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Layer-by-Layer Assembly of Amine-Reactive Multilayers Using an Azlactone-Functionalized Polymer and Small-Molecule Diamine Linkers

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Abstract

We report the reactive layer-by-layer assembly of amine-reactive polymer multilayers using an azlactone-functionalized polymer and small-molecule diamine linkers. This approach yields crosslinked polymer/linker-type films that can be further functionalized, after fabrication, by treatment with functional primary amines, and provides opportunities to incorporate other useful functionality that can be difficult to introduce using other polyamine building blocks. Films fabricated using poly(2-vinyl-4,4-dimethylazlactone) (PVDMA) and three model non-degradable aliphatic diamine linkers yielded reactive thin films that were stable upon incubation in physiologically relevant media. In contrast, films fabricated using PVDMA and varying amounts of the model disulfide-containing diamine linker cystamine were stable in normal physiological media, but were unstable and eroded rapidly upon exposure to chemical reducing agents. We demonstrate that this approach can be used to fabricate functionalized polymer microcapsules that degrade in reducing environments, and that rates of erosion, extents of capsule swelling, and capsule degradation can be tuned by control over the relative concentration of cystamine linker used during fabrication. The polymer/linker approach used here expands the range of properties and functions that can be designed into reactive PVDMA-based coatings, including functionality that can degrade, erode, and undergo triggered destruction in aqueous environments. We therefore anticipate that these approaches will be useful for the functionalization, patterning, and customization of coatings, membranes, capsules, and interfaces of potential utility in biotechnical or biomedical contexts and other areas where degradation and transience are desired. The proof of concept strategies reported here are likely to be general, and should prove useful for the design of amine-reactive coatings containing other functional structures by judicious control of the structures of the linkers used during assembly.

TOC Image

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Supporting Information. Supporting information including the results of additional physical characterization of multilayer-coated silicon substrates and hollow multilayer capsules can be found in online version at doi:

Introduction

Covalent or ‘reactive’ layer-by-layer assembly—a process in which two mutually reactive building blocks are alternately and iteratively deposited on a surface—presents a useful framework for the fabrication of thin, polymer-based multilayer films.^{1–4} In contrast to conventional approaches to layer-by-layer assembly, which typically lead to multilayers assembled through multivalent weak interactions (e.g., ionic interactions, hydrogen bonding, etc.),^{5–9} reactive assembly leads to covalently crosslinked polymer films that are generally more stable in harsh media and chemically complex environments.^{1–4} This approach to assembly also leads to coatings that contain residual reactive functionality that can serve as a useful chemical ‘handle’ to immobilize additional functionality or tune bulk and interfacial properties.^{1–4} The physical, chemical, and mechanical properties of these covalent assemblies, the extents to which they can be modified post-fabrication, the nature of the chemical crosslinks, and the conditions under which they remain stable or can be induced to degrade are all, in large measure, dependent upon the functional properties of the reactive building blocks used during fabrication and the nature of the reactive groups used to drive assembly.^{1–4} A variety of reactive materials have been investigated to manipulate these variables, with the majority of past studies focused on the investigation of mutually reactive macromolecular building blocks to fabricate polymer/polymer-based multilayers of interest and potential utility in many different fundamental and applied contexts.^{1–4}

The work reported here was motivated by past studies in our group on reactive layer-by-layer assembly using the azlactone-functionalized polymer poly(2-vinyl-4,4-dimethylazlactone) (PVDMA; Figure 1) as a reactive building block.^{10–17} Azlactones react rapidly, through ring-opening reactions, with primary amine-functionalized nucleophiles under mild conditions and without the generation of reaction byproducts.^{13,18} When paired with a polymer building block containing primary amines, PVDMA thus provides a useful synthon for reactive assembly. In past studies, we have used poly(ethyleneimine) (PEI) as a model amine-functionalized polymer.^{10,13} This approach leads to PEI/PVDMA-based multilayer films that are covalently crosslinked by unique ‘amide/amide’-type bonds that are hydrolytically stable.^{10,13} This approach also leads to coatings that contain residual azlactone functionality that can be used for further functionalization (e.g., by simple treatment with other amine-based nucleophiles) to install or pattern new chemically or biologically relevant surface features.^{14,19} This combination of features renders PEI/PVDMA multilayers useful as platforms for the design of new types of functional and customizable nano/biointerfaces that are stable in harsh and chemically complex environments (e.g., in high ionic strength media, at extremes of pH, and in the presence of high concentrations of surface-active species).^{11,14,17,19}

While the stability of these materials is useful in many potential applications, the hydrolytically stable ‘amide/amide’ bonds that comprise the crosslinks in these coatings can serve as an obstacle to their use in other applications where degradation, erosion, or environmental transience would be useful. As a step toward the design of new azlactone-functionalized multilayers that degrade in aqueous environments, we recently reported a backbone degradable azlactone-functionalized copolymer that can be used as a building block for covalent assembly (in this case, reactive assembly leads to the formation of hydrolytically stable amide/amide-type bonds, and subsequent polymer backbone hydrolysis promotes film degradation and disassembly).²⁰ As an alternative approach, we also recently reported the fabrication of degradable multilayers by the reactive layer-by-layer assembly of non-degradable PVDMA and chemically or enzymatically degradable polyamine building blocks (this approach leads to reactive films that degrade gradually either by chemical hydrolysis or upon the addition of enzymes that can degrade the polyamine components of the films).²¹ These approaches are straightforward to implement, and lead to polymer/polymer-based multilayers with new functional properties well suited for applications in biological, biomedical, biotechnological, or environmental contexts.

