Published in final edited form as:

Biomacromolecules. 2012 May 14; 13(5): 1523–1532. doi:10.1021/bm300234q.

Reactive Polymer Multilayers Fabricated by Covalent Layer-by-Layer Assembly: 1,4-Conjugate Addition-Based Approaches to the Design of Functional Biointerfaces

Shane L. Bechler and David M. Lynn

Department of Chemical and Biological Engineering, 1415 Engineering Drive, University of Wisconsin-Madison, Madison, WI 53706

Abstract

We report on conjugate addition-based approaches to the covalent layer-by-layer assembly of thin films and the post-fabrication functionalization of biointerfaces. Our approach is based on a recently reported approach to the ‘reactive’ assembly of covalently-crosslinked polymer multilayers driven by the 1,4-conjugate addition of amine functionality in poly(ethyleneimine) (PEI) to the acrylate groups in a small-molecule pentacrylate species (5-Ac). This process results in films containing degradable β -amino ester crosslinks and residual acrylate and amine functionality that can be used as reactive handles for the subsequent immobilization of new functionality. Layer-by-layer growth of films fabricated on silicon substrates occurred in a supra-linear manner to yield films ~750 nm thick after the deposition of 80 PEI/5-Ac layers. Characterization by AFM suggested a mechanism of growth that involves the reactive deposition of nanometer-scale aggregates of PEI and 5-Ac during assembly. IR spectroscopy studies revealed covalent assembly to occur by 1,4-conjugate addition without formation of amide functionality. Additional experiments demonstrated that acrylate-containing films could be post-functionalized via conjugate addition reactions with small-molecule amines that influence important bio-interfacial properties, including water contact angles and the ability of film-coated surfaces to prevent or promote the attachment of cells *in vitro*. For example, whereas conjugation of the hydrophobic molecule decylamine resulted in films that supported cell adhesion and growth, films treated with the carbohydrate-based motif D-glucamine resisted cell attachment and growth almost completely for up to 7 days in serum-containing media. We demonstrate that this conjugate addition-based approach also provides a means of immobilizing functionality through labile ester linkages that can be used to promote the long-term, surface-mediated release of conjugated species and promote gradual changes in interfacial properties upon incubation in physiological media (e.g., over a period of at least one month). These covalently-crosslinked films are relatively stable in biological media for prolonged periods, but they begin to physically disintegrate after ~30 days, suggesting opportunities to use this covalent layer-by-layer approach to design functional biointerfaces that ultimately erode or degrade to facilitate elimination.

Introduction

Methods for the layer-by-layer fabrication of polymer thin films provide practical and versatile approaches to the design of functional surfaces and interfaces. Many approaches to layer-by-layer assembly take advantage of ionic interactions between oppositely charged polymers (polyelectrolytes) to drive the bottom-up assembly of ‘polyelectrolyte multilayer’

Supporting Information Available. Additional AFM images, fluorescence microscopy images, and plots of film thickness versus incubation time in PBS used to characterize film morphology, cell adhesion, and film stability in aqueous media. This information is available free of charge via the Internet at <http://pubs.acs.org>.

films (PEMs).^{1–4} Practical advantages of these approaches include the ability to exert precise, nanometer-scale control over film thickness and composition and the ability to fabricate uniform, conformal coatings on the surfaces of topographically and topologically complex objects (e.g., curved and porous surfaces, etc.). One potential drawback of these methods, however, is that the structures and stabilities of PEMs can be compromised upon changes in environmental conditions (e.g., pH or ionic strength) that can disrupt the ionic crosslinks or other interactions that stitch these materials together (often resulting in film degradation or other changes in film structure or function).^{5–14} Approaches to the post-fabrication introduction of covalent crosslinks to PEMs (e.g., by thermal treatment,^{6,15–18} exposure to UV radiation,^{16,19–21} or by treatment with reactive crosslinking agents^{22–27}) have been developed to improve the stability of PEMs and/or tune a variety of other important film properties (e.g., stiffness, compliance, etc.).

As an alternative to post-fabrication crosslinking, several groups have reported methods for the direct ‘covalent’ or ‘reactive’ layer-by-layer assembly of crosslinked multilayers.^{28,29} These methods typically exploit covalent bond-forming reactions between mutually reactive polymers (as opposed to the formation of ionic contacts between oppositely charged materials) and, thus, lead to the introduction of covalent crosslinks into multilayer structures *during* layer-by-layer assembly. The most basic requirement for these reactive processes is the availability of two molecular ‘building blocks’ with functionality that can react with each other to form a covalent bond. Caruso and others have reported extensively on the use of “click” chemistry for the layer-by-layer assembly of polymeric multilayers containing triazole-based crosslinks.^{30–39} Our group has exploited rapid reactions between azlactones and primary amines to promote reactive layer-by-layer assembly,^{40–45} and a wide range of other types of covalent bond-forming reactions have been used for this purpose.^{46–58} In addition to increased film stability, one additional practical advantage of these reactive approaches is they also provide films containing residual reactive groups that can be used as platforms for the further functionalization of film-coated surfaces (e.g., by post-fabrication treatment with compounds that can react with these remaining reactive groups). Although it is also possible to functionalize the surfaces of conventional (e.g., ionically-crosslinked) PEMs, the combination of increased film stability and chemical reactivity afforded by covalent assembly can facilitate post-fabrication modification and, in general, provides new opportunities for the design of functional surfaces and interfaces.

The work reported here was motivated by recent reports on the covalent layer-by-layer assembly of thin films using polyamines (e.g., branched polyethyleneimine, PEI) and small molecules containing multiple acrylate groups (e.g., 5-Ac, which can react with the primary amines present in PEI by conjugate addition reactions; see Scheme 1).^{53,55,56,58} These reports demonstrated that conjugate addition chemistry can be used to fabricate PEI/5-Ac multilayers on a variety of surfaces, including planar and particulate objects, and that acrylate and amine functionality in the films could be used to introduce additional functionality post-fabrication (e.g., by treatment of acrylate-functionalized films with primary amine-based nucleophiles, etc.).^{55,56,58} Under certain conditions, and on certain substrates, film growth was reported to proceed in a ‘dendritic’ manner that lead to the generation of nanostructured surfaces and enabled the ‘amplification’ of surface functionality (e.g., the design of hydroxyl-rich surfaces)^{53,56,58} of interest in the contexts of sensing and other applications. Pishko and coworkers have also reported on the assembly of multilayers using polyamines and acrylate end-functionalized poly(ethylene glycol).^{47,54} In general, however, conjugate addition-based approaches have not been explored as widely as several other reaction platforms used to promote covalent layer-by-layer assembly.

