Published in final edited form as:
J Polym Sci A Polym Chem. 2011 April 1; 49(7): 1572–1581. doi:10.1002/pola.24578.

Design and Synthesis of a Fluorescently End-Labeled Poly(β-amino ester): Application to the Characterization of Degradable Polyelectrolyte Multilayers

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Abstract

We report the synthesis of a fluorescently end-labeled analog of a synthetic and degradable cationic poly(β-amino ester) (PBAE; polymer **1**) used in past studies for the delivery of DNA and the layer-by-layer assembly of erodible polyelectrolyte multilayers (PEMs). The synthesis of an analog of polymer **1** having acrylate functionalized end groups provided a platform for the introduction of fluorescent labels by post-polymerization conjugate addition of amine-functionalized fluorophores. This approach enabled the synthesis of fluorescently end-labeled polymer (polymer $\mathbf{1_{FL}}$) with molecular weights and polydispersities (M_n = 18,000; PDI ~1.8) similar to those used in past studies for the fabrication of PEMs using polymer **1**. Layer-by-layer assembly of PEMs using polymer $\mathbf{1_{FL}}$ and poly(styrene sulfonate) enabled characterization of film erosion and, for the first time, direct observation of the release of cationic polymer from these assemblies using fluorescence microscopy and fluorometry. Our results shed new light on the behaviors of the cationic components of these PEMs and could prove useful for the design of thin films for a range of different controlled release applications. Our results also provide new fluorescent cationic polymer probes that could be useful for characterization of the behaviors of PBAEs in other fundamental or applied biotechnological contexts.

Keywords

Cationic polymer; Degradable polymers; Thin films; Polyelectrolytes; Layer-by-layer

Introduction

Fluorescently labeled polyelectrolytes are useful for the characterization of charged polymers in a variety of contexts, ranging from the study of fundamental physical behaviors in solution to the development of new tools for applications in biology and biotechnology. Fluorescently labeled polymers, in general, facilitate characterization of polymer behavior using techniques such as fluorometry, fluorescence microscopy, and flow cytometry that provide information about locations and dynamics that can be impossible (or at least more difficult) to obtain using unlabeled materials. A substantial challenge associated with the design of fluorescently labeled polyelectrolytes, however, is the development of methods for the introduction of fluorescent moieties to these ionic materials in ways that do not perturb the behaviors of the polymers (for example, methods that do not significantly increase or

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Supporting Information Available. Additional details of polymer characterization. Additional supporting information may be found in the online version of this article.

reduce the net charge of a polymer or render it more hydrophobic than an unlabeled polymer, etc.). The work reported here takes a step toward addressing these broader issues through the design of fluorescently end-labeled analogs of a hydrolytically degradable cationic polymer used widely for the design of polyelectrolyte-based assemblies of interest in a variety of biomedical contexts.

This investigation was motivated broadly by our interest in the design of thin films and polyelectrolyte assemblies fabricated using cationic poly(β-amino ester)s (PBAEs).[1-3] PBAEs are a class of synthetic and hydrolytically degradable polyamines originally developed and investigated extensively as cationic polymer agents for the delivery of DNA.[4-6] Past studies by our group and others have demonstrated that these degradable polyelectrolytes can also be used to fabricate ultrathin multilayered polyelectrolyte films (or 'polyelectrolyte multilayers', PEMs) on surfaces by the alternating, layer-by-layer deposition[7] of these polyamines with a range of different anionic polymers. Because PBAEs are hydrolytically degradable, this approach to film fabrication can also be used to design thin, polymer-based coatings that erode in aqueous media and promote the release of incorporated anionic polymers.[1-3] This general approach has been used to design PBAE-containing films that promote the controlled and surface-mediated release of DNA,[8-16] peptides and proteins,[13,17-19] and a range of other agents.[20-29] Other approaches to the design of PEMs capable of mediating the release of biological molecules and other agents have been reviewed comprehensively.[30-32]

Many of the past studies mentioned above describing PEMs fabricated using PBAEs have used polymer **1** as a model degradable polycation. Our own work with polymer **1** has focused largely on the fabrication of PEMs using anionic polymers such as plasmid DNA (to design films useful for the localized delivery of DNA to cells)[8-12,15,16] or sodium poly(styrene sulfonate) (SPS; useful as a model for physicochemical characterization and fundamental studies of film erosion).[22-24,26] These past studies have demonstrated that the ester bonds in polymer **1** play an important role in promoting film erosion,[23] and that it is possible to tune film erosion (and the release of film components) over periods ranging from several days to several weeks by changing the structure of the polymer used to fabricate the films (e.g., by increasing the hydrophobicity of the polymer backbone[17,23,24,27,29] or by changing side chain structure[11]).

