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Layer-by-Layer Fabrication of Covalently Crosslinked and Reactive Polymer Multilayers Using Azlactone-Functionalized Copolymers: A Platform for the Design of Functional Biointerfaces

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Abstract

We report a method for modulating the physicochemical properties of surfaces that is based on the reactive layer-by-layer fabrication of covalently crosslinked thin films using azlactone-functionalized copolymers. We demonstrate that copolymers containing different molar ratios of methylmethacrylate (MMA) and 2-vinyl-4,4-dimethylazlactone (VDMA) can be alternately deposited with poly(ethyleneimine) to assemble covalently crosslinked thin films. Characterization using ellipsometry demonstrates that, in general, film growth and thickness decrease as the content of reactive, azlactone functionality in the copolymer used to assemble the film decreases. Reflective infrared spectroscopy experiments demonstrate that films fabricated from MMA:VDMA copolymers contain residual azlactone functionality and that these reactive groups can be exploited to modify film-coated surfaces. Fabricating films from MMA:VDMA copolymers containing different compositions permitted modulation of the density of reactive groups within the films and, thus, the extent to which the films are functionalized by exposure to small molecule amines. For example, functionalization of MMA:VDMA copolymer films with the small molecule D-glucamine resulted in films with water contact angles that varied with the composition of the copolymer used to fabricate the film (e.g., as the azlactone content in the film increased, glucamine-modified films became more hydrophilic). We demonstrate further that treatment of copolymer-containing films with glucamine resulted in changes in the numbers of mammalian cells that grow on the surfaces of the films. Our results suggest the basis of methods that could be used to modulate or tune the density of chemical and biological functionality presented on surfaces of interest in a variety of fundamental and applied contexts.

Introduction

An important goal in the design of synthetic materials for surface coatings and other thin film constructs is the ability to control with precision the molecular architecture and, thus, the physical, chemical, and mechanical properties, of the films. Methods that can be used to fabricate thin films and assemblies with well-defined physicochemical properties are critical for engineering synthetic surfaces for a range of applications, including the design of

substrates for culturing and directing the growth of cells,[1–3] tissue engineering and regeneration,[1,3–5] polymer membranes for separation/filtration or catalysis,[6,7] photovoltaic or solar cell devices,[8–10] and many others. Many approaches to the assembly of thin polymer films on surfaces have been developed and investigated, including the deposition of Langmuir-Blodgett films,[11] spin coating or casting of polymer films,[12] chemical vapor deposition,[13,14] and layer-by-layer assembly.[15,16] Of these methods, layer-by-layer assembly has been recognized as a particularly versatile approach for the fabrication of films and surface-coatings with well-defined, nanostructured architectures and physicochemical properties.[17–21] Layer-by-layer methods for assembling thin films have generally focused on the alternate and repetitive adsorption of oppositely-charged, water-soluble polymers on surfaces to fabricate polymer multilayers assembled through electrostatic interactions.[17–21] Because this assembly process is modular, the thicknesses and chemical compositions of films fabricated using layer-by-layer methods can be readily tuned to yield thin films with a wide range of chemical, physical, and mechanical properties. Consequently, these materials have been investigated extensively for a range of biomedical, biotechnological, and industrial applications.[17–21]

While most past studies using layer-by-layer methods have focused on the assembly of films based on non-covalent intermolecular interactions (e.g., electrostatic or hydrogen-bonding interactions), we and others have recently demonstrated the layer-by-layer assembly of polymers bearing mutually reactive functionality that can react to form covalent bonds as the films are assembled.[22–28] The resulting films exhibit increased chemical and mechanical stability relative to physically crosslinked films.[23] In addition, polymers that are either insoluble in water or that react with water can be incorporated into covalently crosslinked thin films by using organic solvents for film fabrication. These reactive films can be chemically modified post-fabrication to further tune the physicochemical properties of the thin films.

In several previous reports, we demonstrated an approach to the reactive layer-by-layer assembly of polymer films on surfaces that makes use of the versatile reactivity of azlactone-functionalized polymers.[25,29–31] Azlactone-functionalized polymers, such as poly(2-vinyl-4,4-dimethylazlactone) (PVDMA), react rapidly with primary-amine functionalized nucleophiles (Scheme 1)[32] and, thus, can be readily assembled into covalently crosslinked thin films when alternately deposited on surfaces with a primary amine-functionalized polymer [e.g., branched poly(ethyleneimine) (PEI)]. Our past studies demonstrated that residual, unreacted azlactone functionality within the films could be used to modify PEI/PVDMA thin films post-fabrication to tune the physicochemical properties of film-coated surfaces after assembly.[25,29–31] For example, we demonstrated that PEI/PVDMA films can be functionalized with small molecule amines in ways that promote, prevent, or that can be used to pattern, the adhesion of proteins, mammalian cells, or bacteria on film-coated surfaces.[29]

These past studies focused primarily on the use of the azlactone-functionalized homopolymer PVDMA to assemble covalently crosslinked and reactive thin films. Several groups have demonstrated that the monomer 2-vinyl-4,4-dimethylazlactone (VDMA) can be readily copolymerized with many other vinyl monomers (e.g., styrene, a variety of acrylates

and methacrylates, vinylpyrollidone, etc.) to synthesize a range of copolymer structures containing varying amounts of the reactive azlactone group.[32–36] We sought to investigate whether azlactone-based copolymers could be used to fabricate covalently crosslinked and reactive multilayered films, for several reasons. First, the incorporation of copolymers with specific azlactone contents could permit control over the density of reactive groups within the polymer film and, subsequently, the density of functionality introduced into the film post-fabrication. Second, varying the amount of the azlactone functionality in the polymer could provide a means to tune the crosslinking density within the film, and thus, permit modulation of the mechanical properties of the resulting films. Finally, other orthogonally reactive functional groups could be incorporated into the polymer structure and the film and, thus, provide an additional reactive handle that could be used to modify film-coated surfaces with a broad range of chemical and biological functionality at specific densities.