The conjugate addition-based process described above is similar in many ways to conjugate addition-based processes used to synthesize poly(β -amino ester)s,^{59,60} a class of

hydrolytically degradable polyamines investigated widely as biodegradable and biocompatible materials for drug delivery and other applications.^{59–62} It occurred to us that, in addition to imparting initial structural stability to these films, the presence of the degradable β -amino ester crosslinks inherent in PEI/5-Ac multilayers could also open the door to new biomedical and biotechnological applications of reactive layer-by-layer assembly (e.g., for the design of crosslinked/reactive multilayers that erode in aqueous media, or to provide platforms that permit reversible immobilization of active agents, etc.). There are currently few reports on covalent layer-by-layer assembly focused specifically on the design of films containing degradable crosslinks,^{34,35} and, in general, many questions remain regarding the physical and chemical structures of these materials, mechanisms of film growth, and the extent to which crosslinking and post-fabrication processing can be used to tailor film properties. This study sought to characterize physicochemical properties and behaviors of PEI/5-Ac multilayers relevant to the design of biointerfaces and the potential application of these materials in biomedical contexts.

This study is presented in three parts. In the first part, we characterize the growth, structures, and physicochemical properties of multilayers of PEI and 5-Ac fabricated on planar glass and silicon substrates. In the second part, we demonstrate that both residual amine and residual acrylate functionality remaining in these films can be used to functionalize film-coated surfaces using amine- and acrylate-reactive molecules, including functionality that can be used to tailor film wetting properties and prevent or promote the adhesion of mammalian cells *in vitro*. Finally, we characterize the stabilities of these films in physiologically relevant media and demonstrate that the conjugate addition-based reactions used to assemble them also enable the immobilization of amine-functionalized molecules through covalent, hydrolytically degradable ester bonds. Our results demonstrate that this conjugation-based approach to post-fabrication functionalization can be used to design surfaces that release covalently attached small molecule agents from film-coated surfaces for extended time periods or undergo other dynamic changes in their physical properties upon incubation in physiologically relevant environments.

Materials and Methods

Materials

Branched poly(ethylene imine) (PEI; Mn = 10,000; Mw = 25,000), reagent grade DMSO, decylamine, dansyl cadaverine, and dansyl amide were purchased from Aldrich Chemical Co. (Milwaukee, WI). N,N-Diethylethylenediamine was purchased from Acros Organic (Geel, Belgium). D-Glucamine was purchased from TCI America (Portland, OR). Dipentaerythritol pentaacrylate (5-Ac, SR399) was obtained from Sartomer (Exton, PA). Test-grade n-type silicon wafers were obtained from Silicon Inc. (Boise, ID). Glass microscope slides were purchased from Fisher Scientific (Pittsburgh, PA). Dulbecco's modified Eagle medium (DMEM), Calcein AM fluorescent cell stain, phosphate-buffered saline (PBS) used for cell based experiments, tetramethylrhodamine, and tetramethylrhodamine isothiocyanate (TRITC) were purchased from Invitrogen (Carlsbad, CA). PBS used for film stability experiments was prepared by dilution of commercially available concentrate (EM Science, Gibbstown, NJ). African green monkey kidney fibroblasts (COS-7 cells) were obtained from ATCC (Manassas, VA). All materials were used as received without further purification unless noted otherwise. Compressed air used to dry films and coated substrates was filtered through a 0.2 μ m syringe filter.

General Considerations

All silicon and glass substrates were washed with acetone, ethanol, methanol, and deionized water and dried using filtered compressed air prior to film fabrication. The optical

thicknesses of films were determined using a Gaertner LSE Ellipsometer (632.8 nm, incident angle = 70°). Data were processed using the Gaertner Ellipsometer Measurement Program. Relative thicknesses were calculated assuming an average index of refraction of 1.577 for the multilayer films. Atomic force microscopy (AFM) images were collected in tapping mode using a Nanoscope Multimode atomic force microscope (Veeco/Digital Instruments, Santa Barbara, CA), using scan rates of 7.5 – 10 $\mu\text{m/s}$ to obtain 256×256 pixel images. Silicon cantilevers with a spring constant of 40 N/m were used (model NSC15/NoAl, MikroMasch, USA, Inc., Portland, OR). The heights and root mean square roughnesses of features were calculated from image sections using the Nanoscope software (Veeco/Digital Instruments, Santa Barbara, CA). For experiments to evaluate film thickness using AFM, a portion of the film was removed by scratching with a razor blade prior to imaging. Fluorescence microscopy images were collected using an Olympus IX70 microscope and were analyzed using the Metavue version 7.1.2.0 software package (Molecular Devices; Toronto, Canada). For polarization modulation infrared reflectance-absorbance spectroscopy (PM-IRRAS) experiments, 10 nm and 200 nm layers of titanium and gold, respectively, were sequentially deposited onto clean silicon wafers using an electron-beam evaporator (Tek-Vac Industries, Brentwood, NY). PM-IRRAS was conducted in analogy to previously reported methods.⁴⁰ Briefly, gold coated substrates coated with covalently assembled films were placed at an incident angle of 83° in a Nicolet Magna-IR 860 Fourier transform infrared spectrophotometer equipped with a photoelastic modulator (PEM-90, Hinds Instruments, Hillsboro, OR), a synchronous sampling demodulator (SSD-100, GWC Technologies, Madison, WI), and a liquid-nitrogen-cooled mercury cadmium-telluride detector. The modulation was set at 1500 cm^{-1} and 200 scans were collected for each sample at a resolution of 4 cm^{-1} . The differential reflectance infrared spectra were then normalized and converted to absorbance spectra using a previously reported procedure.⁶³ Static water contact angle measurements were made using a Dataphysics OCA 15 Plus instrument. The volume of water used for all water contact angle measurements was 3 μL . Solution fluorescence measurements were made using a Jobin Yvon FluoroMax-3 fluorometer. For characterization of dansyl-based fluorophores, an excitation of 335 nm was used, and the emission was reported as an average of the values collected over the wavelengths from 540 to 550 nm. For characterization of tetramethylrhodamine-based fluorophores, an excitation of 543 nm was used, and the emission was reported as an average of the values collected over the wavelengths from 570 to 580 nm. All fluorescence microscopy images were processed using ImageJ software (NIH).

Fabrication of Polymer Multilayers

Films were fabricated on planar silicon substrates (for characterization of film growth and stability) or on glass substrates (for characterization of cell adhesion and proliferation). 5-Ac solutions used for film fabrication were prepared by dissolving 1.32 g of 5-Ac in 10 mL of ethanol, and PEI solutions were prepared by dissolving 500 mg of PEI in 10 mL of ethanol. Fabrication of films was conducted using a stepwise layer-by-layer procedure according to the following protocol: 1) substrates were immersed in a solution of PEI for 10 seconds, 2) substrates were removed and placed in an initial ethanol rinse bath for 10 seconds followed by a second ethanol bath for an additional 10 seconds, 3) substrates were immersed in a solution of 5-Ac for 10 seconds, and 4) substrates were rinsed as described in step 2. This cycle was repeated until the desired number of PEI and 5-Ac layers (typically 80) were deposited onto the surface. PEI, 5-Ac, and ethanol rinse baths were not replaced with fresh solutions during fabrication unless otherwise noted (see text). Once fabrication was complete, films were rinsed with copious amounts of ethanol and dried under a stream of filtered, compressed air. Films were then either characterized and used in subsequent experiments immediately or stored in a vacuum dessicator until use.

