the morphology of partially eroded films using AFM (Fredin et al., 2005; 2007). Below, we describe experiments designed to further characterize this transformation in films having the structure (LPEI/SPS)₁₀(**1**/DNA)₈ fabricated using one or more fluorescently labeled polyelectrolytes, using AFM in combination with LSCM to provide information about changes in the relative locations of the individual polymer and DNA components of these films.

Characterization of PEMs Fabricated Using Fluorescently Labeled Polyelectrolytes by LSCM and AFM

We conducted a series of initial experiments using fluorescently labeled SPS (referred to hereafter as SPS_{FL}) to determine whether LSCM could be used to visualize and characterize the locations of individual components in these films. SPS_{FL} was synthesized by the radical copolymerization of 4-styrenesulfonic acid sodium salt and methacryloxyethyl thiocarbamoyl

rhodamine B (Dähne et al., 2001). Solutions of SPS used for film fabrication were prepared by mixing unlabeled commercially available SPS with SPS_{FL} in a ratio of 9:1 (w/w). Films having the structure (LPEI/SPS_{FL})₁₀(1/DNA)₈ were fabricated layer-by-layer on silicon substrates using methods described in past studies (Fredin et al., 2005; 2007; Jewell et al., 2005; Zhang et al., 2004), such that SPS_{FL} was incorporated into every layer of the LPEI/SPS bilayers used to form the film. Figure 1C shows a LSCM image of a film having this structure imaged prior to incubation. Inspection of this image reveals a relatively uniform distribution of fluorescence over a 12 μm by 12 μm area. This result suggests that SPS_{FL} was incorporated into the films during layer-by-layer assembly and that it is distributed uniformly throughout the x-y plane of the film. The uniformity of this film is generally consistent with the results of our previous studies using AFM and SEM to characterize the topography of these films prior to incubation in aqueous environments (Fredin et al., 2005; 2007). Figure 1D shows an image of an otherwise identical film after incubation in PBS at 37 °C for six hours. Inspection of this image reveals fluorescence to be confined in punctate structures with sizes on the order of ~200 nm, suggesting that SPS has accumulated in these regions (or, alternatively, that it has been removed or lost from other areas of the film).

The sizes and shapes of the fluorescent structures in Figure 1D are similar to the sizes and shapes of the nanoparticulate features observed in the AFM image of the partially eroded film shown in Figure 1B. We sought to determine whether it was possible to use these LSCM and AFM images to determine the extent to which the locations of punctate fluorescence overlap with the locations of the particulate structures observed by AFM. One significant obstacle to making this comparison is the difficulty of imaging the same precise location of a single film using both methods, because imaging by both LSCM and AFM cannot be performed simultaneously using our instrumentation. To determine whether the punctate fluorescent regions in Figure 1D do correspond to the particulate structures observed in partially eroded films using AFM, we developed a procedure to characterize the same area of a single film using both AFM and LSCM independently. To permit precise registration of LSCM and AFM images, a set of perpendicular lines was scratched into these films prior to imaging to mark a unique location, and the films were characterized by AFM and LSCM at one of the corners formed by these scratches. Figures 2A and 2B show LSCM and AFM images of a (LPEI/SPS_{FL})₁₀(1/DNA)₈ film imaged using this approach. Inspection of these images reveals that the fluorescent regions in the LSCM image have approximately the same sizes and densities as the nanoparticulate topographical features that appear in the AFM images. Figure 2C presents an overlaid image in which this LSCM image is superimposed directly onto the AFM image, and demonstrates further that the punctate fluorescent regions in Figure 2A do correspond to the locations of the particulate features observed in the AFM image in Figure 2B. These results, when combined, suggest that (i) SPS accumulates in these particulate regions during the morphological transformation of the film, (ii) that the regions between these features are relatively free of SPS, and (iii) that, in general, LSCM can be used to characterize the morphological changes that occur when these multilayered films are incubated in aqueous media.

We note here that the cross-sectional thickness used to acquire the LSCM images in the experiments described above was 820 nm. This value is much larger than the thickness of the films themselves (e.g., 100 to 200 nm) or any of the features that develop during the transformation to a particulate morphology. It is therefore unlikely that the non-uniform distribution of fluorescence in Figure 2A results from imaging only a portion of the film in the z direction (e.g., by “slicing through” the topmost portions of the particulate structures, while excluding portions of the film between particles). Additional control experiments performed by adjusting the plane of focus and the section thickness eliminate further the likelihood that the image in Figure 2A could arise from artifacts resulting from the optical settings used to acquire these images (data not shown).