Here, we describe the assembly of covalently crosslinked multilayered films fabricated from PEI and copolymers synthesized from VDMA and the comonomer methyl methacrylate (MMA). In the first part of the work reported here, we characterize the fabrication and chemical and physical properties of the thin films using ellipsometry, infrared spectroscopy, and fluorescence microscopy. We demonstrate that films can be fabricated from azlactone-functionalized copolymers and that these films contain residual, unreacted azlactone functionality after fabrication. This residual azlactone functionality can be exploited to chemically modify the copolymer films post-fabrication and permits modulation of the chemical and physical properties of the films. The second part of this investigation demonstrates that chemically modified copolymer films can be used to tune the interactions of mammalian cells with film-coated surfaces and that the density of reactive groups within the films can be used to tune the density of cells that adhere to these surfaces. The results of the work reported here suggest materials and approaches that could be used to control the physicochemical properties of thin films and surface coatings as well as the ability to mediate the density of chemical or biological functionality presented on surfaces. These characteristics could be useful for many applications in membrane technology, medicine, and biotechnology, as well as fundamental studies related to how cells and other biological systems respond to a variety of chemical and physical cues.

Materials and Methods

Materials

Branched poly(ethyleneimine) (PEI, Mn = 10,000, Mw = 25,000; the ratio of primary:secondary:tertiary amines = 1:1.2:0.76), acetone, DMSO, methyl methacrylate (MMA), and dansyl cadaverine were purchased from Aldrich Chemical Company (Milwaukee, WI). The azlactone-functionalized monomer 2-vinyl-4,4-dimethylazlactone (VDMA) was a kind gift from Dr. Steven M. Heilmann (3M Corporation, Minneapolis, MN). D-Glucamine was purchased from TCI America (Portland, OR). Dulbecco’s modified Eagle Medium (DMEM), Opti-MEM I reduced serum medium, and Calcein AM fluorescent cell stain were purchased from Invitrogen (Carlsbad, CA). African green monkey kidney fibroblasts (COS-7 cells) were obtained from ATCC (Manassas, VA). Test grade n-type silicon wafers were purchased from Si-Tech, Inc. (Topsfield, MA). Glass microscope slides

were purchased from Fischer Scientific (Pittsburgh, PA). 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 0.4 μm membrane syringe filter.

General Considerations

Gel permeation chromatography (GPC) was performed using a GPCmax-VE2001 Solvent/Sample module (Viscotek Corp., Houston, TX) and two PlusPore Organic GPC Columns (Polymer Laboratories, Amherst, MA) equilibrated to 40 °C. THF was used as the eluent at a flow rate of 1.0 mL/min. Data were collected using the refractive index detector of a Viscotek TDA 302 triple detector array and processed using the OmniSEC 4.5 software package. Molecular weights and polydispersities are reported relative to monodisperse polystyrene standards. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AC+ 300 (300.135 MHz) spectrometer. Chemical shift values are given in ppm and are referenced with respect to protons from an internal standard (tetramethylsilane). Silicon (10 mm × 50 mm) and glass (10 mm × 30 mm) substrates were cleaned with acetone, ethanol, methanol, and deionized water and dried under a stream of filtered, compressed air prior to the fabrication of multilayered films. Silicon substrates used for reflective infrared (IR) spectroscopy experiments were prepared by depositing thin layers of titanium (10 nm) and gold (200 nm) sequentially onto clean silicon wafers using an electron-beam evaporator (Tek-Vac Industries, Brentwood, NY). The optical thicknesses of films deposited on silicon 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 refractive index of 1.58 for the thin films. Thicknesses were determined in at least five different standardized locations on each substrate. Static contact angle measurements were made using a Dataphysics OCA 15 Plus instrument and ImageJ. Polarization–modulation infrared reflectance–absorbance spectroscopy (PM–IRRAS) was conducted in analogy to previously reported methods.[37,38] Briefly, gold-coated silicon substrates coated with thin 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 obtained 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.[39] Optical and fluorescence microscopy images were acquired using an Olympus IX70 microscope and analyzed using the Metavue version 4.6 software package (Universal Imaging Corporation).

Synthesis of Poly(2-vinyl-4,4-dimethylazlactone)

VDMA was purified by passage through a phenolic inhibitor removal resin followed by passage through a short plug of silica gel prior to polymerization. The initiator 2,2′-azobisisobutyronitrile (AIBN, 54.4 mg, 0.3313 mmol) was weighed into a 25 mL round-bottomed flask equipped with a stir bar. Ethyl acetate (6 mL) was added and the solution was stirred to dissolve the AIBN. VDMA (2.4011 g, 17.4 mmol) was added to the flask, the flask was capped with a septum, and the solution was purged with N_2 for 10 minutes. The

solution was stirred under N_2 at 60 °C for 24 hours, after which time the viscous reaction mixture was cooled to room temperature and acetone (5 mL) was added to the flask. The polymer was precipitated into hexanes to yield a white solid. The polymer was filtered and washed with hexanes, then redissolved in acetone and precipitated once more in hexanes. PVDMA was isolated as a white solid in 90% yield. ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.37 (br s, (–C***H*****3**)$_2$), 1.62–2.1 (br m, –C***H*****2**CH–), 2.69 (br s, –CH$_2$C***H***–). FT-IR (ATR, cm^{-1}): 2980–2900 (C-H), 1820 (lactone C=O), 1672 (C=N). M_n: 20,394; PDI = 3.2.

Synthesis of Poly(MMA-*co*-VDMA)