Post-Fabrication Functionalization of Polymer Multilayers

Experiments designed to investigate the reactivity of residual acrylate and amine groups present in the polymer multilayers were conducted by fabricating films on gold-coated substrates (for films to be characterized by PM-IRRAS) or on glass substrates (for films to be characterized using fluorescence microscopy). Films to be functionalized with compounds containing amine functionality were first pre-treated by immersion in a 5-Ac solution (equivalent in concentration to the 5-Ac solution used for fabrication, see above) for ~24 hours in the dark at room temperature, followed by rinsing and incubation in ethanol ~24 hours. Films to be functionalized with compounds containing other functionality were treated with 5-Ac and then subsequently treated in a similar manner using a solution of PEI. 5-Ac-terminated films were then incubated in a solution of dansyl cadaverine (1 mg/mL) in DMSO for ~24 hours. PEI-terminated films were incubated in tetramethylrhodamine isothiocyanate (TRITC; 0.5 mg/mL) in DMSO. Solutions of dansyl amide and tetramethylrhodamine (1 mg/mL and 0.5 mg/mL in DMSO, respectively) were used as non-reactive compounds in control experiments. Following functionalization, films were rinsed with copious amounts of acetone, dried under filtered, compressed air, and then soaked in a DMSO rinse for ~24 hours. Experiments to functionalize films with other functionality (e.g., decylamine or D-glucamine) were performed in a similar manner using 50 mM solutions of these compounds in DMSO. Films were subsequently rinsed with acetone, dried under filtered, compressed air, and stored in a vacuum dessicator until used.

Characterization of Release of Covalently Immobilized Functionality and Changes in Contact Angles

For experiments designed to characterize the time-dependent release of covalently immobilized functionality, films 80 bilayers thick fabricated on glass substrates were terminated with either 5-Ac or PEI as described above. Films were then treated with dansyl cadaverine or dansyl amide (for 5-Ac terminated films) or TRITC or tetramethylrhodamine (for PEI terminated films). Film-coated substrates were then placed in a glass vial containing 3 mL PBS (pH 7.4) and incubated in a dark incubator at 37 °C. At predetermined time intervals, substrates were removed, rinsed with water, and dried using compressed air. One milliliter of the incubation solution was transferred to a UV/vis cuvette and the solution fluorescence of dansyl- and tetramethylrhodamine-based fluorophores was measured using fluorometry (see above for excitation and emission wavelengths). Substrates were then transferred to a glass vial containing 3 mL of fresh PBS and returned to the incubator. For experiments designed to evaluate time-dependent changes in surface hydrophobicity, films 80 bilayers thick terminated with 5-Ac or PEI or functionalized with decylamine or glucamine were prepared in triplicate as described above. These films were then immersed in a solution of PBS and stored in a dark incubator at 37 °C. At predetermined times, substrates were removed, rinsed with water, and dried using compressed air. Static water contact angle measurements were then made for a minimum of three 3 μ L drops on each substrate, and substrates were then returned to the incubator. PBS solutions were periodically refreshed during these experiments.

Characterization of Cell Adhesion on Film-Coated Surfaces

For experiments designed to investigate cell adhesion and proliferation on modified and unmodified PEI/5-Ac films, films 80 bilayers thick were fabricated on glass substrates and terminated with either PEI, 5-Ac, decylamine, or glucamine as described above. Prior to cell seeding, a razor blade was used to scrape films from the backside of the coated glass substrates (to yield substrates coated on only one side) to facilitate imaging. Film-coated substrates were placed individually into the wells of tissue culture treated polystyrene culture plates. COS-7 cells in DMEM (supplemented with 10% (v/v) fetal bovine serum, 100 units/mL penicillin, and 100 μ g/mL streptomycin) were then seeded onto films by

carefully adding a small volume of cell-containing media such that the entire added volume was maintained on the film-coated substrate (i.e., to prevent the cell suspension from flowing off of the substrate and spilling onto the surrounding surface of the culture wells). Prior to seeding cells, the concentrations of cells in the culture media was adjusted so that each substrate was exposed to suspensions of ~60,000 cells during the initial seeding process. Cells were allowed to attach for 30 minutes at room temperature and then 2 mL of cell culture media was added to the wells. At pre-determined times, cells were stained using 2 mL of Calcein AM staining solution (1 μ L/mL PBS). After incubation for 30 minutes at 37 °C, the staining solution was aspirated, substrates were transferred to a new culture plate well, and then covered with fresh DMEM (2 mL). Cells were then imaged by phase contrast and fluorescence microscopy without removing the glass substrates from the wells.

Results and Discussion

Characterization of Film Growth and Morphology

We selected branched PEI and the pentacrylate-functionalized small molecule 5-Ac (Scheme 1A) as model reactive building blocks for use in this study on the basis of a past report demonstrating that this polymer/small molecule combination can be used to fabricate covalent multilayers.⁵⁵ That study demonstrated that films fabricated on surfaces coated with a commonly used photoresist compound grew in a ‘dendritic’ manner that lead to highly nanostructured surfaces. All films investigated in our current study were fabricated layer-by-layer on the surfaces of bare silicon and glass substrates to (i) minimize the influence of the substrate on film growth and to (ii) facilitate both physicochemical characterization and evaluation of short-term biocompatibility of film-coated substrates in *in vitro* cell culture experiments. Films were fabricated using solutions of PEI and 5-Ac in ethanol to facilitate conjugate addition. We note here that while the conjugate addition of the primary amine groups of PEI to the acrylate functionality of 5-Ac should proceed to yield β -amino ester crosslinks within the films (i.e., through a generally preferred 1,4-conjugate addition process; see Scheme 1B), primary amine functionality in PEI could also potentially react via a less favored 1,2-addition process with acrylate functionality (to generate acrylamide functionality) or with β -amino ester functionality to yield more stable amide-functionalized interlayer crosslinks. We return to a discussion of these possibilities again in the sections below.

We performed an initial set of fabrication experiments to characterize the growth profiles of PEI/5-Ac films fabricated on the surfaces of reflective silicon substrates. Figure 1 shows a plot of increases in optical film thickness (as determined by ellipsometry) as a function of the number of PEI/5-Ac dipping cycles for films fabricated using two different sets of fabrication conditions. For the first set of conditions (Figure 1, closed circles), solutions of PEI and 5-Ac and ethanol rinse solutions were refreshed every 10 dipping cycles (see Materials and Methods for additional details of fabrication protocols). This protocol was adopted initially because we observed both polymer and rinse solutions to gradually become visually cloudy after approximately 10 consecutive dipping cycles. Inspection of these data reveals the resulting films to grow in a linear manner, albeit extremely slowly under these conditions (e.g., film thicknesses were only ~10 nm thick after 80 dipping cycles. Control experiments performed by eliminating either PEI or 5-Ac solutions during fabrication revealed no significant accumulation of material on these substrates under otherwise identical conditions (as determined by ellipsometry).

