Published in final edited form as:

J Control Release. 2008 November 12; 131(3): 228–234. doi:10.1016/j.jconrel.2008.07.032.

Release of a Model Protein from Biodegradable Self Assembled Films for Surface Delivery Applications

Mara Macdonald¹, Natalia M. Rodriguez², Renee Smith¹, and Paula T. Hammond^{2,*}

¹ Joint Harvard-MIT division of Health Sciences and Technology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139 United States

² Department of Chemical Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139 United States

Abstract

Layer-by-layer (LbL) films have multiple features which make them attractive for drug delivery including the possibility of sequential delivery of growth factors, however, to date, proof of concept has been lacking for protein delivery from such films. Here, LbL polyelectrolyte films constructed with lysozyme (a model protein) and a hydrolytically degradable and biocompatible synthetic polycation are shown to be capable of release attractive for the localized delivery of therapeutic proteins from implanted medical devices. Milligram/cm² scale release with power law or linear profile can be achieved over 3 weeks to 3 months at room temperature. The release rate at 37°C increases in a way that is compatible with a surface erosion mechanism of release from 100 days to 5 days. This time scale of release can be tuned by changing the degradability of the synthetic polycation, and an increase to 34 days of release at 37°C is seen by increasing the hydrophobicity of this degradable polyester. The enzyme released from these films retains 80–100% functionality, underscoring the mild processing conditions that are apt to preserve fragile protein function. These results uncover many possibilities for incorporation of therapeutic proteins to modulate the interaction between implanted surfaces and the cells they contact.

Introduction

Layer-by-layer (LbL) deposition[1,2], a technique in which nanoscale layers of polyanions and polycations are dipped onto a charged surface from aqueous baths, constitutes a powerful tool with applications ranging from electrochemical thin films to biocatalysis[3,4]. LbL's many advantageous characteristics in the context of drug delivery coatings include nano-scale film architecture allowing fine control of film loading and release of multiple drugs, ability to conformally coat difficult geometries, adherence to many surface chemistries, and ease of fabrication. Particularly relevant to protein delivery, LbL deposition has the advantage of utilizing mild aqueous baths which have the potential to preserve fragile protein activity in comparison with the harsh organic solvents typically employed to fabricate protein delivery devices. When LBL films are constructed with surface eroding poly(β-aminoesters), it is furthermore possible to avoid more acidic degradation products of PLGA and PLLA (forerunner polymers in the field) and resultant low pH erosion conditions that cause proteins to denature and deactivate[5]. Finally, LbL enjoys the advantage of being compatible with

*Corresponding Author. Hammond@mit.edu; phone 617 258-7577; fax 617 258-5766.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

existing surface patterning and templating techniques[6], opening interesting avenues to explore the cellular response to protein exposure in localized micro-environments. However, to reach the high potential of LbL in the exploration of protein-surface-cell interactions, more must be done to understand the characteristics of protein-releasing LbL films.

Original research in the LbL drug delivery field focused upon the diffusive release of low molar mass drugs from uniform, non-degradable films. While demonstrating a clear scalability of drug loading with increased numbers of layers, diffusion- or bulk dissociation- based release typically yields “burst” profile characteristics with high drug elution at the beginning of the time course, and very low levels at the end [7]. Rarely is diffusion controlled release from LbL films sustained for more than a few hours. One notable exception to this trend was the use of nanoporous multilayer films to sequester hydrophobic drug, which resulted in release times of up to multiple days in certain cases at room temperature[8]. However, because the release time is impacted by the affinity of the drug for water, it is directly related to the hydrophobic nature of the drug, rather than an externally controlled parameter, and therefore is not a useful strategy for more hydrophilic macromolecules, such as proteins.

A few mechanisms of protein release from LbL films do not require diffusion. It is possible to coat a crystal of protein with a non-diffusive LbL shell for release under significant pH (pH 8 or higher) or ionic strength changes[9], [10] under which the shell dissipates, releasing the protein; however this is impractical for many medical applications in which such large deviations from physiological conditions would be deadly. In other cases, enzymatic breakdown of biological polymers renders release over a period of several hours[11–13]; in one case time-dependent release was shown to be possible[13], which is a positive step towards time-delayed release of protein therapeutics. However, release is dependent on specific cellular adhesion and interaction with the surface, and the loading and release of protein can be influenced by a combination of diffusion and enzymatic degradation that is more difficult to tune for sustained periods. Rather than relying upon diffusion, bulk release, or large pH changes, we hypothesized that a more linear release profile could be attained by utilizing hydrolytic surface degradation of biocompatible and degradable polymers.

The concept of using degradable synthetic polyions in LbL films addresses many of the inherent difficulties encountered with above systems while affording new, very desirable characteristics. These polymers retain biocompatibility and biodegradability while allowing tunability of polymer degradation and therefore release rates, and may have the additional benefit of avoiding the low pH release environment of PLGA or PLLA. Surface erosion allows the possibility of linear release profiles and therefore the ability to exert stronger control over the drug release characteristics of the system. Finally and crucially, through the combination of the nanoscale architecture possible with LbL deposition with surface eroding poly(β -aminoester)s[2,14], it is possible to create sequentially releasing devices in which the composition of individual layers changes through the thickness of the film; different proteins are released as the film erodes from the top down and dissolves layers of differing compositions, yielding exquisite control of the release profile of each incorporated protein. The widespread opportunities in tissue engineering and drug delivery available with sequential delivery require controlled, surface erosion based release, and are inaccessible with diffusion or bulk degradation based release systems.