Copolymers containing MMA and VDMA were polymerized at molar fractions of 0.25:0.75, 0.50:0.50, and 0.75:0.25 MMA:VDMA using procedures similar to those described above for the polymerization of VDMA. MMA and VDMA were purified by passage through a phenolic inhibitor removal resin followed by passage through a short plug of silica gel prior to polymerization. AIBN (0.018 equivalents) was weighed into a 25 mL round-bottomed flask equipped with a stir bar. Ethyl acetate (6 mL) was added and the solution was stirred to dissolve the AIBN. VDMA and MMA were added to the flask, the flask was capped with a septum, and the solution was purged with N_2 for 10 minutes. The solution was stirred under N_2 at 60 °C for 24 hours, after which time the viscous reaction mixture was cooled to room temperature and CH_2Cl_2 (5 mL) was added to the flask. The polymer was precipitated into hexanes to yield a white solid. The polymer was filtered and washed with hexanes, then redissolved in CH_2Cl_2 and precipitated once more in hexanes. MMA/VDMA copolymers were isolated as white solids. Poly(MMA$_{0.75}$-*co*-VDMA$_{0.25}$): AIBN (43.3 mg, 0.263); MMA (1.025 g, 10.2 mmol); VDMA (0.4890 g, 3.5 mmol); ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 0.80–1.27 (br m, (C-C***H*****3**, MMA), 1.37 (br s, –C(C***H*****3**)$_2$, VDMA), 1.50–2.30 (br m, –C***H*****2**CH–, MMA and VDMA), 2.45–2.70 (br m, –CH$_2$C***H***–, VDMA), 3.60 (br t, –OC***H***$_3$, MMA). M_n: 13,292; PDI = 3.4. Poly(MMA$_{0.50}$-*co*-VDMA$_{0.50}$): AIBN (39.2 mg, 0.238); MMA (0.628 g, 6.27 mmol); VDMA (0.8720 g, 6.27 mmol); ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 0.80–1.27 (br m, (C-C***H*****3**, MMA), 1.37 (br s, –C(C***H*****3**)$_2$, VDMA), 1.50–2.23 (br m, –C***H*****2**CH–, MMA and VDMA), 2.40–2.65 (br s, –CH$_2$C***H***–, VDMA), 3.60 (br t, –OC***H***$_3$, MMA). M_n: 12,095; PDI = 3.80. Poly(MMA$_{0.25}$-*co*-VDMA$_{0.75}$): AIBN (36.2 mg, 0.220 mmol); MMA (0.290 g, 2.9 mmol); VDMA (1.2 g, 8.7 mmol); ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 0.80–1.27 (br m, (C-C***H*****3**, MMA), 1.38 (–C(C***H*****3**)$_2$, VDMA), 1.50–2.39 (br m, –C***H*****2**CH–, MMA and VDMA), 2.50–2.60 (br s, –CH$_2$C***H***–, VDMA), 3.60 (–OC***H***$_3$, MMA). M_n: 5,817; PDI = 3.4.

Layer-by-Layer Fabrication of Films

Solutions of PEI, PVDMA, or poly(MMA$_x$-*co*-VDMA$_y$) were prepared in acetone (20 mM with respect to the molecular weight of the polymer repeat unit). Films were deposited manually layer-by-layer on silicon or glass substrates according to the following general protocol: 1) Substrates were submerged in a solution of PEI for 30 seconds, 2) substrates were removed and immersed in an initial acetone bath for 30 seconds followed by a second acetone bath for 30 seconds, 3) substrates were submerged in a solution of PVDMA (or poly(MMA$_x$-*co*-VDMA$_y$) for copolymer films) for 30 seconds, and 4) substrates were rinsed in the manner described above. This cycle was repeated until the desired number of PEI/PVDMA or PEI/poly(MMA$_x$-*co*-VDMA$_y$) layers was reached. Films were either

characterized or used in subsequent experiments immediately, or were dried under a stream of filtered, compressed air and stored in a vacuum desiccator until use. All films were fabricated at ambient room temperature.

Post-Fabrication Functionalization of Thin Films

PEI/PVDMA films and PEI/poly(MMA_x-*co*-$VDMA_y$) films were patterned with small, circular spots of dansyl cadaverine by treatment with a drop (1 μL) of a dansyl cadaverine solution (20 mg/mL in DMSO) for 12 hours. Dansyl-functionalized films were rinsed briefly with DI water, soaked in fresh DMSO for ~2 hours, and rinsed with acetone prior to drying with filtered air. Films functionalized with spots of dansyl cadaverine were imaged in water. Average grayscale intensities of the dansyl cadaverine spots were calculated by measuring the intensities for three different spots on 10-bilayer PEI/PVDMA films and PEI/poly(MMA_x-*co*-$VDMA_y$) films. Films used for contact angle measurements were submerged completely in a solution of glucamine (50 mM in DMSO) for 2.5 days. The films were rinsed by soaking in fresh DMSO for ~2 hours, rinsed with acetone, and dried with filtered air. Static water contact angles were measured and averaged for a total of six water droplets (5 μL) using two different films of each type. Films fabricated for cell adhesion experiments were patterned with small, circular spots of glucamine by treating the films with a drop (1 μL) of a glucamine solution (20 mg/mL in DMSO) for 2 hours. The glucamine-patterned films were rinsed by soaking in DMSO for 2 h, rinsed with EtOH, and dried under air. Functionalized films were used immediately or stored in a vacuum desiccator until further use.

Characterization of Adhesion and Growth of Mammalian Cells on PEI/PVDMA and PEI/poly(MMA_x-*co*-$VDMA_y$) Films

Experiments designed to investigate the attachment and proliferation of mammalian cells on modified PEI/PVDMA and PEI/poly(MMA_x-*co*-$VDMA_y$) films were performed using films fabricated on transparent glass substrates. For these experiments, PEI/PVDMA and PEI/poly(MMA_x-*co*-$VDMA_y$) films were fabricated in duplicate and each film was patterned with two spots of glucamine. All films were sterilized prior to seeding cells by rinsing liberally with EtOH (~10 mL), followed by drying with filtered compressed air. Glucamine-treated films were placed individually into the wells of tissue culture-treated polystyrene culture plates. COS-7 cells were seeded on films at an initial density of 5,000 cells/cm^2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were seeded directly on the films in DMEM and allowed to adsorb to the surface for 20 minutes at room temperature, after which an additional 2–3 mL of the appropriate growth medium was added. Cells were incubated with substrates for at least 24 hours at 37 °C to allow for cell attachment and growth. For imaging, cells were stained with 2 mL of a Calcein AM staining solution (1 μg/mL in PBS) for 30 minutes at 37 °C. Following incubation, the staining solution was aspirated and replaced with 2 mL of DMEM. Cells were stained and imaged by optical light microscopy and fluorescence microscopy without removal of the glass substrates from the culture wells after 2, 7, 10, 14, and 18 days of incubation.

Results and Discussion

In several previous reports, we demonstrated that branched PEI can be assembled on surfaces and at interfaces with the azlactone-containing homopolymer PVDMA using a reactive layer-by-layer approach.[25,29–31] These films contain residual, unreacted azlactone functionality that can be exploited to chemically modify film-coated surfaces post-fabrication. In the work described here, we sought to determine whether azlactone-functionalized copolymers could be used to assemble covalently crosslinked multilayered films and whether fabricating films using azlactone-functionalized copolymers of different composition could be used as an additional means to tune the physicochemical properties of film-coated surfaces (e.g., to influence the ways in which the films interact with or prevent cell adhesion, etc.).