Figure 1 also shows the growth profile of films fabricated without the intermittent refreshing of PEI, 5-Ac, and rinse solutions (closed squares). Under these conditions, film thickness increased in a manner that was supra-linear (or ‘exponential’) after the first 10 dipping cycles, reaching thicknesses of ~120 nm after 50 dipping cycles. Films fabricated under

these conditions transitioned gradually from optically clear to opaque during this process. As a result, films fabricated using more than 50 dipping cycles could not be characterized reliably using ellipsometry (additional characterization of these thicker films is described below).

The onset of rapid film growth in these latter experiments occurred in parallel with a steady increase in the cloudiness of the 5-Ac solution (these solutions also progressed from clear and colorless to opaque/white over the course of the fabrication process). Characterization of these dipping solutions using dynamic light scattering (DLS) revealed the presence of aggregates ~350 nm in size after 15 dipping cycles (data not shown). Solutions of 5-Ac that were not used for dipping did not cloud under otherwise identical conditions. These light scattering experiments cannot be used to characterize the compositions of these aggregates, but the presence of these aggregates is consistent with the formation of solution-based complexes of PEI and 5-Ac that could result from the carry-over of PEI into solutions of 5-Ac during the iterative and repetitive cycling of substrates (e.g., as could result from incomplete removal of unreacted PEI from film-coated surfaces during ethanol rinse steps). Because solution cloudiness and rapid film growth were not observed when solutions were intermittently refreshed (e.g., Figure 1, closed circles), we hypothesized that the supra-linear growth exhibited using this second protocol could result, at least in part, from the iterative deposition and reactive accumulation of PEI/5-Ac aggregates during layer-by-layer assembly. Our results using refreshed dipping solutions (closed circles) suggest that this growth is less likely to result solely from the diffusion of individual film components during assembly, as has been demonstrated for the ‘exponential’ growth of PEM systems fabricated from certain weak polyelectrolytes.^{64,65}

Additional characterization of film growth and morphology by atomic force microscopy (AFM) provided additional support for this hypothesis. Figure 2 shows representative AFM images of PEI/5-Ac films after 15, 30, 50, and 80 dipping cycles. After 15 dipping cycles (Figure 2A), small aggregates ranging from 200 – 500 nm in size are observed on the surface of the substrate. After 30 dipping cycles (Figure 2B), we observed a higher number of aggregates as well as many larger aggregate clusters. These clusters of aggregates grew in size and almost completely covered the surface over an additional 20 dipping cycles (Figure 2C), and after 80 cycles surfaces were completely covered with rough, but continuous, polymer films (Figure 2D; additional AFM images showing larger areas of these film-coated substrates are included in Figure S1 of the Supporting Information). The root-mean-squared roughness of 80-bilayer films was ~275 nm and average physical film thickness, determined by measuring the profile of a scratch that was made prior to imaging, was ~750 nm. Measurements of scratched films obtained after 30 dipping cycles revealed the presence of a smooth and continuous film ~6 nm thick in the flatter regions between aggregate clusters (e.g., similar to the flatter regions seen in the image of the unscratched film shown in Figure 2B). This thickness is consistent with the thicknesses of films fabricated using intermittently refreshed solutions (e.g., see Figure 1 (closed circles) and additional AFM results discussed in Figure S1), and suggests that film growth may occur by both the reactive deposition of soluble PEI and 5-Ac and the reactive accumulation of larger aggregates. Additional evidence for a ‘reactive’ (covalent bond-forming) mechanism of film growth is discussed below. Although it should prove possible to optimize or tune film growth further by manipulation of solution conditions and dipping/reaction times, films fabricated using the protocols described above for these supra-linear films (Figure 1) were sufficient for all other experiments described below.

We used polarization modulation infrared reflectance-absorbance spectroscopy (PM-IRRAS) to characterize the structures and compositions of PEI/5-Ac films fabricated on gold-coated silicon substrates. Figure 3 shows a representative IR spectrum of a 30-bilayer

PEI/5-Ac film, and includes peaks at 1739 cm^{-1} and 1410 cm^{-1} that reveal the presence of unreacted acrylate functionality. These peaks correspond to an ester carbonyl stretch and symmetric deformation of the C-H bond for the β carbon of the vinyl group, respectively, and were identified by comparison to the IR spectrum of pure 5-Ac (not shown).⁶⁶ These IR data do not permit the direct and unambiguous identification of β -amino ester-type crosslinks, but the results shown in Figure 3 are generally consistent with a mechanism of covalent/reactive assembly that proceeds via the 1,4-conjugate addition of PEI to 5-Ac. The IR spectrum of this film does not feature peaks suggesting the presence of significant amounts of amide-based functionality (e.g., as could arise from the reaction of primary amine functionality with existing β -amino ester-type crosslinks).

Post-Fabrication Functionalization of Reactive PEI/5-Ac Films

We next performed experiments to determine the extent to which the amine and acrylate functionality in crosslinked PEI/5-Ac films could be used as reactive handles for the subsequent immobilization of new chemical functionality. Initial experiments were performed using two small-molecule fluorescent agents: dansyl cadaverine (an amine-functionalized agent that can react with acrylate functionality via 1,4-conjugate addition) and tetramethylrhodamine isothiocyanate (TRITC; an agent that can react with primary or secondary amine functionality) (Figure 4). For these experiments, we used 80-bilayer films fabricated on glass substrates pre-treated with an excess of 5-Ac (to increase the number of acrylate groups available on the surface and in the bulk of the films; referred to hereafter as 'acrylate-functionalized films'). Films to be functionalized via residual primary and secondary amines within films were subsequently treated with PEI (to increase the amount of amine functionality; referred to hereafter as 'PEI-functionalized films') prior to functionalization.

Figure 4A shows a fluorescence microscopy image of an acrylate-functionalized film treated with dansyl cadaverine. The inset of this image shows an otherwise identical acrylate-functionalized film treated with dansyl amide, a molecule that does not react readily with acrylate functionality. The presence of bright yellow fluorescence demonstrates that dansyl cadaverine can be immobilized on these films. The absence of fluorescence in the film treated with dansyl amide suggests that the fluorescence in panel A does not arise from the physical adsorption of fluorophore and is consistent with immobilization that occurs by the conjugate addition of dansyl cadaverine to free acrylate groups in the film. Figure 4B shows an image of a PEI-functionalized film treated with TRITC [the inset again shows an otherwise identical film treated with the non-reactive fluorescent analog tetramethylrhodamine (TMR)]. These results also demonstrate that the amine functionality present in these films can be exploited to covalently immobilize amine-reactive molecules.

Additional experiments demonstrated that acrylate-functionalized films could also be functionalized via conjugate addition reactions with functional small-molecule amines that influence important bio-interfacial properties of these materials, including water contact angles and the ability of film-coated surfaces to promote or prevent the attachment of cells. Surfaces coated with acrylate-functionalized films were moderately hydrophobic and exhibited contact angles of $\sim 95^\circ$, as shown in the image in Figure 4C. Treatment of these films with solutions of *n*-decylamine (a model hydrophobic amine) or D-glucamine (a model, amine-functionalized hydrophilic carbohydrate motif), however, resulted in substantial changes in contact angles. Decylamine-treated films (Figure 4D) were more hydrophobic and exhibited water contact angles of $\sim 138^\circ$. Films treated with glucamine were hydrophilic and resulted in instant and almost complete spreading of water droplets during contact angle measurements (Figure 4E).