In general, the studies noted above reporting physicochemical characterization of PEMs fabricated using polymer **1** have focused largely on characterization of (i) changes in film thickness during film fabrication and erosion, (ii) changes in micrometer- and nanometer-scale surface structures, and (iii) changes in the rates at which incorporated agents (such as DNA, SPS, or proteins) are released into solution. In contrast, relatively little information exists about the behaviors and fates of the cationic PBAEs used to fabricate these assemblies. This dearth of knowledge has resulted, at least in part, from the lack of methods for the synthesis of analogs of PBAEs containing labels that facilitate imaging or other forms of physicochemical characterization during or after film assembly. Important questions therefore remain regarding the locations of the cationic polymer components of these films, the rates at which they may be released upon film erosion, and the nature of subsequent interactions between these polymers and the anionic components of the films following release (e.g., whether cationic and anionic components are released as separate entities or whether they are released as electrostatically assembled interpolyelectrolyte complexes; this latter question is particularly relevant, for example, in the context of the development of these films as platforms for the delivery of DNA to cells).[3,33] The ability to probe and/or image directly the locations of PBAEs in solution and at interfaces would contribute broadly to an understanding of the nature of the physical interactions in these multicomponent assemblies and help guide the design of new generations of these materials

for different potential applications. More generally, these methods would also provide new tools useful to other groups investigating PBAEs as non-viral agents for the nanoparticle-based delivery of DNA and other agents to cells.[5,6]

We recently reported an approach to the synthesis of a fluorescently labeled analog of polymer **1** by incorporating a primary amine-functionalized fluorophore into the backbone of polymer **1** during step-growth polymerization.[34] This 'copolymer'-based approach was suitable for characterization of the behavior of polymer **1** during the decomposition of DNA-containing PEMs (e.g., using confocal microscopy). In general, however, it was difficult to define with precision the amounts and locations of the labels incorporated into the polymer backbone, and the introduction of a third co-monomer during synthesis made it difficult to synthesize fluorescent polymer analogs with structures and properties (e.g., molecular weights) that were representative of samples of unlabeled polymer **1** synthesized by conventional routes. This investigation sought to develop an approach to the synthesis of fluorescently labeled analogs of polymer **1** that would (i) permit the incorporation of fluorophores in well defined locations, (ii) limit the number of fluorophores installed per chain (to reduce the potential influence of these labels on polymer behavior), and (iii) provide convenient routes to samples of labeled and unlabeled polymers having similar molecular weights and molecular weight distributions.

This study is presented in two parts. In the first part, we describe methods for the synthesis of fluorescently end-labeled analogs of polymer **1** via post-polymerization functionalization of samples of polymer **1** having reactive acrylate-functionalized end groups. Characterization by gel permeation chromatography and gel electrophoresis reveals that this approach can be used to install fluorescent labels in a well defined manner without significantly altering important parameters such as molecular weight and polydispersity. In the second part, we demonstrate that fluorescently end-labeled polymer **1** can be used to fabricate erodible PEMs and characterize the behaviors of these materials in aqueous environments using a range of different fluorescence-based analytical techniques. The results of this study shed light on the behaviors of the cationic components of these PEMs during assembly and erosion and provide insight that could prove useful for the design of thin films for a range of different controlled release applications. Our results also provide tools that could be useful for characterization of PBAEs in other fundamental and applied contexts.

Materials and Methods

Materials

4,4′-Trimethylenedipiperidine was purchased from Aldrich Chemical Co. (Milwaukee, WI). 1,4-Butanediol diacrylate was purchased from Alfa Aesar Organics (Ward Hill, MA). Sodium acetate buffer was purchased from Accugene (Rockland, ME). Poly(sodium 4-styrene sulfonate) (SPS) was purchased from Acros Organics (Geel, Belgium). Tetramethylrhodamine cadaverine was purchased from Invitrogen (Eugene, OR). Test-grade n-type silicon wafers were purchased from Silicon Inc (Boise, Idaho). Commercially available linear poly(ethylene imine) (LPEI, MW = 25,000) was purchased from Polysciences, Inc. (Warrington, PA). Phosphate-buffered saline (PBS) was prepared by dilution of commercially available concentrate (EM science, Gibbstown, NJ). All materials were used as received without further purification unless noted otherwise. Deionized water (18 MΩ) was used for washing steps and to prepare all buffer and polymer solutions. LPEI and SPS solutions used to fabricate base layers were filtered through a 0.2 μm membrane syringe filter prior to use. Compressed air used to dry films and coated substrates was filtered through a 0.2 μm membrane syringe filter.

General Considerations

^{1}H NMR spectra were recorded on a Bruker AC+ 300 (300.135 MHz) spectrometer. Chemical shift values are reported in parts per million and are referenced to residual protons from solvent. Gel permeation chromatography (GPC) measurements were made using a Waters 515 HPLC pump (Waters Corporation, Milford, MA), a Rheodyne model 7725 injector with a 20 μL injection loop, and two Waters Styragel HT 6E columns in series. Tetrahydrofuran (THF) containing 0.1 M triethylamine was used as the eluent with a flow rate of 1.0 mL/min. Data were collected using a Waters 2410 refractive index detector and processed using the Waters Empower software package. Molecular weights are reported relative to monodisperse polystyrene standards. Silicon substrates used for multilayer film fabrication and erosion experiments were cleaned with acetone, ethanol, methanol, and water and dried using 0.2 μm filtered compressed air. Silicon surfaces were then activated by etching with an oxygen plasma for 5 min (Plasma Etch, Carson City, NV) prior to film fabrication. The optical thicknesses of films 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 thickness was calculated assuming an average index of refraction of 1.577 for the multilayered films. Thickness was measured at five locations on the films and is presented as an average value (with standard deviation) determined from the measurement of three different films. All films were rinsed with water and dried using air passed through a 0.2 μm filter prior to measurement. UV/vis absorbance values for PBS solutions used to characterize the release of SPS from films were recorded using a Beckman Coulter DU520 UV-vis spectrophotometer (Fullerton, CA). Absorbance values for SPS were recorded at a wavelength of 226 nm. Solution fluorescence measurements were made using a Jobin Yvon FluoroMax-3 fluorometer at an excitation wavelength of 543 nm and reporting the emission as the average of values collected at a wavelength of 581 – 585 nm. The pH of buffers used for erosion and hydrolysis experiments was measured using a pH meter.