In this current study we sought to explore the feasibility of using PVDMA to fabricate degradable multilayers by developing strategies that introduce chemically degradable crosslinks. Because azlactones can also react with hydroxyl functionality to yield hydrolytically degradable ‘amide/ester’-type functionality, one possible approach to the design of multilayers with degradable crosslinks would be to pair PVDMA with a polymer containing hydroxyl side groups. Although such an approach is chemically feasible, we note that the ring opening of azlactones by hydroxyl functionality typically requires higher temperatures and the use of a catalyst and, in general, proceeds more slowly than reactions between azlactones and primary amines.^{18,22,23}

As an alternative to that approach, we report here a strategy for the design of covalently crosslinked, azlactone-functionalized multilayers fabricated using PVDMA and small-molecule diamine linkers. This approach represents a departure from past studies on the design of PVDMA-based polymer/polymer multilayers and yields reactive assemblies that are crosslinked by either chemically stable or chemically labile groups depending on the structure of the linker used during assembly. This overall approach thus preserves practical benefits conferred by reactive assembly, but yields new polymer/small-molecule-type coatings that either remain stable or undergo triggered degradation upon exposure to environments that can cleave their labile crosslinks. We demonstrate proof-of-concept of this approach using PVDMA, model non-degradable alkyldiamine linkers, and a model disulfide-containing diamine linker (cystamine). We demonstrate further that this latter approach can be used to design surface coatings or hollow microcapsules that undergo triggered or stimuli-responsive degradation upon exposure to chemical reducing agents that can cleave disulfide bonds. The work reported here extends the range of structures and functions that can be designed into reactive multilayer assemblies using PVDMA, and opens the door to new designs of reactive thin films and degradable coatings, capsules, and biointerfaces that can degrade, erode, or undergo triggered or stimuli-responsive destruction in aqueous environments.

Materials and Methods

Materials

2-Vinyl-4,4-dimethylazlactone (VDMA) was a gift from Dr. Steven M. Heilmann (3M Corporation, Minneapolis, MN). Poly(2-vinyl-4,4-dimethylazlactone) (PVDMA, MW ~18,000; $\bar{D}$ = 3.1) was synthesized by the free-radical polymerization of VDMA, as described previously.¹² Cystamine hydrochloride purum (98.0%), 2,2'-azoisobutyronitrile (AIBN), 3-(aminopropyl)triethoxysilane (APTES; 98.0%), tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 98%), ethylene diamine, butylene diamine, hexamethylene diamine, and acetonitrile were purchased from Aldrich Chemical Company (Milwaukee, WI). Cystamine (Cys) was prepared by the deprotonation of cystamine hydrochloride, as described previously.²⁴ Glutathione (GSH, 98%, reduced) and 3-dimethylaminopropylamine (DMAPA, 99%) were purchased from Acros Organics (New Jersey, USA). SiO₂-Research microparticles (diameter = 5.06 ± 0.44 μ m) were purchased from Bangs Laboratories, Inc. (Indiana, USA). Tetramethylrhodamine (TMR) cadaverine (TMR_{cad}) was purchased from Invitrogen (Oregon, USA). PVDMA labeled with TMR (0.5 mol%; referred to from hereon as PVDMA_{TMR}) was synthesized as described previously.¹² Test grade n-type 100 mm silicon wafers were obtained from Silicon, Inc. (Boise, ID). Compressed air used to dry substrates and films after fabrication was filtered through a 0.2 μ m nylon membrane syringe filter. Phosphate buffer (PB, 0.1 M) used for stability/degradation studies was prepared by mixing 77.4 mL of disodium hydrogen phosphate (Na₂HPO₄, 0.2 M) with 22.5 mL of monobasic sodium phosphate (NaH₂PO₄, 0.2M) and diluting with MilliQ water to a final volume of 1.0 L; the pH of the buffer was found to be 7.42. All materials were used as received without further purification unless otherwise noted.

General Considerations

Gel permeation chromatography (GPC) was performed using a GPCmax-VE2001Solvent/Sample module (Viscotek Corp., Houston, TX) and two PlusPore Organic GPC Columns (Polymer Laboratories, Amherst, MA) in series. For the characterization of PVDMA, 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 OminiSEC 4.5 software package. Molecular weights and dispersities ($\bar{D}$) are reported relative to monodisperse polystyrene standards. Silicon substrates (e.g., 1.0×5.0 cm) were cleaned with acetone, ethanol, methanol, and deionized water and dried under a stream of compressed air prior to the fabrication of multilayer films. Prior to film fabrication, substrates were coated with an aminosilane coating to improve adhesion of the multilayers to their substrates during long-term incubation in aqueous media. Briefly, APTES was deposited onto the silicon surface from a 1% solution in anhydrous toluene for 1 hour, followed by rinsing with toluene and ethanol. Optical thicknesses of films fabricated on silicon substrates were characterized 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 multilayered films. Thicknesses were determined using at least three substrates in at least five different locations on each substrate and are presented as averages with standard deviations. Optical and fluorescence microscopy images were acquired using an Olympus

IX70 microscope and analyzed using MetaMorph Advanced version 7.7.8.0 (Universal Imaging Corporation). Images were processed using NIH Image J software. Polarization modulation infrared reflectance-absorbance spectroscopy (PM-IRRAS) was conducted in analogy to previously reported methods.^{25,26} 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).^{25,26} Coated silicon substrates 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. A BD Accuri C6 flow cytometer (Ann Arbor, MI) was used to characterize the degradation of capsules. All flow cytometry measurements were performed at room temperature. A fixed volume of sample was characterized at each time point, and samples were pumped through the flow cytometer at a flow rate of 14 µL/min.