We next conducted a series of experiments to characterize the locations of the other three polyelectrolyte components of these films (i.e., LPEI, polymer **1**, and DNA; see Materials and Methods for details of synthesis of labeled polyelectrolytes and the preparation of solutions used for film fabrication) using the methods described above. Three films having the structures $(\text{LPEI}_{\text{FL}}/\text{SPS})_{10}(\mathbf{1}/\text{DNA})_8$, $(\text{LPEI}/\text{SPS})_{10}(\mathbf{1}_{\text{FL}}/\text{DNA})_8$, and $(\text{LPEI}/\text{SPS})_{10}(\mathbf{1}/\text{DNA}_{\text{FL}})_8$ were prepared, incubated in PBS for six hours, and imaged by AFM and LSCM using the methods described above. The results of these experiments are summarized in Figure 3. Figures 3A, 3B, and 3C show images of a film containing LPEI_{FL} acquired by AFM and LSCM. The general features of these images are similar to those shown in the corresponding images of the film containing SPS_{FL} in Figure 2. In Figure 3A, fluorescence is, in large part, confined to punctate structures. Figure 3C, which shows an overlay of this LSCM image superimposed on the AFM image in Figure 3B, reveals that the locations of these fluorescent structures also correspond to the locations of the particulate structures shown in the AFM image. These results, when combined with those described above in Figure 2, suggest that LPEI and SPS – which were deposited in alternating fashion to form the bottommost layers of the film, and, as a result, are likely intimately intermixed – are both involved in this transformation and accumulate in the particulate structures that are formed. We note that differences in the shapes and sizes of the particulate structures in the AFM images in Figures 3B, 3E, and 3H reflect normal experiment-to-experiment variations that appear to arise from small differences in film thickness, both from sample to sample and within a single film (e.g., near the edge of the substrate vs. near the center) (Fredin et al., 2007).

We next sought to determine the locations of the polyelectrolytes located in the topmost layers of the film (i.e., the locations of polymer **1** and DNA, deposited in the final eight bilayers of the film during layer-by-layer fabrication) before and after incubation in PBS. LSCM and AFM images of a film fabricated using DNA_{FL} are shown in Figures 3D, 3E, and 3F. The high levels of fluorescence in the image shown in Figure 3D indicate that significant quantities of DNA remain in the film after incubation for six hours. These results confirm that DNA is not released or expelled rapidly from these films over the relatively short time scales over which this initial transformation occurs, and are consistent with the results of our past studies demonstrating that DNA is released gradually over a period of approximately 48 hours as these films erode (Zhang et al., 2004). Further inspection of the fluorescence micrograph in Figure 3D, however reveals significant differences between this image and the images shown in Figures 2A or 3A for films fabricated using LPEI_{FL} and SPS_{FL} . Whereas all three of these images show punctate areas of bright fluorescence, the image in Figure 3D also shows significant levels of fluorescence in the areas of the film that are located between the brighter punctate structures. Registration of this fluorescence micrograph with the topographical features of an AFM image of the same area (see Figure 3F) reveals that the brighter punctate structures in Figure 3D correspond to the particulate structures in Figure 3E. We interpret these results, when combined, to suggest that some accumulation of DNA in the particulate structures occurs as a result of the transformation from a smooth film to a nanoparticulate morphology, but that the distribution of DNA throughout the film is less disrupted by this process than the distributions of LPEI or SPS. This observation of regions of film present in areas between the particulate features is consistent with the results of our past studies on the characterization of scratched films by SEM, which demonstrated that the particulate structures are fused to or embedded in a thin residual film that is present in areas of the substrate between the particles (Fredin et al., 2005). We return to these observations again in the discussion below.