Synthesis of Polymer 1 and Fluorescently Labeled Polymer $\mathbf{1_{FL}}$

To synthesize polymer **1**, 4,4′-trimethylene dipiperidine (3.020 mmol) and 1,4-butanediol diacrylate (3.019 mmol) were weighed out separately and dissolved in anhydrous THF (5 mL). The solution of diacrylate was added to the solution of dipiperidine via pipette, and the resulting reaction solution was heated to 50 °C and stirred. After 48 hrs, approximately 2 mL of the reaction solution was removed and precipitated twice into hexanes (10 mL) and dried under vacuum to yield polymer **1** as a white solid. The remainder of the reaction solution was used to synthesize acrylate end-functionalized polymer. To this end, 1,4-butanediol diacrylate (100 μL, 98.7 mg, 0.498 mmol) was added and the resulting reaction solution was allowed to stir at 50 °C for an additional 10 hours. The reaction products were concentrated by rotary evaporation and precipitated three times into hexanes and dried under vacuum to yield polymer $\mathbf{1_{Acr}}$ as a white solid. For the synthesis of polymer $\mathbf{1_{FL}}$, a sample of polymer $\mathbf{1_{Acr}}$ (101.3 mg) was dissolved in anhydrous THF (1.3 mL) in a screw-capped vial. Tetramethylrhodamine cadaverine (TMR-cad; 3.5 mg) was dissolved in 200 μL of methanol, added to the dissolved polymer via pipette, and the vial was sealed with a Teflon-lined screw cap. The reaction solution was heated to 50 °C and stirred for 10 hours. The product was then precipitated once into hexanes and dried under vacuum overnight. The crude polymer (57.2 mg) was then dissolved in 0.5 M HCl (420 μL) and precipitated into a vigorously stirring aqueous solution of 0.2 M NaOH (2.5 mL). The resulting suspension was centrifuged and the resulting precipitate was washed 5 times with water. The final product was frozen using liquid nitrogen and lyophilized overnight to yield polymer $\mathbf{1_{FL}}$ as a pink solid. The ^{1}H NMR spectrum for polymer $\mathbf{1_{FL}}$ was similar to spectra of polymer **1** in previous reports. ^{1}H-NMR (300 MHz, $CDCl_3$, 25°C): δ = 1.18 (b m, 12H), 1.71 (b m, 8H), 1.94 (b m, 4H), 2.50 (b t, 4H), 2.63 (b t, 4H), 2.88 (b m, 4H), 4.10 (b t, 4H) ppm.

Preparation of Polyelectrolyte Solutions

Solutions of polymer **1** and polymer $\mathbf{1_{FL}}$ (5 mM with respect to the molecular weight of the repeat unit) were prepared in sodium acetate buffer (100 mM, pH = 4.9), as described in past studies.22,23 Solutions of SPS used for the deposition of polymer **1**/SPS and polymer $\mathbf{1_{FL}}$/SPS layers (20 mM with respect to the repeat unit of the polymer) were prepared in 0.067 mM HCl as described in past reports.24 Solutions of LPEI and SPS used for the fabrication of LPEI/SPS base layers (20 mM with respect to the molecular weight of the polymer repeat unit) were prepared using 10 mM NaCl solution in 18 MΩ water. LPEI solutions contained 4 mM HCl to aid polymer solubility.

Agarose Gel Electrophoresis

Solutions of polymer $\mathbf{1_{FL}}$ (2 mg/mL) were prepared in 100 mM acetate buffer and approximately 5-10 μL of these solutions were added to 15 μL of a glycerol:water loading buffer (50:50, v/v). These samples were then loaded into the wells of a 1% agarose gel for analysis (HEPES 20 mM, pH 7.2, 95 V, 1 hr). The resulting gels were visualized using a transilluminator.

Fabrication of Polyelectrolyte Multilayers

Films were fabricated on planar silicon substrates coated with thin multilayered films composed of 10 bilayers (or layer pairs) of LPEI and SPS (terminated with SPS) to provide a suitably charged surface for fabrication of polymer **1**/SPS films as previously reported.22,23 These precursor layers were fabricated using an automated dipping robot (Riegler & Kirstein GmbH, Potsdam, Germany). Multilayered films fabricated using polymer **1** or polymer $\mathbf{1_{FL}}$ were fabricated manually using an alternating dipping procedure similar to that previously described for the fabrication of unlabeled polymer **1**/SPS films.22,23 Briefly: (1) Substrates were submerged in a solution of cationic polymer (e.g., polymer **1** or polymer $\mathbf{1_{FL}}$) for 5 min, (2) substrates were removed and immersed in two sequential water baths for 1 minute each, (3) substrates were submerged in a solution of SPS for 5 min, and (4) substrates were rinsed again as described in step 2. This cycle was repeated until 8 bilayers of cationic polymer and SPS were deposited. For experiments used to characterize stepwise film growth, films were dried with 0.2 μm filtered air after every two deposition cycles for characterization by ellipsometry. Films used in erosion and release experiments were dried with filtered air and stored in a vacuum dessicator prior to use. All films were fabricated at room temperature.