Fabrication of Amine-Reactive 'PVDMA/Linker' Multilayers on Planar Substrates

For the fabrication of non-degradable films, solutions of linker **1**, linker **2**, linker **3**, and PVDMA were prepared in acetonitrile (20 mM; for PVDMA, this concentration was calculated with respect to the molecular weight of the polymer repeat unit). Films were then fabricated by reactive/covalent layer-by-layer assembly on aminosilane-treated silicon substrates or gold-coated silicon substrates using the following general protocol: (i) substrates were submerged in a solution of PVDMA for 5 seconds, (ii) substrates were removed and submerged in succession into two acetonitrile rinse solutions for 5 seconds, (iii) substrates were immersed in a solution of difunctional linker **1**, **2**, or **3** for 5 seconds, and (iv) substrates were removed and rinsed again as described in step (ii). This cycle was repeated until a desired number of PVDMA/linker layer pairs had been deposited. Films fabricated in this manner are denoted using the notation (PVDMA/linker)_x, where x is the number of PVDMA/linker layer pairs deposited. (PVDMA/Cys)_x films were fabricated using the general protocol above, with the difference that in addition to linker **4** (Cys) different concentrations of monofunctional DMAPA (linker **5**) were added to decrease the number of disulfide bonds in the film (see text). Films fabricated in this manner are denoted using the notation (PVDMA/Cys_z)_x, where z denotes the mole percentage (i.e., 25%, 50%, 75%, and 100%) of Cys in the Cys/DMAPA solution mixture used to fabricate the film. After fabrication, films were immediately rinsed with a stream of acetonitrile, dried under a stream of filtered air, and stored in a vacuum desiccator prior to characterization or use in other experiments. For experiments aimed at characterization of film growth profiles by ellipsometry, films were dried after every 5 or 10 cycles of the above procedure using filtered compressed air. Samples to be used in film stability and erosion experiments were either used immediately or were dried under a stream of filtered compressed air and stored in a vacuum desiccator until use. All film fabrication procedures were performed at ambient room temperature.

Fabrication of Hollow Multilayer Microcapsules

Solutions of PVDMA_{TMR} and Cys/DMAPA were prepared in acetonitrile (20 mM; for PVDMA_{TMR}, this concentration was calculated with respect to the molecular weight of the polymer repeat unit). SiO₂ microparticles were placed into microcentrifuge tubes and rinsed using 1 mL of acetonitrile prior to the fabrication of multilayers. The first layer of PVDMA_{TMR} was deposited onto the SiO₂ particles by adding 1 mL of the PVDMA_{TMR} solution to the particle suspension and manually shaking the particles for 1 minute to allow sufficient time for the polymer to adsorb to the particle surface. The particles were then centrifuged for two minutes at 1500 rpm. The supernatant was removed by pipette and the particles were rinsed two times by re-suspending them in 1 mL of acetonitrile and vortexing. After each rinse in acetonitrile, the particles were centrifuged for two minutes at 1500 rpm and the supernatant was removed. The second layer of the multilayered film was fabricated by adding 1 mL of a Cys/DMAPA solution to the particle suspension and shaking for 30 seconds to allow sufficient time for the small molecules to react with the previously deposited PVDMA_{TMR} layer. The particles were then rinsed two times with 1 mL of acetonitrile. Subsequent layers were fabricated by repeating this process, alternately adding PVDMA_{TMR} or Cys/DMAPA solutions and allowing each layer to react for 30 seconds, until the desired number of (PVDMA_{TMR}/Cys)_x layer pairs (typically 8) were deposited onto the particle surface. After every two layer pairs, particles were placed in a new microcentrifuge to minimize aggregation. After film fabrication, coated particles were washed with acetonitrile and placed into 1 mL of DMAPA in acetonitrile (20 mM) with gentle shaking for 1 hour. Coated particles were then rinsed with acetonitrile three times and finally dispersed in 1 mL of deionized water prior to etching the silica particle templates. The functionalized polymer-coated silica template particles were treated with 5 M HF for 10 minutes to etch the silica cores. *Warning! Extreme care should be taken when handling HF. HF solutions and vapors are extremely poisonous and corrosive and may cause extreme burns that are not immediately painful. Handle with extreme caution in a chemical fume hood, and use appropriate protective equipment (gloves, face/eye protection, laboratory coat, etc., and neutralize waste appropriately).* The resulting functionalized polymer capsules were washed five times with water and characterized by fluorescence microscopy.