LSCM and AFM images of films fabricated using polymer $\mathbf{1}_{\text{FL}}$ are shown in Figures 3G, 3H, and 3I. Inspection of the LSCM image in Figure 3G reveals punctate regions of brighter fluorescence similar to the images in Figures 2A, 3A, and 3D, corresponding to films fabricated using SPS_{FL} , LPEI_{FL} , and DNA_{FL} . The superimposed LSCM and AFM images shown in Figure 3I confirm again that the bright regions in the LSCM image correspond to the locations

of the particles from the AFM image. Closer inspection of areas between the bright regions of fluorescence in Figure 3G, however, also reveals the presence of low levels of fluorescence that were not observed in the corresponding images for SPS_{FL} or LPEI_{FL} (Figures 2A and 3A), suggesting that polymer **1** may also be present both in the particulate structures and in regions of the film between these features. We considered the possibility that what appears to be fluorescence in the areas between the particles could be an artifact arising from the settings used to acquire these images. The results of additional control experiments, however, are not consistent with this possibility. We used LSCM to acquire an image of the boundary between the film fabricated using polymer **1**_{FL} and the portion of the substrate in which the film was scratched away. Inspection of this image, shown in Figure 4, reveals that the intensity of fluorescence in areas between bright punctate areas is greater than the intensity in the scratched portion of the surface. If these low levels of fluorescence were artifacts arising from the acquisition or processing of the images, the scratched area should appear equally bright. We interpret these results to suggest that polymer **1** is present both in the particulate structures and in areas of the film between them, albeit to different extents.

The results described above, when combined, can be interpreted in ways that provide insight into the processes by which the morphologies of these films change upon incubation in aqueous buffer. One possibility is a process in which the bottommost layers of the film, composed of LPEI and SPS, reorganize to form particulate features while the topmost layers of the film, composed of polymer **1** and DNA, remain more intact. Such a process leads to a physical picture of a nanoparticulate morphology consisting of a less disturbed portion of film composed of polymer **1** and DNA juxtaposed over an array of particulate structures composed predominantly of LPEI and SPS. The results of additional physical characterization experiments that provide support for this interpretation are discussed in the following section. We note here, however, that this interpretation rests on the conclusion (as discussed above) that polymer **1** is located both in areas where particles are located and in regions of the film between the particles. An alternative process, in which LPEI, SPS, and polymer **1** (but not DNA) accumulate predominantly in the particulate structures, seems less likely on the basis of the charge compensation requirements of these materials, and would likely lead to the release of DNA from these materials at rates that are significantly faster than those that are observed.

Characterization of Partially Eroded Films Using SEM and EDX

Figure 5A shows an SEM image of a film having the structure $(\text{LPEI}/\text{SPS})_{10}(\text{1}/\text{DNA})_8$ after incubation in PBS for 1.5 hours. This image shows an array of nanometer-scale particulate structures on the surface of the film that is similar to that shown in the AFM image in Figure 1B and the results of SEM characterization of partially eroded films in our past studies (Fredin et al., 2005; 2007; Jewell et al., 2006). Figures 5B–D show several additional SEM images of regions of partially eroded films that were scratched prior to imaging. These images show regions of the scratched edges of these films that are lifted up and/or inverted and, thus, permit imaging of the undersides of these films. Figure 5B shows an image of a scratched area of the same film shown in Figure 5A. Inspection of the image in Figure 5B reveals what appear to be raised particulate structures under the film that appear at the same size and density as features observed from a top-down perspective of the film surface (Figure 5A). Figures 5C and 5D show images of $(\text{LPEI}/\text{SPS})_{10}(\text{1}/\text{DNA})_8$ and $(\text{LPEI}/\text{SPS})_{10}(\text{1}/\text{DNA})_{20}$ films, respectively, after incubation in PBS for five hours. These images also show portions of scratched edges where the films were lifted up, and reveal the presence of particulate structures on the surface of the silicon substrate. These images are consistent with the physical picture presented above, in which particulate features are formed in the bottommost layers of the film near the silicon substrate, with a film covering both the particles themselves and the areas of the silicon substrate located between the particulate features. We note in this context that although these films were deposited layer-by-layer in a sequence of two distinct polyelectrolyte pairs (i.e., ten

bilayers of LPEI and SPS followed by eight bilayers of polymer **1** and DNA), several past studies have demonstrated that many PEM systems exhibit degrees of interpenetration between polymer chains deposited in adjacent or nearby layers (Decher, 1997; Losche et al., 1998). As a result of this interpenetration, a polyelectrolyte deposited in a given film layer may also be in contact with chains deposited during previous and subsequent steps of the layer-by-layer fabrication process. It is therefore likely that the fabrication of the films used in this present study results in areas of the film along the *z*-axis of the film (i.e., normal to the substrate surface) in which all four components are present and intimately mixed. Despite the possibility of such intermixing, however, it seems likely that the portion of the film closest to the silicon substrate could consist primarily of, or be enriched in, LPEI and SPS (with little or no polymer **1** or DNA present) and that the topmost portion of the film would be composed primarily of polymer **1** and DNA (and be relatively free of LPEI and SPS).