We note again that while 1,4-conjugate addition is the favored pathway for the reaction of primary amines with acrylate functionality, the primary amines in decylamine and glucamine could also potentially react via 1,2-addition with existing β -amino ester functionality in these films to result in immobilization via amide linkages. Support for conjugation via 1,4-addition was provided by IR spectroscopy characterization of films treated with decylamine. Figure 5 shows normalized IR spectra of a 30-bilayer, acrylate-functionalized film prior to (dotted curve) and after (solid curve) treatment with decylamine. Inspection of these data reveals the peak at 1410 cm^{-1} , associated with the vinyl bond of the acrylate functionality as discussed above, to decrease significantly upon treatment with decylamine. The disappearance of this peak is consistent with immobilization of decylamine by 1,4-conjugate addition (and inspection of the carbonyl region of the spectrum for the treated film does not reveal the presence of significant amount of new amide functionality). Additional evidence in support of this view is provided in other sections below.

Characterization of Cell Adhesion and Growth on Functionalized Films

D-Glucamine has been demonstrated in past studies of self-assembled monolayers (SAMs) to confer anti-fouling properties when immobilized on gold-coated surfaces.^{67,68} Other recent studies have demonstrated that this anti-fouling behavior can be preserved when glucamine is covalently immobilized on reactive, azlactone-functionalized polymer multilayers.^{41,44} We performed a series of cell-based studies to (i) characterize the short-term biocompatibility of these surfaces *in vitro* and (ii) determine the extent to which the covalent, post-fabrication modification of PEI/5-Ac films could be used to control the attachment and growth of mammalian cells. For these initial studies, we used African green monkey kidney fibroblasts (COS-7 cells) as a model cell line.

Figure 6 shows fluorescence microscopy images of COS-7 cells seeded on the surfaces of glass microscope slides coated with 80-bilayer PEI/5-Ac films functionalized by treatment with either decylamine (A–C) or glucamine (D–F) as described above. Cells were seeded in serum-containing cell culture medium for 30 minutes (see Materials and Methods for additional details) and then allowed to grow in serum-containing medium for an additional seven days. Culture medium was exchanged periodically during the course of these experiments, and cells were stained periodically with the fluorescent cell-viability stain Calcein-AM (a small molecule that fluoresces green upon internalization by live cells) to provide a measure of cell viability and assist in identifying the locations and characterizing the morphologies of cells using fluorescence microscopy.

Inspection of Figure 6A–C reveals that decylamine-functionalized surfaces supported significant levels of cell adhesion and growth. One hour after seeding, cells attached to these surfaces appeared rounded and punctate but were viable, as indicated by the observation of bright green fluorescence in nearly every cell (Figure 6A; inspection of corresponding phase contrast images (not shown) revealed the absence of other non-viable cells on these surfaces). These cells adopted a more spread morphology and grew to nearly complete monolayers of cells after approximately two days (Figure 6B). After seven days, cells had completely covered the surfaces of these decylamine-functionalized substrates (Figure 6C; phase contrast microscopy images (not shown) suggested the presence of cell multilayers many cell layers thick at this time point). The films used in these studies did not visibly detach or delaminate during the course of these experiments. Although additional experiments will be required to characterize more completely the longer-term biocompatibility of these PEI/5-Ac films, the results of these initial experiments demonstrate that these decylamine-functionalized films support the attachment and growth of cells and do not appear to influence cell viability significantly over a period of at least one week.

In contrast to results on decylamine-functionalized surfaces, cells did not attach initially (Figure 6D) or subsequently grow (Figure 6E–F) significantly on films functionalized with glucamine. While some cells are observed to have attached in each of these images, the numbers of cells are significantly lower than those in Figure 6A–C, and the cells that did attach generally remained rounded and did not grow to cover film-coated surfaces for up to seven days. These results are consistent with the results of past studies demonstrating that surfaces presenting glucamine can prevent the attachment of mammalian cells (in part, by preventing the non-specific adsorption of serum proteins that can help promote subsequent cell attachment).^{41,44,67,68} Many differences exist between well-ordered and densely packed SAMs and the surfaces of the glucamine-treated multilayers investigated here (and, also, between these PEI/5-Ac films and azlactone-functionalized multilayers investigated in past studies). Our current results, however, demonstrate clearly that these PEI/5-Ac films can be functionalized with glucamine at levels that are sufficient to prevent the attachment and subsequent growth of mammalian cells in serum-containing media. Additional studies using surfaces coated with acrylate-functionalized or PEI-functionalized multilayers (i.e., not functionalized with glucamine) demonstrated that untreated films did support significant attachment and growth of cells for up to seven days (similar to decylamine-treated films; see Figure S2 of the Supporting Information). We conclude on the basis of these additional experiments that the cell resistant properties of glucamine-treated films arise from the presence of immobilized glucamine, and not from other background physicochemical properties of the PEI/5-Ac films themselves. Finally, we note that these results also suggest opportunities to pattern the surfaces of these films using decylamine and glucamine (either alone, or in combination with other amine-functionalized moieties, such as cell-adhesion peptides) to further control or confine the growth of cells on surfaces coated with these materials.

Characterization of Film Stability and the Release of Immobilized Agents

As discussed above, these PEI/5-Ac films were physically robust and did not delaminate upon incubation in cell culture media for up to one week. We attribute this stability, at least in part, to the covalently-crosslinked nature of these films. As also described above, however, the 1,4-conjugate addition of amines to acrylate functionality results in crosslinks containing ester functionality (Scheme 1B and Figure 3) that could also hydrolyze and promote subsequent film erosion. We performed experiments to characterize the longer-term stability of 50-bilayer films on silicon substrates upon incubation in PBS at 37 °C. The results of these experiments revealed these films to be stable, in general, for ~1 month under these conditions (e.g., as reflected by the absence of substantial decreases in film thickness, see Figure S3 of Supporting Information). In many cases, however, we observed films to partially delaminate from their underlying substrates and/or begin to physically disintegrate after ~30 days (by visual inspection). These physical changes did not occur uniformly across large areas of all substrates, but were sufficient to complicate quantitative characterization of time-dependent changes in film thickness over longer time periods. Overall, these results reveal these films to be relatively stable in PBS, and suggest that the hydrolysis of the ester functionality in these films is sufficiently slow under these conditions to prevent significant large-scale film erosion for at least one month. While it is unclear whether the film disintegration observed here is a direct consequence of the slow hydrolysis of ester-based crosslinks, these results suggest new opportunities to design covalent/reactive layer-by-layer films that do disintegrate and/or facilitate elimination over more extended periods.^{34,35}

We also discovered during the course of the studies described above that these generally stable PEI/5-Ac films could promote the controlled and long-term release of covalently attached small-molecule agents. Figure 7A shows the results of a series of incubation experiments using two 80-bilayer films functionalized by either (i) post-fabrication

treatment of acrylate-functionalized films with dansyl cadaverine (as described above; see Figure 4), or (ii) by post-fabrication treatment of PEI-functionalized films with TRITC (as also described above; Figure 4). The reaction of dansyl cadaverine with acrylate-functionalized films (by 1,4-conjugate addition) should lead to covalent immobilization of this fluorophore through an ester bond; treatment of PEI-functionalized films with TRITC should result in immobilization via reaction with available primary amine groups to result in thiourea linkages). Figure 7A also includes the results of two additional control experiments using acrylate- and PEI-functionalized films treated with the two non-reactive fluorophores dansyl amide and TMR. The presence (or absence) of fluorescence in these treated films was confirmed by fluorescence microscopy prior to use in these experiments (e.g., as shown in Figure 4). These films were incubated in PBS (pH 7.4, 37 °C) and the fluorescence of the incubation solutions was monitored over time using fluorometry.