Post-Fabrication Functionalization of Reactive Polymer Multilayers

Azlactone-containing multilayers were covalently functionalized by treatment with solutions of primary amine-containing nucleophiles. For functionalization with the fluorophore TMR_{cad}, film-coated substrates were patterned in small circular regions by treatment with small droplets (1 µL) of a TMR_{cad} solution (1 mg/mL in DMSO). An identical treatment protocol was used in control experiments for films treated with solutions of non-reactive TMR. For functionalization with propylamine, film-coated substrates were immersed in solutions of propylamine (50 mM in acetonitrile) for two hours followed by rinsing with acetonitrile. For experiments designed to characterize film stability and/or degradation, all reactive films were further functionalized with DMAPA (50 mM in acetonitrile) for one hour at room temperature, followed by rinsing with fresh acetonitrile. Films characterized by PM-IRRAS fabricated on gold-coated substrates were 50 layer pairs thick.

Characterization of Film Stability and Erosion Profiles

Experiments designed to characterize the stability of multilayers in various environments were performed in the following general manner. Degradation was characterized in the presence and absence of GSH (5 mM) and TCEP (1 mM) in phosphate buffer (pH = 7.4) at 37 °C. For experiments using planar substrates, film-coated silicon substrates were placed in a UV-transparent cuvette and 1.0 mL of the corresponding aqueous solution was added to completely cover the film-coated region of the substrates. The optical thicknesses of the films were then measured using ellipsometry at pre-determined time intervals, and the samples were returned to the cuvette. For experiments to characterize the degradation of (PVDMA_{TMR}/Cys) microcapsules, capsules were suspended in MilliQ water and TCEP was added. At specified time intervals, the number of capsules in a defined volume of the capsule suspension was quantified using flow cytometry and fluorescence microscopy, in analogy to methods described previously.^{27,28}

Results and Discussion

Our approach to the covalent assembly of polymer multilayers containing chemically degradable crosslinks exploits reactions between the azlactone groups in PVDMA and the amine functionality in non-polymeric, small-molecule diamines (Figure 1A). This overall approach yields reactive azlactone-containing multilayers that contain hydrolytically stable ‘amide/amide’ bonds (formed by reactions between the diamines and the azlactone functionality in PVDMA; Figure 1B) connected by short ‘linkers’ (denoted as ‘R’ groups in Figure 1B) with properties and chemical stabilities that can be tuned via changes to the structures of the diamines. As noted above, this strategy presents a departure from past studies on the use of polymeric amines as building blocks for the reactive assembly of polymer/polymer-based multilayers using PVDMA,^{10–12,14,29} and creates opportunities to fabricate PVDMA/linker-type multilayer coatings with new structures and functional properties. This study sought to (i) explore the feasibility of this PVDMA/linker approach to design crosslinked and stable amine-reactive multilayer coatings, and (ii) develop approaches to the design of crosslinked and reductively degradable amine-reactive multilayers by using small diamines containing reductively-degradable disulfide bonds.

Characterization of Film Growth and Reactivity using PVDMA and Aliphatic Small-Molecule Diamine Linkers

Past studies have reported the reactive layer-by-layer assembly of crosslinked multilayers using combinations of reactive polymers and small, non-polymeric linkers functionalized with mutually reactive functional groups.^{30–33} It was not clear at the outset of these studies, however, whether this approach could be used to promote and sustain multilayer growth using the azlactone-based chemistry associated with PVDMA, or whether films fabricated using this polymer/linker-based approach would contain reactive azlactone functionality sufficient for post-fabrication functionalization and the installation of new surface features. To explore the feasibility of this approach, we conducted an initial series of proof of concept studies using PVDMA and three model diamines containing 2-, 4-, or 6-carbon aliphatic spacers (linkers 1–3; Figure 1A).

We performed a series of experiments to characterize the growth profiles of PVDMA/linker films fabricated on the surfaces of reflective silicon substrates. Figure 2A shows a plot of optical thickness, as determined by ellipsometry, as a function of linker structure and the number of PVDMA/linker deposition cycles deposited on planar silicon substrates (for these experiments, all silicon substrates were pre-treated with APTES to install primary amine groups and promote the covalent immobilization of PVDMA; see Materials and Methods and past studies for additional details). Inspection of these results reveals film growth to occur in manner that was approximately linear for all three linkers. Films fabricated using the linker with the shortest aliphatic spacer (linker **1**; closed diamonds) were substantially thinner (~60 nm thick after 50 PVDMA/linker cycles) than coatings fabricated using longer linkers **2** (closed squares) and **3** (closed triangles), which reached thicknesses of ~135 nm and ~100 nm, respectively, after 50 PVDMA/linker deposition cycles.

These results reveal linker length to have an influence on layer-by-layer growth. Although the reasons for these differences are not entirely clear, this influence could arise, at least in part, from differences in the degree to which these difunctional linkers may be able to react non-productively (with respect to promoting film growth) with two surface-immobilized azlactone groups during each deposition cycle, which would reduce the number of free amine groups available to support the subsequent reactive deposition of a new incoming layer of PVDMA (and, thereby, impact the overall extent of covalent crosslinking in a film). In the cases of longer linkers **2** and **3**, at least, these observed differences in film growth are less likely to result from differences or changes in the nucleophilicities of the amine groups, either before or after reaction with PVDMA. We note that the thicknesses of films fabricated using all three PVDMA/linker combinations investigated here are substantially lower than those of analogous PVDMA/polymer-based films reported in past studies (e.g., films fabricated using PVDMA and the primary amine-containing polymer poly(ethyleneimine) (PEI) are ~60 nm thick after the deposition of only 10 PVDMA/PEI layer pairs,¹⁰ an outcome that we attribute to the larger size and multivalent nature of that polymeric building block compared to the small-molecule linkers used here).