Characterization of Film Erosion and Release Profiles

Film-coated substrates were placed in a plastic UV-transparent cuvette containing 1 mL of PBS (pH = 7.4, 137 mM NaCl; an amount sufficient to cover the film-coated portion of the substrates). Samples were then incubated at 37 °C and removed at predetermined intervals for characterization of film thickness using ellipsometry. Substrates were rinsed with water and dried with filtered air prior to measuring film thickness. The buffer solutions were characterized at each time point using UV/vis spectrophotometry (absorbance at 226 nm) and fluorometry (excitation: 543 nm and emission: 581 – 585 nm) to characterize the release of SPS and polymer $\mathbf{1_{FL}}$, respectively. Film-coated substrates were then placed in a new cuvette containing fresh PBS and returned to the incubator.

Characterization of Films Using Fluorescence Microscopy

Polymer $\mathbf{1_{FL}}$/SPS films eight bilayers thick were fabricated on the surfaces of planar glass microscope slides and imaged dry on a fluorescence microscope. A spot was then photobleached using the 40x objective until fluorescence reached a minimum. Films were imaged dry prior to erosion and then placed into a cuvette containing 1 mL of PBS. Low

magnification (4X) images of these submerged films were acquired at predetermined times during these experiments. ImageJ (NIH) was used to analyze and quantify the fluorescence intensity on a line drawn through the center of the photobleached spot at each time point.

Results and Discussion

Several past studies have used fluorescently labeled polyelectrolytes to characterize the behaviors of the cationic and anionic polymer components of PEMs. For example, labeled polyelectrolytes have been used to characterize vertical polymer diffusion during layer-by-layer assembly and thereby provide critical insight into mechanisms of exponential film growth observed for some weak polyelectrolyte systems.35-39 Fluorescently labeled polyelectrolytes have also permitted visualization and quantification of lateral diffusion of polyelectrolytes within PEMs,40-43 as well as the release or expulsion of polyelectrolytes from PEMs.11,44-46 In each of these past studies, fluorescently labeled polyelectrolytes have broadened the range of analytical techniques that could be used to characterize these materials and thus contributed to a more complete understanding of the structures and properties of these multicomponent assemblies.

In the context of designing tools to understand polyelectrolyte behavior, an ideal fluorescently labeled polyelectrolyte would (i) have a molecular weight and polydispersity similar to that of related unlabeled analogs it is designed to mimic, and (ii) contain a sufficient number of labels to allow it to be readily observed, but not so many as to alter significantly the behavior of the polymer by changing net charge or impacting other important properties (such as hydrophobicity or radius of gyration, etc.). Many of the past studies noted above have used fluorescently labeled polyelectrolytes synthesized by the post-polymerization conjugation of fluorescent labels to the backbones of conventional (unlabeled) polyelectrolytes.11,35,36,38-41,43-46 This approach has the important advantage of providing access to labeled polymers with molecular weights that are similar to samples of unlabeled polymer. However, it can be difficult to control with precision the number and distribution of fluorescent labels introduced to a polymer backbone using this approach (and one recent study demonstrates how this approach has the potential to lead to large differences in polyelectrolyte behavior during layer-by-layer assembly47). Alternative methods based on the direct incorporation of fluorescently labeled co-monomers during polymerization can also be limited in their ability to control the numbers and distributions of fluorescent labels incorporated into the structure of a polymer.

As part of a broader effort to address several of the issues noted above and develop methods for the synthesis of well-defined, fluorescently labeled polyelectrolytes, we recently reported an approach to the synthesis of fluorescently end-labeled samples of poly(acrylic acid) (PAA).48 This past work exploited methods for the controlled/living free-radical polymerization of vinyl monomers to design well-defined, end-labeled anionic polymers useful for characterization of the diffusion of PAA during the layer-by-layer assembly of PEMs. One important outcome of this past study was the observation that the incorporation of a single fluorescent label as an end group did not appear to influence significantly the behavior of the polymer (relative to unlabeled PAA) during layer-by-layer assembly.48 With respect to the goals of this current study, however, we note that this particular synthetic approach to the end-labeling of chain growth polymers cannot be used to design fluorescently labeled PBAEs because these degradable polymers are synthesized by step growth polymerization.

Synthesis and Characterization of Fluorescently End-Labeled Polymer 1

To develop an approach to the synthesis of high molecular weight samples of fluorescently labeled polymer **1**, we adopted and further modified an approach reported previously for the

synthesis of end-functionalized PBAEs.49 This past report used stoichiometric imbalances of diamine and diacrylate monomers (with an excess of diacrylate) during step growth polymerization to synthesize acrylate end-functionalized polymers. Subsequent studies demonstrated that this approach could be used to further modify polymer end groups by the conjugate addition of primary amine-functionalized molecules to the terminal acrylate functionality of the polymers.50¯52 This approach was also used to synthesize large libraries of different PBAE structures having amine-functionalized end groups useful for the delivery of DNA to cells.50¯52 This synthetic approach is, in general, also suitable for the introduction of a range of other amine-functionalized molecules (such as fluorophores) as polymer end groups. We note, however, that the use of stoichiometric imbalances during step growth polymerizations can serve to limit significantly the molecular weights of the resulting materials.53 While this past approach generated materials well-suited for polyplex-mediated DNA delivery, we sought to synthesize samples of polymer **1** with high molecular weights (e.g., M_n ~10,000 – 20,000) and PDIs that were more closely matched to those of polymers used in our past studies of polymer **1**-based PEMs.