Inspection of the results in Figure 7A (closed squares) reveals that films functionalized with dansyl cadaverine released fluorophore into solution gradually over a period of at least 35 days. No significant increases in fluorescence were observed during the incubation of otherwise identical films treated with dansyl amide (open squares), suggesting that the release of dansyl cadaverine did not arise from the desorption of fluorophore that was simply physically or nonspecifically bound. These results are, in general, consistent with gradual hydrolysis of the ester groups through which dansyl cadaverine was covalently immobilized (e.g., Scheme 1B), followed by resulting release of the conjugated fluorophore. Additional support for this view was provided by the results of experiments using TRITC-treated films (Figure 7A; closed triangles), which did not reveal similar increases in solution fluorescence over the same extended 35-day period. This result suggests that increases in fluorescence arising from dansyl cadaverine-treated films do not result from larger scale film erosion or other degradation processes (i.e., processes that would otherwise result in the release of fluorescently-labeled PEI). More broadly, these results suggest additional opportunities to exploit the two different types of reactive groups in these films (i.e., amines and acrylate groups) to functionalize or pattern films with multiple agents through combinations of stable and labile bonds (that is, to decorate these films with different functionality designed to be either a 'permanent' or a 'temporary' fixture of these materials).

Further support for this ester-cleavage hypothesis was provided by studies using decylamine-treated films incubated in PBS (Figure 7B). As described above, decylamine-treated films are hydrophobic, and have water contact angles of $\sim 138^\circ$. As shown in Figure 7B, however, the contact angles of these films decreased gradually, reaching values of $\sim 20^\circ$ after incubation for ~ 22 days (that is, these initially very hydrophobic films gradually transitioned to become quite hydrophilic over time). This gradual decrease in contact angle occurs over a time scale (three weeks) that is also, generally, on the order of the time scales over which fluorophore is released from dansyl cadaverine-treated films (Figure 7A). Although we cannot explicitly rule out other possibilities on the basis of this experiment, these results are also consistent with a process of gradual ester hydrolysis that slowly results in the loss of hydrophobic decyl groups and the concomitant unmasking of hydroxyl functionality (e.g., see Scheme 1B) that would render the surfaces of these films significantly more hydrophilic.

We note here that the hydrolysis of the β -amino ester bonds through which the functionality in the experiments above is attached to these films results in the subsequent release of material with a chemical structure that is slightly different from the structure of the primary amine-based agent that was initially used to functionalize the film (e.g., see Scheme 1B; hydrolysis results in the creation of secondary amine functionality bearing an additional β -amino acid-type structure). In the context of drug delivery, the practical utility of this approach to the immobilization and surface-mediated release of active agents would thus

likely be limited to situations where the presence of this added functionality would not influence the activity or function of the released agent. However, in the broader context of designing coatings and bio-interfaces that undergo other dynamic and time-dependent changes in physical properties upon exposure to aqueous environments (e.g., changes in contact angle and gradual transitions from hydrophobic to hydrophilic character), these small changes in the structure of these 'leaving groups' would be of little consequence.

Finally, we note that the release of fluorophore shown in Figure 7A proceeds gradually over a period of ~35 days, but does not appear to be complete over this time period. Unfortunately, characterization of release over longer time periods was complicated by the physical disintegration and partial delamination of these dansyl-functionalized films after ~45 days. As discussed above, the origins of this physical deterioration are unclear. However, it is also worth noting that the covalent/reactive approach to assembly used in this study should offer opportunities to prevent or reduce delamination, if needed, by fabricating these multilayers on the surfaces of reactive amine- or acrylate-functionalized surfaces. In general, increasing the stability of layer-by-layer films by covalent attachment of the bottom-most layers of a film directly to their underlying substrates is something that is more difficult to accomplish using conventional methods for the assembly of polyelectrolyte-based multilayers (i.e., PEMs, which are stitched together in the bulk by weak interactions that are more easily disrupted).

Summary and Conclusions

We have reported on conjugate-addition based approaches to the layer-by-layer fabrication and subsequent chemical functionalization of amine-reactive polymer multilayers. Covalent/reactive assembly of PEI and 5-Ac leads to covalently-crosslinked thin films that are stable for prolonged periods in contact with physiologically relevant media (e.g., PBS and serum-containing cell culture media) and provide a reactive platform for the post-fabrication attachment of additional functional groups (e.g., through additional conjugate addition reactions with residual acrylate functionality in the films). This post-fabrication approach can be used to modify important bio-interfacial properties of these materials, including water contact angles and the ability of the films to either prevent or promote the attachment and growth of mammalian cells *in vitro*. The results reported here demonstrate proof of concept of this approach, but this conjugate addition-based approach, in combination with the many well-known practical benefits associated with layer-by-layer assembly, should also be useful for the immobilization and patterning of a wide range of other useful chemical and biological functionality (e.g., containing amine or thiol functionality, etc.) on a variety of different types of substrates and materials. In addition, the presence of residual amine functionality in these films provides a second reactive 'handle' for the chemically orthogonal immobilization of other amine-reactive functionality useful in biomedical and biotechnological contexts.

The conjugate addition approach used here leads to films that inherently contain ester-functionalized crosslinks. Our results reveal that these films are generally stable in aqueous environments, but that they begin to physically disintegrate after ~30 days, suggesting opportunities to use this covalent/reactive layer-by-layer approach to design interfaces that ultimately erode or degrade to facilitate elimination. Our results also demonstrate that functional groups immobilized via conjugate addition are released slowly (over at least 30 days) into solution. Thus, in addition to promoting covalent assembly and facilitating chemical modification of interfacial properties, this conjugate addition-based approach may also prove useful in the contexts of surface-mediated drug delivery and the design of surfaces that undergo dynamic changes in interfacial properties (e.g., gradual decreases in contact angle, etc.). More broadly, these covalently-crosslinked coatings should also provide

opportunities for the more conventional loading and diffusion-controlled, surface-mediated release of small-molecule agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support for this work was provided by the Wisconsin Alumni Research Foundation, the National Institutes of Health (R01 EB006820), and the Vilas Trust at the University of Wisconsin – Madison. The authors are grateful to Adam Broderick for assistance with AFM experiments and to Dr. Maren Buck for many helpful discussions.