Synthesis of MMA:VDMA Copolymers

The azlactone-functionalized monomer 2-vinyl-4,4-dimethylazlactone (VDMA) can be readily copolymerized with a number of vinyl comonomers and has been used to synthesize a range of reactive random and block copolymers.[32–36] For the experiments described here, we selected MMA as the comonomer for several reasons: i) the copolymerization of MMA and VDMA has been well-characterized and copolymerization is reported to yield random copolymers,[32,33] ii) MMA does not react readily with primary amines used to functionalize our polymer films post-fabrication, and iii) poly(methyl methacrylate) is commonly used for cell culture and other biomedical applications.[40]

We synthesized a series of copolymers containing various ratios of MMA:VDMA (Scheme 2) according to previously reported procedures.[33] The polymers were obtained in nearly quantitative yields using conventional free radical polymerization in ethyl acetate at 60 °C and AIBN as the initiator. Table 1 summarizes the results of the polymerizations and the characteristics of the copolymers. The relative ratios of MMA and VDMA in the copolymers were in good agreement with the mole fractions in the feed, as evaluated using ^{1}H-NMR spectroscopy. In addition, the polydispersities of the four polymers are similar and are comparable to polydispersities reported previously for MMA:VDMA copolymers synthesized using conventional free radical polymerization.[33] The molecular weights of the polymers ranged between 5,000 and 20,000 g/mol relative to monodisperse polystyrene standards. It is possible that these differences in molecular weight could influence film properties (e.g., film thickness), however, conventional free radical polymerization was determined to be adequate for all studies described below. We note that living radical polymerization methods can be used to synthesize azlactone-functionalized polymers with controlled molecular weights and narrow polydispersities,[34,35,41,42] and could be used for future studies investigating the influence of molecular weight and polydispersity on film properties.

Fabrication and Characterization of Copolymer-Containing Thin Films

We have demonstrated in previous reports that the homopolymer PVDMA can be readily assembled into covalently crosslinked thin films,[25,29–31] but it was not clear at the outset of these studies whether copolymers with reduced azlactone content could also be used for

reactive layer-by-layer assembly. To determine whether the copolymers described above could be used to fabricate thin films, we assembled four different films, each composed of PEI and one of the polymers listed in Table 1. Alternate and repetitive immersion of silicon substrates for 30 seconds in dilute solutions (in acetone) of PEI and these polymers resulted in the step-wise growth of ultrathin polymer films. Figure 1 shows a plot of the ellipsometric thickness of each film as a function of the number of PEI/PVDMA and PEI/poly(MMA_x-*co*-$VDMA_y$) bilayers (the term 'bilayer', as used here, refers to one PEI/azlactone-functionalized polymer layer pair). The data shown in Figure 1 reveal that the thicknesses of these films increase in a linear manner after the deposition of the first two bilayers, which is generally consistent with our past results reported for films fabricated on silicon substrates using the homopolymer PVDMA and PEI.[25]

The thicknesses of the films after the deposition of 10 bilayers depended significantly on the relative content of azlactone functionality in the polymer. In general, the growth and overall thicknesses of the copolymer-containing films decreased as the azlactone content of the polymer decreased. For example, the thicknesses of 10-bilayer PEI/poly(MMA_{25}-*co*-$VDMA_{75}$) films (~160 nm) were significantly greater than 10-bilayer poly(MMA_{50}-*co*-$VDMA_{50}$) and poly(MMA_{75}-*co*-$VDMA_{25}$) films (~40 and 18 nm, respectively). As the number of reactive azlactone groups in the polymer decreases, it is possible that less material adsorbs and reacts covalently with the surface during each dipping step, resulting in films that are thinner than when polymers with more reactive groups are used. Control experiments in which PEI was alternately deposited with the non-reactive homopolymer PMMA (rather than an azlactone-containing copolymer) onto silicon substrates resulted in no increase in film thickness, as shown in Figure 1. These results suggest that the azlactone functionality is necessary for film assembly and that the relative quantity of reactive groups influences the amount of material that is adsorbed and bound to the surface.

Finally, we note that 10-bilayer PEI/poly(MMA_{25}-*co*-$VDMA_{75}$) films are also thicker than films fabricated from the homopolymer PVDMA (~100 nm). As described above, the differences in thickness observed for films fabricated from PEI and each of these four different azlactone-functionalized copolymers could arise from differences in the amount of material adsorbed to the surface. It is also possible that the homo- and copolymers adopt different conformations in solution (e.g., extended, rod-like chain or globular morphology) and, consequently, yield differences in thickness based on the morphology or hydrodynamic radius of the polymers when adsorbed to the surface. We return to additional discussion of these observations again in the sections below.

Fabrication of PEI/PVDMA and PEI/poly(MMA_x-*co*-$VDMA_y$) films on gold-coated silicon substrates permitted characterization of the chemical structures of these copolymer-containing films using polarization-modulation infrared reflectance-absorbance spectroscopy (PM-IRRAS). Figure 2 shows the carbonyl region of IR spectra for four different 10-bilayer films, each fabricated from PEI and one of the polymers listed in Table 1. Inspection of these spectra reveals strong absorbance peaks at 1828 cm^{-1} for all films, which corresponds to the carbonyl peak for the azlactone functionality present in PVDMA and each of the copolymers.[32] The peak at 1730 cm^{-1} in each of the copolymer films corresponds to the carbonyl peak of the methacrylate groups. Comparison of the intensities of the azlactone

carbonyl peak at 1828 cm^{-1} in each of the copolymer films relative to the methyl methacrylate carbonyl peak at 1730 cm^{-1} in the same film shows a decrease in the intensity of the peak at 1828 cm^{-1} and a simultaneous increase in the peak at 1730 cm^{-1} as the azlactone content in the copolymer decreases. These results correlate qualitatively with the copolymer compositions determined for the as-synthesized copolymers as discussed above. We note that the intensities of peaks in PM-IRRAS spectra are dependent in part on the thickness of the film and, thus, peak intensities cannot be directly compared between films. However, the relative trend in peak intensities for the azlactone carbonyl peak for each of the films correlates with the trend in film thickness measured using ellipsometry (i.e., film thickness decreases from PEI/poly(MMA_{25}-*co*-$VDMA_{75}$) films to PEI/poly(MMA_{75}-*co*-$VDMA_{25}$) films). The presence of a peak at 1828 cm^{-1} for all copolymer films also indicates that there is residual, unreacted azlactone functionality in the films and that not all reactive groups are consumed by the formation of amide/amide crosslinks during film fabrication.