Scheme 1 shows our general approach to the synthesis of high molecular weight, fluorescently end-labeled polymer **1**. We began with a conventional approach to the synthesis of polymer **1** by reacting stoichiometrically equivalent amounts of the diamine monomer 4,4′-trimethylene dipiperidine and the diacrylate monomer 1,4-butanediol diacrylate in THF at 50 °C.4 After 48 hours, a portion of the reaction mixture was precipitated into hexanes to yield polymer **1**. Characterization of this polymer by gel permeation chromatography (GPC; see Figure 1 and Table 1) revealed this polymer to have a number average molecular weight (M_n) of 21,900 and a polydispersity of 2.27.

In view of the mechanism of step growth polymerization, the end groups of each chain of the sample of polymer **1** isolated above should be functionalized with either an acrylate group or an amine group, or both (as denoted in Scheme 1 by the asterisks at the terminal ends of the polymer; we note that the possibility of backbone hydrolysis and the presence of impurities could also contribute to greater end group diversity). To convert all remaining diamine end groups to diacrylate functionality, the remainder of the reaction mixture described above was treated with an approximate 20% excess of diacrylate monomer (relative to the initial amount of diamine added) and allowed to stir for an additional 10 hours at 50 °C (Scheme 1). The resulting polymer was then precipitated into hexanes and dried under vacuum to yield a white polymer precipitate (referred hereafter as polymer $\mathbf{1_{Acr}}$). We used a large excess of diacrylate in this step of the reaction to promote the rapid reaction of amine-terminated polymer chains with free diacrylate and thus prevent, as much as possible, additional step growth polymerization that would result in changes in M_n and PDI relative to the samples of polymer **1** described above. As shown in Figure 1 and summarized in Table 1, the molecular weight of polymer $\mathbf{1_{Acr}}$ (M_n = 15,900) was similar to, albeit slightly lower than, that of the sample of polymer **1**, with a similar, broad molecular weight distribution (PDI = 2.22). This end-modified polymer was used directly for the synthesis of fluorescently end-labeled polymer.

Polymer $\mathbf{1_{Acr}}$ was dissolved in THF and treated with an excess of the amine-functionalized fluorophore tetramethylrhodamine cadaverine (TMR-cad) to synthesize fluorescently end-labeled polymer $\mathbf{1_{FL}}$ (Scheme 1). This reaction was allowed to proceed for 10 hours at 50 °C, and the resulting product was precipitated into hexanes and dried to yield a dark red solid. Initial characterization of this reaction product by agarose gel electrophoresis (see Figure S1 of the Supporting Information) revealed the presence of fluorescently labeled polymer as well as a large amount of unreacted TMR-cad that was not removed during precipitation. Dissolving the polymer in methylene chloride and washing the polymer solution several times with water failed to extract significant amounts of unreacted

fluorophore from the sample. However, we found that dissolving the crude reaction product in an aqueous solution containing 1.5 equivalents of HCl (with respect to the number of amine groups present in the polymer backbone) and then adding this solution to a cold and vigorously stirred solution of aqueous NaOH was sufficient to precipitate the polymer and remove all unreacted fluorophore. The precipitated polymer was washed multiple times with water and lyophilized to yield polymer $\mathbf{1_{FL}}$ as a light pink powdery solid. Characterization of this polymer by ^{1}H NMR spectroscopy did not reveal clearly defined aromatic resonances associated with TMR. However, additional characterization by agarose gel electrophoresis coupled with UV detection demonstrated that TMR was linked covalently to the polymer and that these samples were free of excess, unreacted fluorophore (see Figure S1 of the Supporting Information).

Characterization of polymer $\mathbf{1_{FL}}$ by GPC revealed average molecular weights and molecular weight distributions (M_n = 18,400; PDI = 1.83; Figure 1 and Table 1) that were generally similar to those of polymer $\mathbf{1_{Acr}}$. These results demonstrate that the aqueous precipitation and purification process used above (which exposed the polymer briefly to both acidic and alkaline environments) did not promote significant backbone hydrolysis or otherwise result in large changes in molecular weight that could impact the usefulness of this fluorescently labeled polymer as a model in additional studies described below. We comment here that, in general, our multi-step approach to the synthesis, isolation, and subsequent functionalization of polymer **1** provides opportunities to prepare end-functionalized PBAEs with molecular weights that are higher than those prepared using stoichiometric imbalances of monomer during polymerization as a means to define end group functionality.

Finally, we note that while we used TMR as a model fluorophore in the experiments described above, our modular approach can be used to synthesize different analogs of polymer **1** that have similar molecular weights, but different fluorescent end labels. We have extended this procedure, for example, to the synthesis of polymer **1** labeled with the fluorophore Oregon Green 488 by treatment of polymer $\mathbf{1_{Acr}}$ with Oregon Green-cadaverine (data not shown). This general approach could thus be used as a platform for the synthesis of collections of end-labeled PBAEs with a range of different spectral properties.