References

1. Decher G. *Science*. 1997; 277:1232–1237.
2. Bertrand P, Jonas A, Laschewsky A, Legras R. *Macromol Rapid Commun*. 2000; 21:319–348.
3. Decher, G.; Schlenoff, JB. *Multilayer Thin Films: Sequential Assembly of Nanocomposite Materials*. Wiley-VCH; Weinheim: 2003.
4. Peyratout CS, Dahne L. *Angew Chem, Int Ed*. 2004; 43:3762–3783.
5. Sukhishvili SA, Granick S. *J Am Chem Soc*. 2000; 122:9550–9551.
6. Mendelsohn JD, Barrett CJ, Chan VV, Pal AJ, Mayes AM, Rubner MF. *Langmuir*. 2000; 16:5017–5023.
7. Dubas ST, Farhat TR, Schlenoff JB. *J Am Chem Soc*. 2001; 123:5368–5369. [PubMed: 11457408]
8. Gao CY, Leporatti S, Moya S, Donath E, Mohwald H. *Langmuir*. 2001; 17:3491–3495.
9. Schuler C, Caruso F. *Biomacromolecules*. 2001; 2:921–926. [PubMed: 11710050]
10. Dubas ST, Schlenoff JB. *Langmuir*. 2001; 17:7725–7727.
11. Antipov AA, Sukhorukov GB, Leporatti S, Radtchenko IL, Donath E, Mohwald H. *Colloids Surf, A*. 2002; 198:535–541.
12. Kovacevic D, van der Burgh S, de Keizer A, Stuart MAC. *Langmuir*. 2002; 18:5607–5612.
13. Nolte AJ, Takane N, Hindman E, Gaynor W, Rubner MF, Cohen RE. *Macromolecules*. 2007; 40:5479–5486.
14. Ott P, Trenkenschuh K, Gensel J, Fery A, Laschewsky A. *Langmuir*. 2010; 26:18182–18188. [PubMed: 21033763]
15. Harris JJ, DeRose PM, Bruening ML. *J Am Chem Soc*. 1999; 121:1978–1979.
16. Yang SY, Rubner MF. *J Am Chem Soc*. 2002; 124:2100–2101. [PubMed: 11878948]
17. Tong WJ, Gao CY. *Polym Adv Technol*. 2005; 16:827–833.
18. Jang W-S, Jensen AT, Lutkenhaus JL. *Macromolecules*. 2010; 43:9473–9479.
19. Sun JQ, Wu T, Liu F, Wang ZQ, Zhang X, Shen JC. *Langmuir*. 2000; 16:4620–4624.
20. Chen JY, Luo GB, Cao WX. *Macromol Rapid Commun*. 2001; 22:311–314.
21. Pastoriza-Santos I, Scholer B, Caruso F. *Adv Funct Mater*. 2001; 11:122–128.
22. Leporatti S, Voigt A, Mitlohner R, Sukhorukov G, Donath E, Mohwald H. *Langmuir*. 2000; 16:4059–4063.
23. Richert L, Boulmedais F, Lavalle P, Mutterer J, Ferreux E, Decher G, Schaaf P, Voegel JC, Picart C. *Biomacromolecules*. 2004; 5:284–294. [PubMed: 15002986]
24. Zhang YJ, Guan Y, Zhou SQ. *Biomacromolecules*. 2005; 6:2365–2369. [PubMed: 16004484]
25. Francius G, Hemmerle J, Ohayon J, Schaaf P, Voegel JC, Picart C, Senger B. *Microsc Res Tech*. 2006; 69:84–92. [PubMed: 16456840]
26. Zelikin AN, Quinn JF, Caruso F. *Biomacromolecules*. 2006; 7:27–30. [PubMed: 16398494]
27. Boudou T, Crouzier T, Auzely-Velty R, Glinel K, Picart C. *Langmuir*. 2009; 25:13809–13819. [PubMed: 20560550]

28. Quinn JF, Johnston APR, Such GK, Zelikin AN, Caruso F. *Chem Soc Rev.* 2007; 36:707–718. [PubMed: 17471396]
29. Bergbreiter DE, Liao K-S. *Soft Matter.* 2009; 5:23–28.
30. Such GK, Quinn JF, Quinn A, Tjijto E, Caruso F. *J Am Chem Soc.* 2006; 128:9318–9319. [PubMed: 16848452]
31. Such GK, Tjijto E, Postma A, Johnston APR, Caruso F. *Nano Lett.* 2007; 7:1706–1710. [PubMed: 17530811]
32. Bergbreiter DE, Chance BS. *Macromolecules.* 2007; 40:5337–5343.
33. Vestberg R, Malkoch M, Kade M, Wu P, Fokin VV, Sharpless KB, Drockenmuller E, Hawker CJ. *J Polym Sci, Part A: Polym Chem.* 2007; 45:2835–2846.
34. De Geest BG, Van Camp W, Du Prez FE, De Smedt SC, Demeester J, Hennink WE. *Macromol Rapid Commun.* 2008; 29:1111–1118.
35. Ochs CJ, Such GK, Staedler B, Caruso F. *Biomacromolecules.* 2008; 9:3389–3396. [PubMed: 18991459]
36. Huang C-J, Chang F-C. *Macromolecules.* 2009; 42:5155–5166.
37. Zhang Y, He H, Gao C, Wu JY. *Langmuir.* 2009; 25:5814–5824. [PubMed: 19374339]
38. El Haitami AE, Thomann J-S, Jierry L, Parat A, Voegel J-C, Schaaf P, Senger B, Boulmedais F, Frisch B. *Langmuir.* 2010; 26:12351–12357. [PubMed: 20568818]
39. Rydzek G, Thomann J-S, Ben Ameer N, Jierry L, Mesini P, Ponche A, Contal C, El Haitami AE, Voegel J-C, Senger B, Schaaf P, Frisch B, Boulmedais F. *Langmuir.* 2010; 26:2816–2824. [PubMed: 19950954]
40. Buck ME, Zhang J, Lynn DM. *Adv Mater.* 2007; 19:3951–3955.
41. Buck ME, Breitbach AS, Belgrade SK, Blackwell HE, Lynn DM. *Biomacromolecules.* 2009; 10:1564–1574. [PubMed: 19438231]
42. Buck ME, Schwartz SC, Lynn DM. *Chem Mater.* 2010; 22:6319–6327. [PubMed: 21151704]
43. Buck ME, Lynn DM. *ACS Appl Mater Interfaces.* 2010; 2:1421–1429. [PubMed: 20402471]
44. Broderick AH, Azarin SM, Buck ME, Palecek SP, Lynn DM. *Biomacromolecules.* 2011; 12:1998–2007. [PubMed: 21504222]
45. Tocce EJ, Broderick AH, Murphy KC, Liliensiek SJ, Murphy CJ, Lynn DM, Nealey PF. *J Biomed Mater Res, Part A.* 2012; 100A:84–93.
46. Liu YL, Bruening ML, Bergbreiter DE, Crooks RM. *Angew Chem, Int Ed Engl.* 1997; 36:2114–2116.
47. Russell RJ, Sirkar K, Pishko MV. *Langmuir.* 2000; 16:4052–4054.
48. Chan EWL, Lee DC, Ng MK, Wu GH, Lee KYC, Yu LP. *J Am Chem Soc.* 2002; 124:12238–12243. [PubMed: 12371865]
49. Major JS, Blanchard GJ. *Chem Mater.* 2002; 14:2574–2581.
50. Serizawa T, Nanameki K, Yamamoto K, Akashi M. *Macromolecules.* 2002; 35:2184–2189.
51. Liang ZQ, Wang Q. *Langmuir.* 2004; 20:9600–9606. [PubMed: 15491192]
52. Hou SF, Wang JH, Martin CR. *Nano Lett.* 2005; 5:231–234. [PubMed: 15794602]
53. Weatherspoon MR, Dickerson MB, Wang G, Cai Y, Shian S, Jones SC, Marder SR, Sandhage KH. *Angew Chem, Int Ed.* 2007; 46:5724–5727.
54. Dyer MA, Ainslie KM, Pishko MV. *Langmuir.* 2007; 23:7018–7023. [PubMed: 17506587]
55. Ford J, Marder SR, Yang S. *Chem Mater.* 2009; 21:476–483.
56. Wang GJ, Fang YN, Kim P, Hayek A, Weatherspoon MR, Perry JW, Sandhage KH, Marder SR, Jones SC. *Adv Funct Mater.* 2009; 19:2768–2776.
57. Seo J, Schattling P, Lang T, Jochum F, Nilles K, Theato P, Char K. *Langmuir.* 2010; 26:1830–1836. [PubMed: 19761256]
58. Fang YN, Berrigan JD, Cai Y, Marder SR, Sandhage KH. *J Mater Chem.* 2012; 22:1305–1312.
59. Lynn DM, Langer R. *J Am Chem Soc.* 2000; 122:10761–10768.
60. Lynn, DM.; Anderson, DG.; Akinc, A.; Langer, R. Degradable Poly(beta-amino ester)s for Gene Delivery. In: Amiji, M., editor. *Polymeric Gene Delivery: Principles and Applications.* CRC Press; New York: 2004. p. 227-241.