Characterization of these films by SEM also provides opportunities to characterize the elemental compositions of nanometer- or micrometer-scale regions of the films using energy-dispersive X-ray spectroscopy (EDX) (Garratt-Reed and Bell, 2003). We sought to determine whether EDX could be used to observe differences in composition between the particulate structures and the residual film between the particles in partially eroded films. We note here that, in the films investigated in this report, only two elements can be unambiguously associated with a unique polyelectrolyte species: sulfur, which is found in SPS, and phosphorus, which is found in DNA (that is, the elements C, H, N, and O are found in two or more of the polyelectrolytes used to fabricate these films and, thus, cannot be used as unique identifiers). We conducted experiments using EDX to collect X-ray spectra in several locations of a (LPEI/SPS)₁₀(1/DNA)₈ film after incubation for six hours by focusing the electron beam for each spectrum either on the centers of particles or on regions between the particles.

Elemental compositions (excluding contributions from the silicon substrate and the gold layer deposited to permit imaging by SEM) were calculated from X-ray spectra and averaged over 15 particles and 15 areas between particles. Figure 6A shows the mean atom percents of phosphorus and sulfur for areas of the film centered in the particles and for areas centered in the spaces between particles. Inspection of these data reveals that sulfur composes a significantly greater fraction of material in the particulate structures than it does in the areas between particles. The atomic percent of phosphorus, on the other hand, is higher in areas between particles than within the particles. Figure 6B shows the ratios of the atom percentages (all calculated excluding contributions from silicon) of phosphorus and sulfur to the atom percent of gold, which is assumed to cover the surface of the polyelectrolyte film evenly (i.e., the absolute amounts of gold on top of particulate structures and on areas of the film between these structures should be equal). While in Figure 6A the data are presented as molar fractions of the material present in the respective locations, the data in Figure 6B are a normalized measure of the absolute molar quantity of each element in the particles and in the areas between particles. The two representations of the data shown in Figures 6A–B yield information that is similar, but they permit different interpretations and comparisons due to differences between the heights of the particulate structures and the thickness of the film in areas between particles (i.e., the electron beam passes through more polyelectrolyte material when focused on a particle than when focused on an area between the particulate structures). Inspection of Figure 6B reveals that sulfur is present in larger quantities in the particles than in areas between the particles and that the amount of phosphorus is approximately the same where particles are located as in areas between particles.

We note that EDX is often used under conditions that result in the penetration of the electron beam several micrometers into the material being characterized, yielding composition from a volume of interaction of cubic micrometers (Garratt-Reed and Bell, 2003). This depth is much greater than the thickness of the films investigated here (e.g., ~150 nm). To minimize the

detection of X-rays arising from interactions between the primary electron beam and the silicon substrate in our samples, we used a relatively low accelerating voltage of 3.5 kV (see Materials and Methods section for additional details of acquisition of EDX spectra). However, decreasing the accelerating voltage also decreased the overall signal, and this resulted in spectra with greater background noise and less well-defined peaks. For these reasons, quantitative information extracted from the results of these experiments should be viewed with requisite caution. In the context of this current study, however, these results do provide additional support for the conclusions arising from our experiments using LSCM and AFM. Specifically, the results shown in Figures 6A–B suggest that (i) SPS is more abundant in the particulate features and comprises a greater fraction of the material found therein than in areas between particles, and that (ii) the amount of DNA is roughly constant over the surface of the film (but that it forms a higher fraction of the film between the particles than it does in the particulate structures). These results provide support for the view that the transformation from a smooth morphology to a particulate topography involves the formation of particles arising from the accumulation of LPEI and SPS deposited in the bottommost part of the films, and that the topmost portion of the film (consisting of polymer **1** and DNA) remains at least partially intact and covers these particulate structures.

Summary and Conclusions

We have used fluorescence-based information provided by LSCM in combination with topographic information acquired by AFM to characterize nanoscale morphological transformations that occur when ultrathin polyelectrolyte multilayers fabricated from plasmid DNA are incubated in physiologically relevant media. We demonstrate that registration of AFM and LSCM images acquired from the same locations of films fabricated using fluorescently labeled polyelectrolytes allows the spatial distribution of each individual polyelectrolyte species in these materials to be determined relative to the locations of specific topographic features that are formed during the transformation from a smooth to particulate morphology. The combination of these two different microscopy techniques thus provides insight into the structures and dynamics of these assemblies that cannot be achieved using either of these characterization techniques independently.