Fabrication of PEMs Using Polmyer 1_{FL} and Characterization of Film Erosion

Our past studies demonstrate that polymer **1** can be used to fabricate ultrathin PEMs that erode gradually upon incubation in physiologically relevant media and thereby promote the release of incorporated anionic components (such as DNA or SPS).[1-3] The release of DNA and SPS in these past studies was characterized using UV/vis spectrophotometry (or, in cases where fluorescently labeled DNA or SPS were used, by fluorometry). To date, however, detailed characterization of the behavior of polymer **1** during film erosion has not been reported. As described briefly in the Introduction, we recently reported on the use of confocal microscopy to characterize PEMs fabricated using DNA and a backbone-labeled fluorescent analog of polymer **1**.[34] While these past studies provided insight into changes in film morphology during the first few hours of incubation in physiologically relevant media, this past study did not characterize the behavior of polymer **1** during film erosion or the nature of its release into aqueous media. In the section below, we demonstrate that polymer $\mathbf{1_{FL}}$ can be used to characterize film erosion and the release of polymer **1** from ultrathin PEMs fabricated using SPS as a model polyanion.

All PEMs used in our initial studies were fabricated layer-by-layer on the surfaces of planar silicon substrates to facilitate characterization of film growth and thickness during fabrication and erosion using ellipsometry. Figure 2 shows a plot of the optical thicknesses of films fabricated using SPS and either polymer **1** or polymer $\mathbf{1_{FL}}$ (referred to hereafter as ‘polymer **1**/SPS films’ or ‘polymer $\mathbf{1_{FL}}$/SPS films’, respectively) as a function of the

number of bilayers (or layer pairs) of polyamine and SPS deposited. Inspection of these data reveals film growth to be linear in both cases and to result in films ~100 to 120 nm thick after the deposition of eight bilayers. These results demonstrate that the fluorescent labels in polymer $\mathbf{1_{FL}}$ do not influence film growth significantly relative to films fabricated using samples of unmodified polymer **1**. Characterization of films fabricated using polymer $\mathbf{1_{FL}}$ by fluorescence microscopy revealed red fluorescence distributed uniformly over film-coated portions of the silicon substrates (as discussed in further detail below), providing additional visual evidence of the incorporation of polymer $\mathbf{1_{FL}}$ into the structures of these films.

We next performed a series of experiments to characterize film erosion and determine whether polymer $\mathbf{1_{FL}}$ could be used to characterize the release of polyamine when these films were incubated in aqueous environments. For these experiments, substrates coated with polymer **1**/SPS or polymer $\mathbf{1_{FL}}$/SPS films eight bilayers thick were incubated in phosphate-buffered saline (PBS) at 37 °C. Our past studies demonstrate that these conditions generally promote film erosion and the release of SPS over periods of ~48 hours.[22-24] Figure 3 shows changes in film thickness and the release of film components into solution as a function of time. As shown in Figure 3A, the thicknesses of the polymer **1**/SPS films (closed squares) and polymer $\mathbf{1_{FL}}$/SPS films (closed circles) both decreased by ~35% within the first hour and then continued to decrease in a more gradual and linear manner over an additional ~75 hours. The rapid initial decrease in film thickness observed after the first hour was not observed in our past studies on the erosion of polymer **1**/SPS films, but the subsequent gradual erosion of these films is otherwise generally consistent with the results of our prior reports.[22-24] The similarity in the erosion profiles for the polymer **1**/SPS and polymer $\mathbf{1_{FL}}$/SPS films suggests again that the fluorescent labels incorporated into polymer $\mathbf{1_{FL}}$ do not influence significantly the behavior of these PEMs.

Figure 3B shows plots of both the absorbance (at 226 nm, the absorbance maximum of SPS) and fluorescence (at 581–585 nm, the maximum for emission from TMR) of PBS solutions versus time measured during the film erosion experiments described above. These data permitted simultaneous characterization of the release of SPS and polymer $\mathbf{1_{FL}}$ during these erosion experiments. Inspection of the absorbance data (closed symbols) reveals that both polymer **1**/SPS and polymer $\mathbf{1_{FL}}$/SPS films released SPS into solution gradually over a period of ~75 hours (these data also reveal an initial burst release of SPS over the first hour of incubation that correlates with the initial drops in film thickness discussed above). These results are generally consistent with the results of our past studies.[22-24] Further inspection of the fluorescence data (open circles), however, reveals that polymer $\mathbf{1_{FL}}$ was also released gradually into solution over this same time period with a general profile (an initial burst followed by more gradual release) that is similar to those observed for the release of SPS (we observed no significant increases in fluorescence during the release of SPS from films fabricated using unlabeled polymer **1**; Figure 3B, open squares).