61. Little SR, Lynn DM, Ge Q, Anderson DG, Puram SV, Chen JZ, Eisen HN, Langer R. *Proc Natl Acad Sci U S A*. 2004; 101:9534–9539. [PubMed: 15210954]
62. Zhang JT, Chua LS, Lynn DM. *Langmuir*. 2004; 20:8015–8021. [PubMed: 15350066]
63. Frey, BL.; Robert, MC.; Weibel, SC. *Handbook of Vibrational Spectroscopy*. Chalmers, J.; Griffiths, PR., editors. Vol. 2. Wiley; New York: 2002. p. 1042
64. Picart C, Mutterer J, Richert L, Luo Y, Prestwich GD, Schaaf P, Voegel JC, Lavalle P. *Proc Natl Acad Sci U S A*. 2002; 99:12531–12535. [PubMed: 12237412]
65. Garza JM, Schaaf P, Muller S, Ball V, Stoltz JF, Voegel JC, Lavalle P. *Langmuir*. 2004; 20:7298–7302. [PubMed: 15301518]
66. Roeges, N. *A Guide to the Complete Interpretation of Infrared Spectra of Organic Structures*. John Wiley & Sons; New York: 1994.
67. Ostuni E, Chapman RG, Holmlin RE, Takayama S, Whitesides GM. *Langmuir*. 2001; 17:5605–5620.
68. Orner BP, Derda R, Lewis RL, Thomson JA, Kiessling LL. *J Am Chem Soc*. 2004; 126:10808–10809. [PubMed: 15339142]

Figure 1.

Plot of ellipsometric thickness versus the number of PEI/5-Ac dipping cycles for films fabricated on silicon substrates. Data correspond to films fabricated using either (i) solutions of PEI or 5-Ac and ethanol rinse solutions that were refreshed after every ten cycles (filled circles), or (ii) films fabricated without refreshing any of these solutions (filled squares; see text).

Figure 2.

Representative tapping mode AFM images ($5\ \mu\text{m} \times 5\ \mu\text{m}$) of PEI/5-Ac films fabricated on bare silicon substrates after (A) 15, (B) 30, (C) 50, and (D) 80 PEI/5-Ac dipping cycles. These films were fabricated without refreshing of PEI, 5-Ac, or ethanol rinse solutions during fabrication (see text). The scale in the z direction for each image is $1.2\ \mu\text{m}$.

Figure 3. PM-IRRAS spectrum of a 30-bilayer PEI/5-Ac film fabricated on a gold-coated silicon substrate.

Figure 4.

Top: Structures of dansyl cadaverine, TRITC, decylamine, and D-glucamine used for post-fabrication modification of PEI/5-Ac films. (A) Fluorescence microscopy image of an acrylate-functionalized 80-bilayer PEI/5-Ac film functionalized post-fabrication by treatment with dansyl cadaverine. The inset shows an image of an otherwise identical film treated with the non-reactive fluorophore dansyl amide (see text). (B) Fluorescence microscopy image of a PEI-functionalized film functionalized by treatment with TRITC. The inset shows an image of an otherwise identical film treated with the non-reactive fluorophore TMR. (C) Representative image showing water contact angle of an acrylate-functionalized 80-bilayer PEI/5-Ac film. (D–E) Images showing the contact angles of otherwise identical films after treatment with (D) decylamine or (E) glucamine.

Figure 5. PM-IRRAS spectra for a 30-bilayer acrylate-functionalized film prior to (dotted line) and after (solid line) treatment with decylamine.

Figure 6.

Fluorescence microscopy images of COS-7 cells growing on (A–C) decylamine-functionalized and (D–F) glucamine-functionalized 80-bilayer PEI/5-Ac films (A, D) 1 hour, (B, E) 2 days, and (C, F) 7 days after initial cell seeding. Cells were stained using the live cell stain Calcein-AM immediately prior to imaging. Scale bars = 100 μm .

Figure 7.

(A) Plot of solution fluorescence versus time during the incubation of substrates coated with fluorophore-treated PEI/5-Ac films 80 bilayers thick in PBS (pH 7.4, 37 °C). Data correspond to results using (i) acrylate-functionalized films treated with either dansyl cadaverine (filled squares) or dansyl amide (open squares), and (ii) PEI-functionalized films treated with either TRITC (closed triangles), or TMR (open triangles). (B) Plot of changes in water contact angle versus time for decylamine-functionalized PEI/5-Ac films incubated in PBS. Values and error bars are reported as the average and standard deviation, respectively, for measurements made using three different films.

Scheme 1.

(A) Representative structure of branched poly(ethylene imine) (PEI) and the structure of the small molecule dipentaerythritol pentaacrylate (5-Ac) used in this study. (B) Schematic showing the 1,4-conjugate addition of a representative primary amine with a representative acrylate group and subsequent hydrolysis of the ester bond. This conjugate addition reaction plays a role in both covalent film assembly and the post-fabrication modification of the resulting films (see text).