Post-Fabrication Chemical Modification of PEI/PVDMA and PEI/Poly(MMA_x-co-$VDMA_y$) Films

We have demonstrated in several past reports that PEI/PVDMA films can be chemically modified post-fabrication by treatment with a range of different small molecule amines.[25,29–31] Our next experiments sought to determine whether the copolymer-containing films described above could be modified post-fabrication, and whether the copolymer composition influenced the extent to which the film could be functionalized with small molecule amines. To this end, we fabricated 10-bilayer films on silicon substrates and treated each film with a small, concentrated drop of the amine-functionalized fluorophore dansyl cadaverine in DMSO for 12 hours followed by rinsing the films in fresh DMSO for 3 hours. The images in Figures 3A–3D show fluorescence microscopy images of 10-bilayer PEI/PVDMA and PEI/poly(MMA_x-*co*-$VDMA_y$) films treated with dansyl cadaverine. Inspection of these images reveals circular areas of fluorescence on each of the azlactone-containing films. We quantified the fluorescence intensity of the spots on each film using computer image analysis of the circular regions; the measured grayscale intensities (arbitrary units) are shown in the upper right corner of each image in Figure 3.

In general, the fluorescence intensity of the dansyl-functionalized area decreases as the azlactone content in the *copolymer* used to fabricate the film decreases (Figures 3B–3D). We note that the PEI/poly(MMA_{25}-*co*-$VDMA_{75}$) film (Figure 3B) shows brighter fluorescence than the homopolymer film (Figure 3A). These observations suggest that more fluorophore was incorporated into the PEI/poly(MMA_{25}-*co*-$VDMA_{75}$) film than into the homopolymer-containing film. These results further suggest that PEI/poly(MMA_{25}-*co*-$VDMA_{75}$) films contain more azlactone groups that are available or accessible for reaction post-fabrication. The differences in intensity observed in the films shown in Figures 3A and 3B could arise from i) differences in the amount of material, and, thus, the total quantity of azlactone functionality, adsorbed to the surface, ii) differences in the ability of dansyl cadaverine to penetrate into and react with available azlactone groups within the bulk of homopolymer versus copolymer films, or a combination of these factors. These results also correlate with the increase in thickness observed for PEI/poly(MMA_{25}-*co*-$VDMA_{75}$) films relative to

homopolymer films, as shown in Figure 2. We note, in this context, that evaluating the fluorescence intensity of 10-bilayer films functionalized with dansyl cadaverine using fluorescence intensity provides an indication of the extent to which the entire film is modified (i.e., the extent to which the fluorescent labels are distributed on the surface as well as in the bulk of the film). Because these 10-bilayer films each have different thicknesses depending upon the structure of the copolymer used to fabricate the films (as described above and in Figure 2), these measurements do not necessarily reflect changes in the chemical composition of the film and are complicated by concomitant changes in the thicknesses of the films. However, the results of these experiments do demonstrate that these copolymer-containing films can be chemically modified post-fabrication, and that levels of functionalization are influenced significantly by the compositions of the copolymers used to fabricate the films.

Our next set of experiments sought to determine whether the incorporation of azlactone-functionalized copolymers into the films described above could be used to modulate the physical properties (e.g., surface wettability) of film-coated surfaces by post-fabrication modification. For these initial studies, we investigated changes in the wettability of films functionalized with the hydrophilic small molecule D-glucamine, which we have shown in past studies to lead to hydrophilic surfaces when immobilized on PEI/PVDMA films.[29] We fabricated four different types of 10-bilayer films on glass substrates, each film composed of PEI and one of the azlactone-functionalized polymers listed in Table 1. The films were subsequently treated with glucamine by immersing the film-coated substrates in a solution of glucamine (50 mM, in DMSO). Figure 4 shows a plot of the water contact angles of these films immediately after fabrication (dark gray bars) and after treatment with glucamine (light gray bars). The average contact angles for the azlactone-containing films prior to treatment with glucamine were measured to be between 55° and 65°, which is similar to the contact angles reported previously for PEI/PVDMA films.[25]

The average contact angles of PEI/PVDMA films treated with glucamine were significantly reduced and were measured to be ~14°, which is consistent with our previous reports.[29] The water contact angles of films containing each of the copolymers poly(MMA_x-*co*-$VDMA_y$) also decreased after modification with glucamine, however, to a lesser extent than films fabricated from PVDMA. As shown by the light gray bars in Figure 4, the contact angles of glucamine-treated films depended on the relative content of MMA and VDMA in the polymer used to fabricate the film. As the MMA content in the polymer increased, the contact angles of glucamine-treated films increased, which reflects an increase in the content of the hydrophobic MMA component (and a similar decrease in the hydrophilic, glucamine-functionalized component) in the film. These results are consistent with a decrease in the amount of glucamine presented at the surface of the copolymer films as the content of the unreactive component (i.e., MMA) in the copolymer increases, and suggest that the surface properties of these films can be modulated by changes in the content of reactive groups in the films.

Cell Adhesion on Copolymer-Containing Multilayered Films

The ability to control how cells and other biological systems interact with surfaces is of significant interest for many applications in medicine, tissue engineering, and other biotechnological applications. In a past study, we demonstrated that mammalian cells grow and proliferate readily on the surfaces of substrates coated with PEI/PVDMA films.[29] In contrast, treatment of PEI/PVDMA films with glucamine prevents the adhesion of mammalian cells for at least one month in culture. Here, we sought to determine whether PEI/PVDMA and PEI/poly(MMA_x-co-$VDMA_y$) films functionalized with glucamine resulted in differences in the ability of mammalian cells to adhere and grow on film-coated substrates.

For these experiments, we patterned films with small, circular areas of glucamine by placing a small drop of a glucamine solution (1 μL of a 20 mg/mL solution in DMSO) on the surfaces of each of the films for 2 hours, followed by liberal rinsing with DMSO and ethanol (the areas around the glucamine-treated spots were left unmodified). Using this experimental approach, we were able to characterize the attachment and growth of cells both on untreated areas of the films (i.e., areas presenting azlactone and methyl methacrylate functionality) and on glucamine-treated areas of the film. Figure 5 shows a series of fluorescence micrographs of COS-7 cells attached and growing on glucamine-spotted films (cells were stained with Calcein AM prior to imaging to aid in characterization of the viability, locations, and morphologies of the cells).[29] Changes in the numbers and behavior of cells as a function of the azlactone content are shown from left to right in the figure. Changes in the numbers and locations of these cells on glucamine-spotted films as a function of time are shown from top to bottom in the figure.