Characterization of films incubated in PBS revealed LPEI and SPS to be located predominantly in the particulate structures that form, and that DNA and polymer **1** were distributed more uniformly throughout the x-y plane of the film (that is, both in areas where particles are located and in areas between these particles). These results suggest a transformation from multilayered films that are relatively smooth to a nanoparticulate morphology consisting of a relatively less disturbed portion of film composed of polymer **1** and DNA juxtaposed over an array of particulate structures composed predominantly of LPEI and SPS. This physical picture is supported further by the results of additional experiments to characterize scratched films using SEM and EDX. The work presented here provides additional insight into the dynamic processes that occur when these complex, multicomponent nanomaterials are incubated in aqueous media, and could prove useful for the design of new materials for the surface-mediated delivery of DNA.

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Figure 1.

(A,B) Representative $10\ \mu\text{m} \times 10\ \mu\text{m}$ AFM images of $(\text{LPEI/SPS})_{10}(\mathbf{1}/\text{DNA})_8$ films (A) prior to and (B) following incubation at $37\ ^\circ\text{C}$ in PBS for 5 hours (range in z direction = 150 nm). (C,D) Representative $10\ \mu\text{m} \times 10\ \mu\text{m}$ LSCM micrographs of $(\text{LPEI/SPS}_{\text{FL}})_{10}(\mathbf{1}/\text{DNA})_8$ films (C) prior to and (D) following incubation in PBS at $37\ ^\circ\text{C}$ for 6 hours. See Materials and Methods section for additional details of film erosion experiments and the acquisition of images by AFM and LSCM.

Figure 2.

12 $\mu\text{m} \times 12 \mu\text{m}$ images acquired using (A) LSCM and (B) AFM over approximately the same area of a $(\text{LPEI}/\text{SPS}_{\text{FL}})_{10}(\text{1/DNA})_8$ film after incubation at 37 °C in PBS for 6 hours. (C) An overlaid composite of the images in A and B; the AFM image in (B) was converted to grayscale and inverted, and the LSCM image in (A) was rendered semi-transparent and superimposed on the AFM image to enable registration of the features in the two images. See Materials and Methods section for additional details of film erosion experiments and the acquisition of images by AFM and LSCM.

Figure 3.

12 $\mu\text{m} \times 12 \mu\text{m}$ images acquired using LSCM (column I) and AFM (column II) of films having the structure $(\text{LPEI}/\text{SPS})_{10}(\mathbf{1}/\text{DNA})_8$, fabricated using LPEI_{FL} (row 1), DNA_{FL} (row 2), and polymer $\mathbf{1}_{\text{FL}}$ (row 3), after incubation at 37 °C in PBS for 6 hours. Figures in column III were processed in a manner analogous to Figure 2C (see caption of Figure 2 for additional details). See Materials and Methods section for additional details of film erosion experiments and the acquisition of images by AFM and LSCM.

Figure 4. LSCM image of a $(\text{LPEI/SPS})_{10}(\text{1}_{\text{FL}}/\text{DNA})_8$ film after incubation at 37 °C in PBS for 6 hours, showing the boundary between the intact film and a location where the film was scratched away (scale bar = 1 μm). The dotted line is a guide to indicate the approximate location of the edge of the scratch. See Materials and Methods section for additional details of film erosion experiments and the acquisition of images by LSCM.

Figure 5.

SEM images of films having the structure $(\text{LPEI/SPS})_{10}(\text{1/DNA})_n$ after incubation in PBS at 37 °C for (A,B) 1.5 and (C,D) 5 hours (scale bars = 1 μm). For (A–C), $n = 8$; for (D), $n = 20$. Images in (B–D) show the boundaries between intact films and areas where the films were scratched away. See Materials and Methods section for additional details of film erosion experiments and the acquisition of images by SEM.

Figure 6.

Mean composition data of a (LPEI/SPS)₁₀(1/DNA)₈ film after incubation at 37 °C in PBS for 6 hours, acquired by EDX microanalysis of 15 particulate structures (gray bars) and 15 areas between particulate structures (white bars). (A) Atom percents of phosphorus (P) and sulfur (S) calculated by excluding contributions from the silicon substrate. (B) Normalized molar quantities of P and S calculated as the ratio of the atom percents of P and S to the atom percent of Au (see text). See Materials and Methods section for additional details of film erosion experiments and EDX microanalysis.