These fluorescence data reveal that the release of SPS from these films during erosion occurs over the same time scale as the release of polymer **1**. While this general physical picture of film erosion could be inferred from the results of past studies of this polymer **1**/SPS system (e.g., on the basis of gradual decreases in ellipsometric film thickness over time), these results provide, for the first time, direct physical evidence that the release of SPS and polymer **1** occur simultaneously. This observation provides additional fundamental insight into the nature of film disassembly and provides information that could prove useful in the design of polymer **1**-based PEMs for the delivery of DNA (e.g., in applications for which the presence of polymer **1** could contribute to the internalization and processing of DNA by cells).[3,33]

The incorporation of polymer $\mathbf{1_{FL}}$ into these films also permitted characterization of film erosion by monitoring time-dependent decreases in the fluorescence of polymer $\mathbf{1_{FL}}$/SPS films using fluorescence microscopy. Figure 4 shows a series of low-magnification fluorescence microscopy images of a polymer $\mathbf{1_{FL}}$/SPS film fabricated on the surface of a planar glass substrate, and reveals red fluorescence to decrease gradually over a period of ~12 hours (additional control experiments demonstrated that this gradual decrease in fluorescence did not arise from photobleaching of the fluorophore over time). The images in Figures 4B-I also show a dark spot in the film that was created by intentional photobleaching in this location. This spot was created to characterize the potential for the translational diffusion or transport of polymer $\mathbf{1_{FL}}$ in these films (e.g., in the plane of the film) during incubation and film erosion.

Quantitative characterization of changes in the intensity of fluorescence in this spot as a function of time did not reveal evidence of substantial fluorescence recovery after photobleaching (FRAP) under these conditions (see Figure 4J). We note that levels of fluorescence in this spot did rise slightly during the early stages of erosion (as shown qualitatively in Figure 4B-C and quantitatively in the plot in Figure 4J). However, further inspection of these data reveals this increase in fluorescence to occur rapidly and uniformly across the spot rather than at the edges of the spot, as would be expected if polymer were to be transported into this region from surrounding areas of the film.[40-43] The reasons for this uniform increase in fluorescence are not clear, but it could potentially arise from the re-deposition of previously released polymer (i.e., from solution) onto the surface of the photobleached spot (or on the glass surface of the dish in which film-coated samples were placed for imaging). In addition, we note that time-dependent reductions in the intensity of fluorescence in areas surrounding this spot (e.g., see Figures 4G-I) complicated interpretations of these data at longer time points. Additional experiments will be required to characterize the potential for lateral transport of polymer **1** during incubation and film erosion. In the context of this current study, however, the results shown in Figure 4 demonstrate that the incorporation of polymer $\mathbf{1_{FL}}$ into PEMs provides opportunities to image and characterize time-dependent changes in these assemblies using methods such as fluorescence microscopy. This approach should therefore also facilitate more detailed investigation and characterization of other processes (such as processes that lead to the nanoscale and microscale decomposition of polymer **1**/DNA films[34,54] and the presence or absence of polymer diffusion) that can occur when films fabricated using PBAEs are exposed to more complex aqueous environments.

Summary and Conclusions

We have reported an approach to the synthesis of a fluorescently end-labeled PBAE and demonstrated the use of this labeled polymer as a tool for the characterization of erodible PEMs. The synthesis of analogs of polymer **1** having acrylate functionalized end groups provided a general platform for the introduction of fluorescent labels by post-polymerization treatment with primary amine-functionalized fluorophores. Relative to previously reported one-pot methods reported for the synthesis of end labeled PBAEs that make use of stoichiometric imbalances of monomer to define end group functionality, the approach used here permits the synthesis of higher molecular weight polymers similar to those used in past studies on the fabrication of PEMs using polymer **1**. Fabrication of PEMs using polymer $\mathbf{1_{FL}}$ and the model polyanion SPS permitted characterization of film behavior, film erosion, and the gradual release of polymer using fluorometry and fluorescence microscopy. This general approach makes possible direct characterization of the behavior of the polycationic components of these assemblies, and provides a platform for characterization of other physical and functional properties of these multicomponent materials using other fluorescence-based analytical methods. Additional investigations into the potential of

polymer $\mathbf{1_{FL}}$ to provide additional insight into the behaviors and intracellular fates of erodible PEMs fabricated using plasmid DNA are currently underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support for this work was provided by the National Institutes of Health (R01 EB006820) and the Alfred P. Sloan Foundation. We are thankful to the NSF (CHE-92084663) and the NIH (NIH 1 S10 RR0 8389-01) for support of the UW NMR facilities. We thank Maren Buck for assistance with collecting and analyzing ^{1}H NMR spectra.

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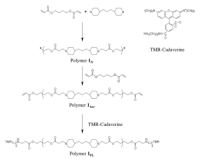

Scheme 1.
Synthesis of a Fluorescently End-Labeled Poly(β-amino ester)

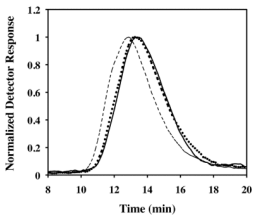

Figure 1.

Superimposed gel permeation chromatography (GPC) traces of polymer **1** (dashed line), polymer $\mathbf{1_{Acr}}$ (dotted line), and polymer $\mathbf{1_{FL}}$ (solid line). All polymers were synthesized in the same experiment as depicted in Scheme 1 as described in the text (i.e. polymer $\mathbf{1_{Acr}}$ is an end-modified derivative of polymer **1** and polymer $\mathbf{1_{FL}}$ is an end-modified derivative of polymer $\mathbf{1_{Acr}}$).