Figures 5A–5D show fluorescence micrographs of COS-7 cells acquired 48 hours after seeding cells on glucamine-treated films. These images clearly show circular regions in which fewer cells have attached relative to the surrounding areas (which, in contrast, reveal a population of cells that is nearly confluent). Closer inspection of these images reveals that the numbers of cells within the circular glucamine-treated regions increases as the relative azlactone-content in the film decreases. Glucamine-treated regions on PEI/PVDMA (homopolymer) films (Figure 5A) showed no cells adhered within the treated area, which is consistent with our past reports.[29] The glucamine-functionalized areas of PEI/poly(MMA_{25}-co-$VDMA_{75}$) and PEI/poly(MMA_{50}-co-$VDMA_{50}$) films showed increased cell attachment relative to the homopolymer film (Figures 5B and 5C, respectively), however, these cells were generally rounded and did not exhibit a spread morphology typical of well-adhered cells, such as those on the unmodified regions of the film outside of the glucamine-treated spot. PEI/poly(MMA_{75}-co-$VDMA_{25}$) films exhibited the largest number of cells attached within the glucamine-treated spot (Figure 5D); most cells within the glucamine-modified areas of these films were not rounded and were generally well-spread. These results suggest that decreasing the content of reactive azlactone groups within the polymer films reduces the density of glucamine functionality presented at the surface and effectively creates surfaces that are less resistant to cell adhesion.

To investigate the longer-term behavior of cells growing on these glucamine-treated, copolymer-containing films, we allowed the cells shown in Figures 5A–5D to grow and

proliferate continually on film-coated substrates for two weeks. For these experiments, cell culture media was exchanged periodically and cells were stained every four or five days with Calcein AM to visualize the locations, numbers, and viabilities of the cells. Figures 5E–5H show representative fluorescence microscopy images of cells cultured on each of the glucamine-treated films for seven days. In all cases, cells have continued to grow in the untreated areas on each of the films. The glucamine-modified regions of the PEI/PVDMA and PEI/poly(MMA_{25}-*co*-$VDMA_{75}$) films continue to resist the adhesion and overgrowth of cells into the treated region (Figures 5E and 5F). In contrast, cells have completely overgrown the glucamine-treated regions on both PEI/poly(MMA_{50}-*co*-$VDMA_{50}$) and PEI/poly(MMA_{75}-*co*-$VDMA_{25}$) films (Figures 5G and 5H) after seven days in culture. After 10 days, treated areas of PEI/poly(MMA_{25}-*co*-$VDMA_{75}$) films (Figure 5J) remain largely devoid of cells, however, cells have begun to penetrate and overgrow the boundary between the glucamine-treated and untreated areas. After two weeks, cells have completely overgrown the glucamine-treated spot on PEI/poly(MMA_{25}-*co*-$VDMA_{75}$) films and formed nearly a monolayer of cells on the treated area (Figure 5N). As shown in Figures 5I and 5M, the glucamine-modified areas of PEI/PVDMA films continue to resist the adhesion and overgrowth of cells within the treated region for two weeks, as previously reported.[29]

The results of these cell-based experiments demonstrate that thin films fabricated using copolymers containing various ratios of MMA:VDMA and functionalized with the small molecule D-glucamine result in surfaces that exhibit varying degrees of resistance to cell adhesion. The numbers of cells that attach initially to the surfaces are influenced by the relative content of methacrylate and azlactone functionality in the polymer used to fabricate the reactive multilayered films. In particular, as the number of reactive groups present in the polymer used to fabricate the film increases, film-coated surfaces become more resistant to the attachment of cells when functionalized with glucamine. These results suggest that increasing the azlactone content (and decreasing the quantity of unreactive, hydrophobic MMA groups) in the polymer used to fabricate the films results in a higher density of glucamine presented at the surface, thus permitting more efficient resistance to cell adhesion. In addition, these treated polymer films exhibit differences in the rates at which cells overgrow the modified regions as a function of time. These experiments were conducted in serum-containing media and it is possible that, over time, copolymer-containing films do not have a sufficiently high density of glucamine to resist long-term fouling by proteins in the media, which, in turn, could promote the overgrowth of cells. We are currently investigating mechanisms for these differences in cell adhesion and growth over time. However, the results of these current experiments demonstrate that it is possible to control the numbers of cells both spatially and temporally by fabricating reactive polymer multilayers using azlactone-functionalized copolymers.

Summary and Conclusions

We have reported an approach to the fabrication of reactive polymer multilayers that is based on the reactive layer-by-layer assembly of azlactone-functionalized copolymers and the primary amine functionalized polymer branched PEI. We demonstrated that the growth and thicknesses of films fabricated from MMA:VDMA copolymers is dependent upon the number of reactive groups in the azlactone-functionalized polymer. In general, as the

azlactone content in the polymer decreases, film growth and thickness decrease. The number of azlactone groups within the film also determines the extent to which the films can be modified post-fabrication. For example, functionalization of these copolymer-containing thin films with the hydrophilic small molecule glucamine results in surfaces with properties (such as wettability and resistance to the adhesion of cells) that are determined by the relative ratio of reactive azlactone groups to unreactive MMA groups in the copolymer. In addition, glucamine-functionalized copolymer-containing films show differences in the rates at which cells overgrow treated areas and, thus, the fabrication of copolymer-containing films may provide an approach to controlling the locations and numbers of cells on coated surfaces.

Our results demonstrate that reactive copolymers can be used to fabricate thin films with controllable physicochemical properties. The ability to incorporate copolymers into these reactive multilayers presents opportunities to further tune the physical, chemical, and mechanical properties of these thin films. We are currently expanding the range of materials that can be incorporated into these thin films to provide fundamental insights into this system and the capacity to precisely control surface properties of interest in a broad range of applications.