We used polarization-modulation infrared reflectance-absorbance spectroscopy (PM-IRRAS) to characterize PVDMA/linker films fabricated on gold-coated silicon substrates. Figure 2B shows representative spectra for PVDMA/linker **1**, PVDMA/linker **2**, and PVDMA/linker **3** films, and reveals an absorbance peak at 1826 cm⁻¹ in each film corresponding to the carbonyl group of the azlactone ring in PVDMA.¹⁰ Further inspection also reveals a peak in each spectrum at ~1650 cm⁻¹ that is characteristic of the amide bond that forms when PVDMA reacts with primary amines.¹⁰ When combined, these results are consistent with covalent crosslinking, and demonstrate that assembly occurs in a manner that leaves a significant amount of unreacted azlactone functionality. The results of additional experiments demonstrated that this unreacted functionality can be used to covalently immobilize additional functionality, post-fabrication, by simple treatment with solutions containing primary amine-based compounds.

Figure 2C shows a representative fluorescence microscopy image of a silicon substrate coated with a PVDMA/linker **1** film after treatment with a small droplet of the fluorophore TMR_{cad} in DMSO (see Materials and Methods for additional information). The presence of

bright red fluorescence in this image is consistent with the covalent immobilization of this amine-functionalized fluorophore (the intensity of red fluorescence did not diminish after repeated additional rinsing with solvent). Figure 2D shows an otherwise identical control film that was treated exhaustively with propylamine to consume all remaining azlactone functionality prior to treatment with TMR_{cad}. The absence of fluorescence in that film further suggests that the fluorescence observed in Figure 2C arises from covalent immobilization, and not from physisorption of the fluorophore. These results are generally consistent with the behaviors of azlactone-functionalized polymer/polymer-type multilayer coatings fabricated using PEI as a building block,¹⁰ and suggest opportunities to pattern new chemical motifs or tune the interfacial (e.g., wetting) behaviors of these reactive PVDMA/linker-based coatings using a range of other amine-based nucleophiles.

Fabrication of PVDMA/Linker Coatings Using a Disulfide-Containing Diamine Linker

The diamine linker approach used above yielded multilayers that were physically stable for at least seven days upon incubation in physiologically relevant media (PBS, pH = 7.4, 37 °C; see Figure S1 of the Supporting Information) owing to the non-degradable nature of the PVDMA backbone and the hydrolytically stable nature of the amide/amide-type bonds (e.g., Figure 1B) that form during reactive assembly. We hypothesized that this linker-based approach could also be used to design films containing degradable crosslinks by using small diamine building blocks that contain degradable linkages (e.g., as opposed to the non-degradable aliphatic linkages in linkers **1–3** used above). To explore the potential of this approach, we conducted a series of experiments with films fabricated using PVDMA and the model disulfide-containing diamine cystamine (Cys; linker **4**; Figure 1A). Based on the results above, we reasoned that this approach would yield reactive azlactone-functionalized multilayers crosslinked by disulfide bonds (Figure 1B, R = —S—S—) that would be stable in non-reducing aqueous environments, but degrade upon exposure to chemical reducing agents to promote film erosion (Figure 3A). This disulfide-based strategy for the design of reductively degradable crosslinked multilayers has been used in many past studies to design environmentally responsive materials.^{34–37} Several of those past studies have relied on the use of thiol- or disulfide-functionalized polymers that can form disulfide bonds during the assembly of polymer/polymer films³⁶ or subsequently be crosslinked, after assembly, by exposure to oxidizing agents.^{35,37} Other approaches have relied on the post-fabrication treatment of hydrogen-bonded multilayers with short disulfide-containing linkers to introduce stabilizing crosslinks that can be cleaved by reducing agents to promote film erosion and release incorporated agents.^{38–41}

Figure 3B shows a plot of optical thickness versus the number of layers of PVDMA deposited during the fabrication of PVDMA/Cys multilayers (closed circles; as indicated in Figure 3B, we also refer to coatings fabricated in this manner as ‘(PVDMA/Cys₁₀₀) multilayers’, for reasons described in greater detail below). These results reveal films fabricated using this disulfide-containing linker to grow in a linear manner, similar to that exhibited using aliphatic linkers **1–3**, to a thickness of ~40 nm after 20 PVDMA/Cys deposition cycles. The results of additional experiments shown in Figure S2 demonstrate that these (PVDMA/Cys₁₀₀) films contain residual reactive azlactone functionality and can be functionalized by treatment with amine-functionalized fluorophores in a manner similar to

that shown in Figure 2C. Interestingly, films fabricated in this manner remained stable and did not decrease in thickness when they were exposed to aqueous solutions containing the chemical reducing agent GSH (5 mM) for up to 24 hours at 37 °C. The stability of these films over the first 120 minutes of incubation in the presence of GSH is shown in the results plotted in Figure 3C; closed circles; these (PVDMA/Cys₁₀₀) films also remained stable upon exposure to higher concentrations of GSH (for up to 12 hours at concentrations as high as 50 mM) and upon addition of stronger reducing agents (e.g., TCEP; data not shown).