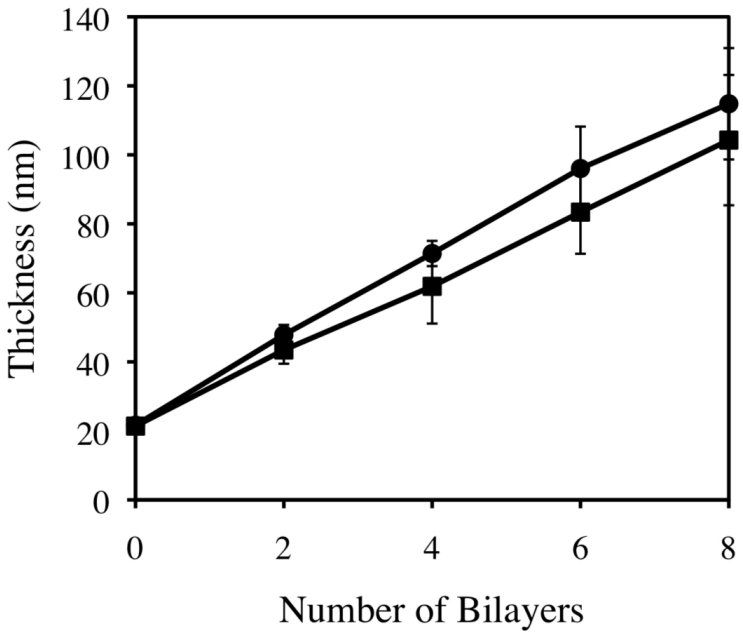

Figure 2.
Plot of ellipsometric film thickness versus the number of polymer **1**/SPS (squares) and polymer $\mathbf{1}_{\mathbf{FL}}$/SPS (circles) bilayers deposited on silicon substrates. Substrates used in these experiments were precoated with 10 bilayers of LPEI/SPS (~20 nm thick) prior to the fabrication of polymer **1**-containing films (see text).

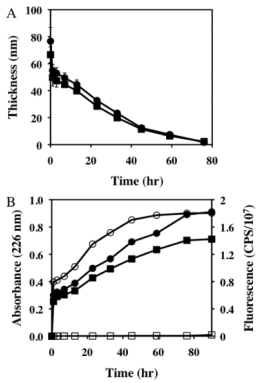

Figure 3.

(A) Plot of ellipsometric thickness versus time for polymer **1**/SPS films (squares) and polymer $\mathbf{1_{FL}}$/SPS films (circles) incubated in PBS buffer at 37 °C (pH = 7.4). (B) Plot of absorbance at 226 nm (closed symbols) and fluorescence (Ex: 543 nm, Em: 581 – 585 nm, open symbols) versus time corresponding to the polymer **1**/SPS films (squares) and polymer $\mathbf{1_{FL}}$/SPS films (circles) incubated in PBS buffer at 37 °C in (A).

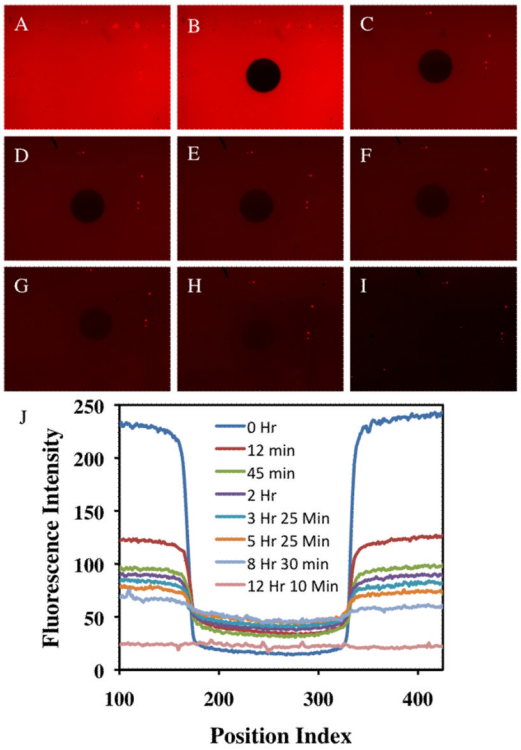

Figure 4.

A series of low magnification fluorescence microscopy images (4x) showing the time-dependent decrease in fluorescence intensity of a polymer $\mathbf{1}_{\mathbf{FL}}$/SPS film incubated in PBS buffer at 37 °C (pH = 7.4). A spot was photobleached into the film using a 40x objective to characterize the potential for lateral transport of polymer during the erosion process (see text; diameter of spot = 600 μm). Images (A) and (B) correspond to the same area of the film immediately (A) before and (B) after photobleaching. Subsequent images show the same region of the film after (C) 12 minutes, (D) 45 minutes, (E) 2 hours, (F) 3 hours 25 minutes, (G) 5 hours 25 minutes, (H) 8 hours 30 minutes, and (I) 12 hours 10 minutes incubation in PBS buffer at 37 °C (pH = 7.4). (J) A plot showing the fluorescence intensity of a line drawn through the center of the photobleached spot in images A – I using ImageJ software. All images were acquired using an exposure time of 35 ms.

Table 1

Molecular Weight Data for Polymer **1** and End-Functionalized Derivatives.[a]

Sample	M_n	M_w	PDI
Polymer **1**	22,000	49,800	2.27
Polymer $\mathbf{1}_{Acr}$	15,900	35,300	2.22
Polymer $\mathbf{1}_{FL}$	18,400	33,600	1.83

[a] Molecular weight relative to polystyrene standards as determined by gel permeation chromatography.