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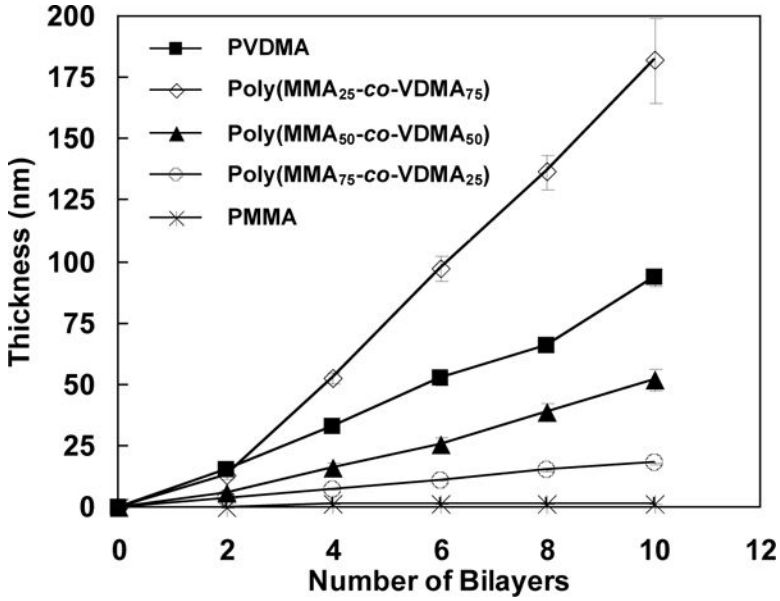


Figure 1.

Plot of ellipsometric thickness versus the number of bilayers for PEI/PVDMA, PEI/Poly(MMA_x-*co*-$VDMA_y$), and PEI/PMMA films deposited on silicon.

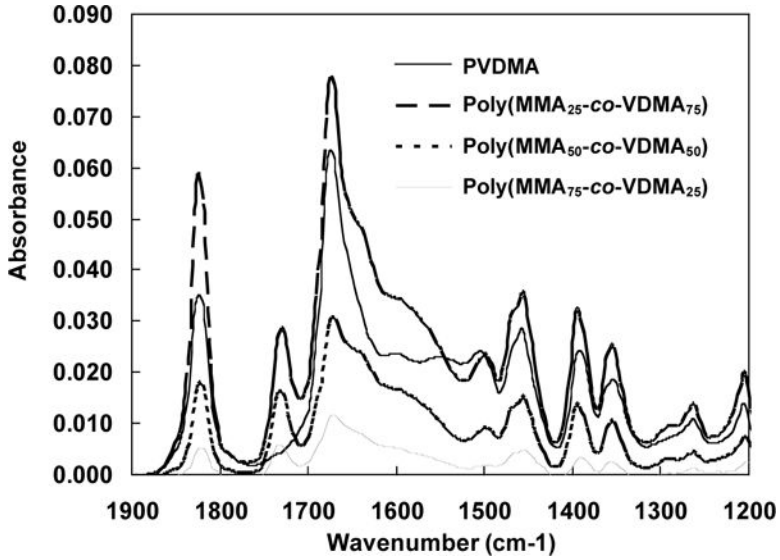


Figure 2.

PM-IRRAS spectra of 10-bilayer PEI/PVDMA and PEI/poly(MMA_x-*co*-$VDMA_y$) films deposited on gold-coated silicon.

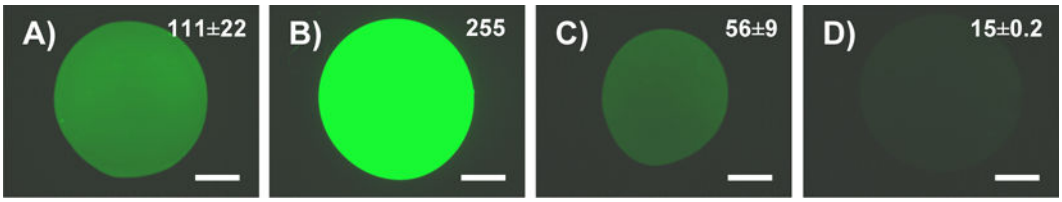


Figure 3.

A–D) Fluorescence micrographs of (A) a PEI/PVDMA film, (B) a PEI/poly(MMA_{25}-*co*-$VDMA_{75}$) film, (C) a PEI/poly(MMA_{50}-*co*-$VDMA_{50}$) film, and (D) a PEI/poly(MMA_{75}-*co*-$VDMA_{25}$) treated with a small drop of a concentrated solution of the amine-functionalized fluorophore dansyl cadaverine. The exposure time was selected such that the fluorescent spot in D was visible and could be compared to the spots shown in A, B, and C. This adjustment resulted in the spot shown in image B, which contained more fluorophore (see text), appearing saturated relative to those shown in images A, C, and D. The value shown in the upper right corner of each image represents the average grayscale intensity measured for three different dansyl cadaverine spots on each film. Scale bars = 500 μm.

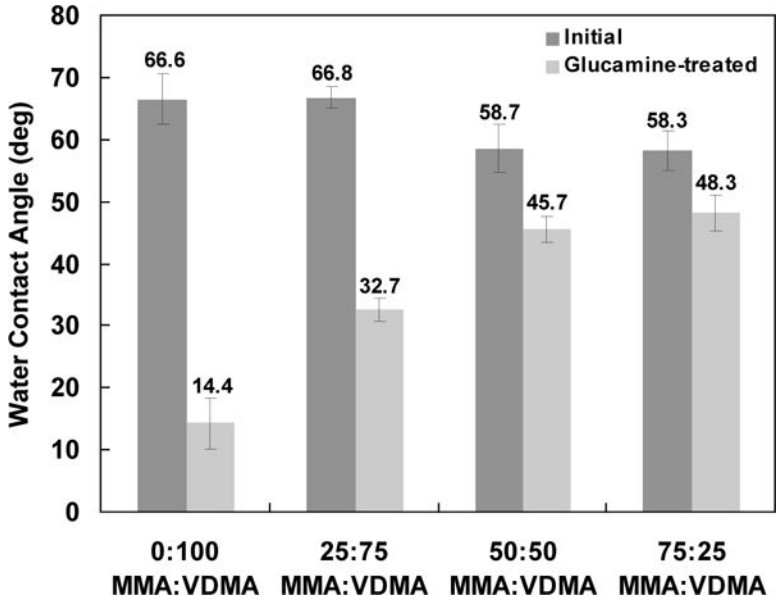


Figure 4.

Static water contact angles measured for PEI/PVDMA and PEI/poly(VDMA$_x$-*co*-MMA$_y$) films. Contact angles were measured for 10-bilayer films prior to (dark gray bars) and after (light gray bars) functionalization with glucamine. Contact angles were measured using a 5 μL water droplet.