The reasons for the apparent stability of these (PVDMA/Cys₁₀₀) films in reducing media are not clear, but this behavior could result, at least in part, from restricted or hindered access of these reducing agents into these crosslinked materials. Subsequent experiments demonstrated that reductively-degradable multilayers could be fabricated—and that relative rates of film erosion could also be tuned—by adopting an alternate assembly strategy in which films were fabricated using solutions of (i) PVDMA and (ii) mixtures of Cys and a second mono-functional amine (instead of fabricating films using PVDMA and Cys alone, as described above). In all other experiments described below, we used linker **5** (DMAPA; Figure 1A) as a model monofunctional amine; this compound contains only one primary amine capable of reacting with the azlactone groups of PVDMA, and thus does not act as a covalent crosslinking agent during reactive assembly. Films fabricated using this approach are identified from here on using the notation (PVDMA/Cys_z)_x, where ‘z’ denotes the mole percentage of Cys in the Cys/DMAPA solution mixtures used to fabricate the film, and ‘x’ indicates the number of PVDMA/linker deposition steps. For example, a film having the nominal structure (PVDMA/Cys₇₅)₂₀ indicates a film fabricated by 20 cycles of deposition using a solution of PVDMA and a second solution of linker containing 75 mol% Cys and 25 mol% DMAPA.

The results shown in Figure 3B show that films fabricated using PVDMA and mixtures of Cys/DMAPA grow in a linear manner, and that this approach leads to films that increase in thickness more rapidly (reaching ~100 nm after 20 deposition cycles) than those fabricated using Cys alone (~40 nm thick after 20 PVDMA/Cys deposition cycles, as described above; Figure 3B). Variation of the mol% of Cys in these Cys/DMAPA solutions from 25% to 75% did not lead to significant differences in overall film thickness (Figure 3B), but did result in large differences in the rates at which the resulting films eroded upon incubation in solutions of GSH (5 mM; 37 °C; Figure 3C). For example, as shown in Figure 3C, (PVDMA/Cys₂₅)₂₀ films (closed squares), fabricated using the lowest concentration of Cys, eroded almost completely over a period of ~30 minutes. In contrast, films fabricated using higher amounts of Cys [both (PVDMA/Cys₅₀)₂₀ and (PVDMA/Cys₇₅)₂₀; closed triangles and closed diamonds, respectively] degraded more slowly. Overall, these results are consistent with the reductive cleavage of disulfide crosslinks in these materials and subsequent processes of physical film erosion that lead to film disassembly (additional control experiments revealed the thicknesses of these films to remain stable for up to a week when incubated under otherwise identical conditions in the absence of GSH; see Figure S3 of the Supporting Information).

We note here that it is possible that the primary amines in Cys and DMAPA may react with the azlactones in PVDMA at different rates during film fabrication. It must therefore be

borne in mind, in this context, that the relative amounts of Cys and DMAPA (and, thus, the number of disulfide crosslinks) in the multilayers reported here may be different from those of the linker solutions used during film assembly. Our current results do not permit quantitative or absolute measures of the mole percentages of Cys and DMAPA that are incorporated into these materials using this solution-mixture approach. Nevertheless, our results demonstrate that manipulation of the compositions of the Cys/DMAPA solutions used to fabricate these materials provides useful means to exert control over the functional properties of these materials (e.g., to tune erosion profiles, with films fabricated using lower concentrations of Cys eroding more rapidly than films fabricated using higher concentrations of this reductively degradable crosslinker; Figure 3C). These results are consistent with the view that changes in the relative concentrations of Cys and DMAPA during assembly lead to changes in the amount of crosslinking present in the films. Finally, we note that the degradation and erosion of these disulfide-crosslinked multilayers can also be triggered by the addition of other agents that are capable of reducing disulfide bonds. Additional experiments characterizing the influence of TCEP on the stability and degradation of hollow (PVDMA/Cys)_Z)_X multilayer microcapsules are described in the section below.

Fabrication of Reactive and Reductively Degradable Multilayer Microcapsules

We performed a final series of experiments to characterize the behaviors of hollow, disulfide crosslinked microcapsules fabricated by the reactive assembly of (PVDMA/Cys)_Z)_X films on sacrificial silica microparticle templates (Figure 4A). For these experiments, we used solutions of PVDMA and Cys/DMAPA similar to those used for fabrication on planar substrates, with the exception that PVDMA labeled with TMR was used to facilitate characterization of layer-by-layer film growth using fluorescence microscopy. Figure 4B shows fluorescence intensity as a function of the number of deposition cycles during the fabrication of (PVDMA/Cys)₅₀)₈ films on the surfaces of the microparticles (the insets show representative fluorescence micrographs of film-coated microparticles; see Figure S4 of the Supporting Information for additional fluorescence micrographs of microparticles characterized in these experiments). The increases in fluorescence intensity observed here are consistent with layer-by-layer film growth.

Etching of the microparticle templates yielded hollow (PVDMA/Cys)_Z)_X multilayer microcapsules that swelled, when suspended in deionized water, to sizes that correlated with differences in the mole percentage of Cys used during fabrication. As shown in Figure 5A, capsules fabricated using solutions of Cys alone [e.g., (PVDMA/Cys)₁₀₀)₈ films] did not swell substantially; these capsules had average sizes of ~5 μm (Figure 5E) that were similar to the silica microparticle templates on which they were fabricated (diameter = 5.06 ± 0.44 μm). However, capsules fabricated using decreasing concentrations of Cys [e.g., 75, 50, and 25 mol%; (PVDMA/Cys)₇₅)₈, (PVDMA/Cys)₅₀)₈, and (PVDMA/Cys)₂₅)₈ films, respectively], swelled to average sizes of 8 μm, 9.6 μm, 10.7 μm (Figures 5B–E). We attribute these differences in swelling to differences in the relative levels of covalent crosslinking (as discussed above), as well as the likelihood of repulsive interactions among covalently bound tertiary amine groups (which arise from the reaction of DMAPA with PVDMA during assembly, and that would be partially protonated under these conditions).

These (PVDMA/Cys₂)₈ capsules remained stable upon incubation in aqueous media for up to 10 days, as determined by the absence of changes in swelling and the shapes and integrities of the microcapsules as monitored by visual inspection using fluorescence microscopy (Figure S5). With the exception of capsules fabricated using (PVDMA/Cys₁₀₀)₈ films, however, these capsules swelled and dissolved rapidly (within several minutes) when TCEP was added (at a concentration of 1 mM; at RT), as depicted in the micrographs shown in Figure 6A–D for representative (PVDMA_{TMR}/Cys₅₀)₈ capsules (changes in swelling as a function of time determined by characterization of these fluorescence microscopy images are also shown in Figure 6E). We also characterized suspensions containing large populations of (PVDMA_{TMR}/Cys₅₀)₈ capsules using a flow cytometry protocol used in past studies to characterize changes in the number of degradable multilayer capsules as a function of time.^{27,28} The results of those experiments, shown in Figure 6F, reveal the percentage of capsules in a defined volume of suspension to decrease gradually, and almost completely, over a period of 10 minutes after the addition of TCEP (1 mM; at RT). These results are also consistent with the rapid, TCEP-triggered degradation of these capsules.

The results of experiments to characterize the TCEP-promoted swelling and degradation of capsules having other compositions [(PVDMA/Cys₁₀₀)₈, (PVDMA/Cys₇₅)₈, (PVDMA/Cys₅₀)₈, and (PVDMA/Cys₂₅)₈] are shown in Figures S6 and S7 of the Supporting Information. In general, the results of those studies reveal rates of capsule degradation to correlate to relative degrees of Cys used during fabrication, with (PVDMA/Cys₂₅)₈ capsules degrading more rapidly than (PVDMA/Cys₇₅)₈ capsules. Capsules fabricated using Cys alone [(PVDMA/Cys₁₀₀)₈ films] did not swell (as noted above) or degrade substantially over a period of up to 30 minutes under these conditions (Figure S6J–K). These results are generally consistent with the results of studies described above and shown in Figure 3C using GSH and (PVDMA/Cys₁₀₀)₂₀ films fabricated on planar substrates.

Overall, our results demonstrate that this degradable-linker approach can be used to design amine-reactive organic multilayers that are stable in physiologically relevant media, but degrade rapidly upon exposure to reducing agents that can cleave the disulfide crosslinks in these materials. The work reported here was designed to demonstrate proof of concept of this approach using a model azlactone-functionalized polymer and a model disulfide-containing diamine linker. This overall strategy, however, is likely to be general. We anticipate that this linker-based approach could also be exploited to fabricate amine-reactive coatings crosslinked by hydrolytically degradable units (e.g., by using diamine analogs that contain amine-stable acetal groups) or by using difunctional, small-molecule linkers that contain terminal hydroxyl or terminal thiol groups (which can also react with azlactone functionality to yield cleavable amide/ester- or amide/thioester-type bonds²⁰ that are structural analogs of the more stable amide/amide-type bonds shown in Figure 1B and exploited in this study). Finally, we note that the layer-by-layer and sacrificial core-based approach to the design of hollow capsules used here has been used in numerous past studies to encapsulate, transport, and deliver many different types of macromolecular agents.^{42,43} The materials and approaches reported here thus also provide new tools for the design of capsules that could promote the active, passive, or triggered release of encapsulated agents, for example in response to reducing environments encountered in biological systems (e.g., in

intracellular environments)^{34,44} or in response to the active addition of reducing agents.^{27,28,35}

Conclusions

We have reported the reactive layer-by-layer fabrication of polymer multilayers using an amine-reactive azlactone-functionalized polymer and small-molecule diamine linkers. This approach leads to thin azlactone-containing polymer/linker-type films that can be further functionalized, post-fabrication, by treatment with primary amine-functionalized nucleophiles, in analogy to polymer/polymer-based coatings reported in past studies, but also provides opportunities to incorporate new functionality that can be difficult to introduce using polyamine building blocks. For example, whereas films fabricated using PVDMA and non-degradable diamine linkers were stable upon incubation in physiologically relevant media, films containing varying amounts of the model disulfide-containing diamine linker cystamine were (i) stable in normal physiological media, but (ii) unstable upon exposure to chemically reducing media. The addition of GSH or TCEP, two reducing agents that can rapidly cleave disulfide bonds, lead to rapid film degradation and erosion. This approach can also be used to design hollow, crosslinked, and degradable polymer microcapsules that swell in aqueous media and degrade upon exposure to reducing environments. Our results demonstrate that rates of film erosion, extents of capsule swelling, and rates of capsule degradation can be tuned by control over the relative concentration of cystamine used during film fabrication.