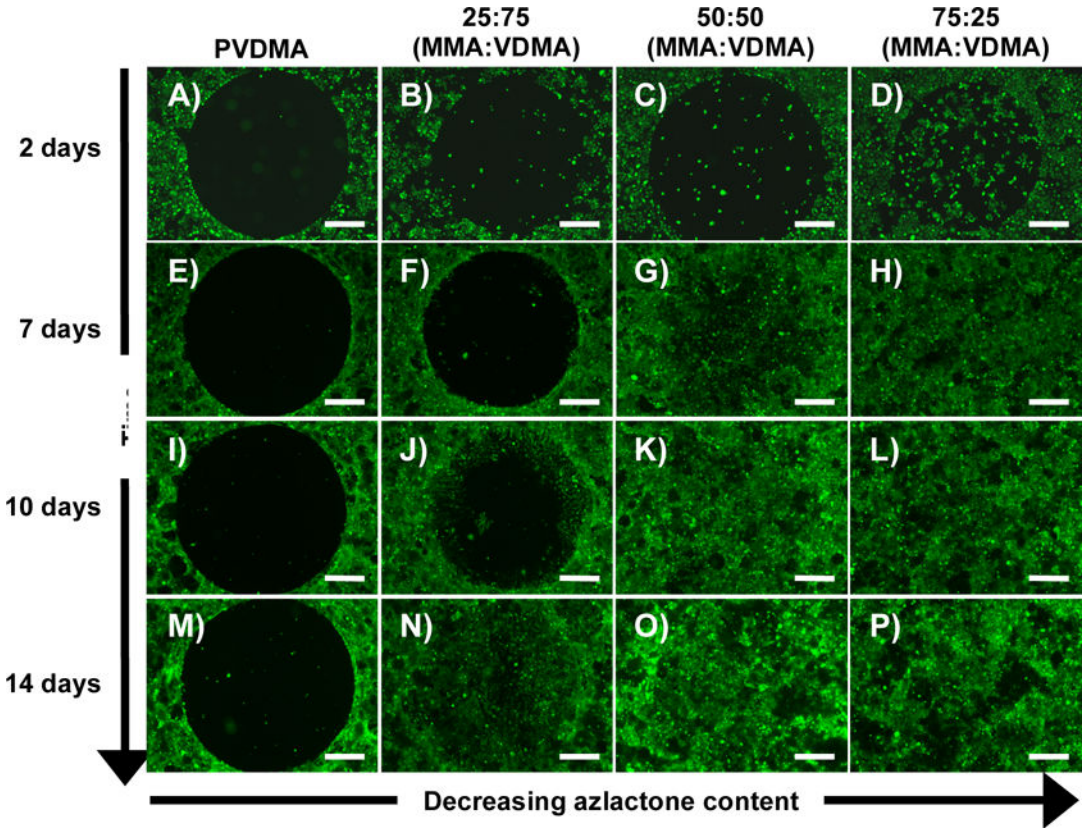
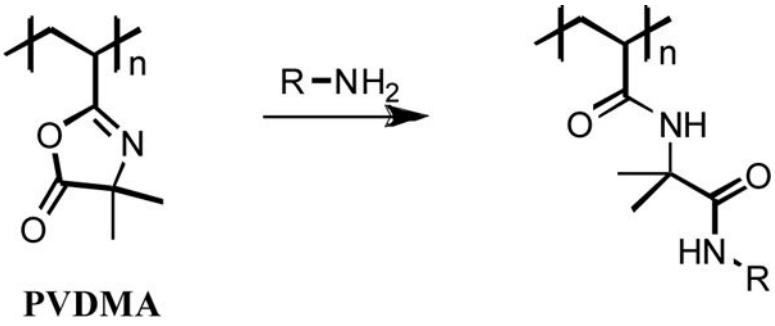


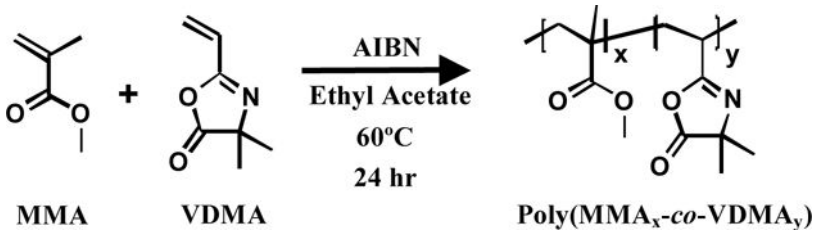
Figure 5.

Fluorescence micrographs of COS-7 cells seeded on a PEI/PVDMA film (A,E,I,M), a PEI/poly(MMA_{25}-*co*-$VDMA_{75}$) film (B,F,J,N), a PEI/poly(MMA_{50}-*co*-$VDMA_{50}$) film (C,G,K,O), and a PEI/poly(MMA_{75}-*co*-$VDMA_{25}$) film (D,H,L,P). All films were patterned with a small drop of glucamine (~1 μL) prior to seeding cells. The cells were stained with Calcein AM and imaged periodically over the course of 2 weeks. Note: The content of reactive azlactone groups within the films decreases from left to right and changes in the numbers and locations of cells over time is shown from top to bottom. Scale bars = 500 μm.



PVDMA

Scheme 1.
Reaction of PVDMA with a primary amine.



Scheme 2.
Conventional free radical polymerization of MMA and VDMA.

Table 1

MMA/VDMA Copolymerization Characteristics

Polymer	[MMA]/[VDMA] In the feed	[MMA]/[VDMA] Ratio in polymer[a]	M_n[b]	PDI[b]
PVDMA	0/100	0/100	20,400	3.2
Poly(MMA_{25}-*co*-$VDMA_{75}$)	25/75	27/73	5,800	3.3
Poly(MMA_{50}-*co*-$VDMA_{50}$)	50/50	45/55	12,100	3.8
Poly(MMA_{75}-*co*-$VDMA_{25}$)	75/25	82/18	13,300	3.4

[a] ^{1}H-NMR spectroscopy was used to calculate the ratio of MMA/VDMA in the copolymers by comparison of the peak areas of the peaks centered at 3.60 ppm (–OCH_3 of MMA) and 2.5 ppm (–C(CH) from VDMA).

[b] Molecular weights and polydispersities were determined using GPC and measured relative to poly(styrene) standards.