Past studies demonstrate that amine-reactive multilayers fabricated using PVDMA and non-degradable amine-containing building blocks can be useful for the functionalization and patterning of surfaces and biointerfaces that are stable in physiological media. The polymer/linker-type approach used here creates opportunities to expand the range of properties and functions that can be designed into PVDMA-based coatings, and provides new means to design reactive materials that degrade chemically, erode physically, and undergo triggered destruction in aqueous media or biological environments. We therefore anticipate that these approaches will be useful for the design of new types of reactive and customizable coatings, membranes, capsules, and interfaces of potential utility in biotechnical or biomedical applications (e.g., for active or passive over the release of active agents) and in other areas where degradation and environmental transience are useful. The proof of concept strategies reported here have the potential to be general, and should also be useful for the design of amine-reactive coatings containing hydrolytically degradable groups or other functional structures by judicious choice of the difunctional linkers used during assembly.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) Chemical structures of the azlactone-containing polymer (PVDMA) and the primary amine-containing bifunctional linkers [non-degradable linkers **1** (ethylenediamine), **2** (butylenediamine), and **3** (hexamethylenediamine); disulfide-containing linker **4** (cystamine); and monofunctional linker **5** (DMAPA)] used for covalent/reactive layer-by-layer assembly. (B) Schematic illustration showing a covalent crosslink between two PVDMA chains formed by the ring opening of the azlactone groups of PVDMA with a diamine linker.

Figure 2.

(A) Plot of ellipsometric thickness versus the number of PVDMA layers deposited for the fabrication of PVDMA/Linker 1 (◆), PVDMA/Linker 2 (■) and PVDMA/Linker 3 (▲) films on aminosilane-treated silicon substrates. (B) Plot showing representative PM-IRRAS spectra for (PVDMA/Linker 1)₅₀ (dotted line), (PVDMA/Linker 2)₅₀ (dashed line), and (PVDMA/Linker 3)₅₀ (solid line) films deposited on gold-coated silicon substrates. (C–D) Fluorescence microscopy images of (C) a native (azlactone-containing) PVDMA/Linker 1 film and (D) a propylamine-treated PVDMA/Linker 1 film, both fabricated on silicon

substrates, after treatment with drop of a solution containing the amine-functionalized fluorophore TMR_{cad} (red). Scale bars represent 500 μm .

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Figure 3.

(A) Schematic illustration showing a covalently-crosslinked and azlactone-functionalized polymer multilayer (shown in green) fabricated by reactive/covalent layer-by-layer assembly using an azlactone-functionalized polymer PVDMA and disulfide-containing diamine linker **4**; the reduction of the disulfide bonds present in the linker **4** component of the covalently crosslinked films results in film disruption and erosion. (B) Plot of ellipsometric thickness versus the number of PVDMA layers deposited during the fabrication of (PVDMA/Cys₂₅) (■), (PVDMA/Cys₅₀) (◆), (PVDMA/Cys₇₅) (▲), and (PVDMA/Cys₁₀₀) (●) films on aminosilane-treated silicon substrates. (C) Plot of normalized ellipsometric thickness versus time for (PVDMA/Cys₂₅)₂₀ (■), (PVDMA/Cys₅₀)₂₀ (◆), (PVDMA/Cys₇₅)₂₀ (▲), and (PVDMA/Cys₁₀₀)₂₀ (●) films incubated in GSH solutions (5 mM) at 37 °C. Error bars represent the standard deviation of measurements obtained using three identically prepared substrates.

Figure 4.

(A) Schematic illustration showing a silica microparticle (black) coated with a (PVDMA_{TMR}/Cys) multilayer film functionalized by treatment with linker **5** (red), followed by removal of the silica core template to yield hollow disulfide-crosslinked capsules. (B) Plot showing the relative fluorescence intensity of (PVDMA_{TMR}/Cys)_x coated silica microparticles as a function of the number of PVDMA_{TMR} layers deposited. Inset images show representative fluorescence microscopy images of (PVDMA_{TMR}/Cys)_x microcapsules where $x = 4, 6$, and 8 . Error bars represent the standard deviations of measurements obtained from characterization of at least 50 different capsules.

Figure 5. (A–D) Representative fluorescence microscopy images of hollow (A) (PVDMA_{TMR}/Cys₁₀₀)₈, (B) (PVDMA_{TMR}/Cys₇₅)₈, (C) (PVDMA_{TMR}/Cys₅₀)₈, and (D) (PVDMA_{TMR}/Cys₂₅) microcapsules in MilliQ water. (E) Plot showing the diameters of the microcapsules versus the mole percentage of cystamine in the films (see text). Scale bars = 10 μm. Error bars represent the standard deviations of measurements obtained by analyzing the diameters of at least 50 different capsules.

Figure 6.

(A–D) Representative fluorescence microscopy images of (PVDMA_{TMR}/Cys₅₀)₈ microcapsules at various times after the addition of TCEP. Capsule degradation was initiated by the addition of TCEP (1 mM) to a dispersion of the microcapsules in MilliQ water. (E) Plot showing the increase in the diameters of the microcapsules as a function of time after the addition of TCEP. (F) Plot showing the percentage of microcapsules remaining in a defined volume of a microcapsule suspension, as characterized using flow cytometry, as a function of time after the addition of TCEP. All experiments were performed at room

temperature. Scale bars = 10 μm . Error bars represent the standard deviations of measurements obtained by analyzing the diameters of at least 50 different capsules.

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