Degradable poly(β -aminoesters) such as Poly1 and Poly2, shown in Scheme 1, are attractive for this purpose due to their ability to slowly degrade via ester hydrolysis at physiologically relevant pH [15], their positive charge (which allows for film construction), and because such poly(β -aminoesters) have been shown to be biocompatible in a number of different applications [14], [16], [17]. Previous work with poly(β -aminoesters) has yielded devices capable of delivering anticoagulants (heparin sulfate), anti-inflammatory agents (chondroitin sulfate[18,

19], DNA for gene therapy[20], and demonstrated proof of concept for sequential delivery [18,21]. Attempting to incorporate proteins in such films is a natural next step; proteins delivered from synthetic LbL films would benefit from the mild processing conditions, elimination of polymers using acidic byproducts, fine control of protein incorporation, and ease of fabrication. This approach is also strongly motivated by a well-recognized need in the fields of tissue engineering and implantable medical devices to sequentially release growth factors, which can be achieved with surface erosion from LbL films. In this work, lysozyme was utilized as a model protein due to its similarity in size and shape to many growth factors. Lysozyme has a molar extinction coefficient of 38,010 and molecular weight of 14.4 kDa, and a high isoelectric point (pI 9), and also has the advantage of being a well studied biomolecule with many assays for detection of concentration and functionality.

Finally, both lysozyme and Poly1 are cationic, necessitating a tetralayered repeat unit architecture with an anionic polymer in between Poly1 and lysozyme. Two anionic biopolymers, heparin sulfate and chondroitin sulfate, were investigated as candidates for film construction. Heparin was selected due to its well known anti-clotting properties. This is a potentially useful characteristic for a delivery device or implant coating, as clotting is a serious concern with any device that may be put into contact with the bloodstream (ie stents). Failure to address this concern may lead to myocardial infarction, stroke, or ischemia of other vital organs. However, in applications where anticoagulation would be problematic (ie at wound sites), alternative functionalities could become desirable. For example, by providing a matrix-like environment which cells can utilize to begin proliferating, it may be possible to speed the integration of an implanted device with the surrounding host tissue. Chondroitin, a native extracellular matrix component with anti-inflammatory properties, was used with this intent.

Thus here we describe for the first time the construction of polyelectrolyte multilayer films that incorporate a synthetic, hydrolytically degradable polymer capable of controlled release and a model protein of interest, constructed for the purposes of drug delivery and tissue engineering. The incorporation of protein within such constructs is examined, and the subsequent hydrolytic degradation and release of proteins from these films is described. We demonstrate tuned and sustained release of significant protein quantities from micron scale conformal thin films over periods of several days to several weeks at body temperature. Finally, the activity of the protein is addressed upon release. Such films may become significant in the sectors of tissue engineering and medical device coating technology, as increased cellular interactions with devices are sought, and the delivery of therapeutic factors at specific times during the implant lifetime become necessary.

Experimental Methods

Materials

Linear poly(ethylenimine) (LPEI, Mn = 25000) was bought from Polysciences, Inc (Warrington, PA) and poly(sodium 4-styrenesulfonate) (PSS, Mn = 1000000) was purchased from Sigma-Aldrich (St. Louis, MO). Chondroitin sulfate sodium salt (Mn = 60000) was obtained from VWR Scientific (Edison NJ) and heparin sodium salt was obtained from Celsus Laboratories (Cincinnati, OH). Poly1 was synthesized as previously described[15]. Poly2 was synthesized in a similar manner to Poly1, substituting a 1,6 heptane dioldiacrylate for the 1,4 butane dioldiacrylate used to synthesize Poly1. Lysozyme and *Micrococcus lysodeikticus* bacteria were obtained from Sigma Aldrich (St. Louis MO). All commercial polyelectrolytes were used as received without further purification. A micro-BCA assay was obtained from Pierce (Rockford, IL) and used according to manufacturer instructions. Glass and quartz slides (substrates) were obtained from VWR Scientific (Edison NJ). Deionized water (18.2 M Ω , Milli-Q Ultrapure Water System, Millipore) was used for all washing steps. Dulbecco's PBS buffer was prepared from 10x concentrate available from Invitrogen (Frederick, MD).

Preparation of Polyelectrolyte Solutions

LPEI and PSS were dissolved in deionized water to a concentration of 10mM with respect to repeat unit and pH adjusted to 4.25 and 4.75 respectively. Heparin, chondroitin, Poly1 and Poly2 were prepared in sodium acetate buffer (pH 5.1, 100mM) at a concentration of 2mg/mL. Lysozyme was prepared at a concentration of 0.5 mg/mL in sodium acetate buffer pH 5.1, 100 mM.

Film Construction—Glass substrates or quartz slides (1" × 1/4") were rinsed with methanol and deionized water, dried under a stream of dry nitrogen, and plasma etched in oxygen using a Harrick PDC-32G plasma cleaner on high RF power for 5 minutes. Ten base layers of (LPEI/PSS) were deposited upon plasma etched substrates to create a surface area 0.75" × 1/4" with a Carl Zeiss HSM series programmable slide stainer according to the following protocol: 5 minutes of dipping in LPEI, followed by three washes (10, 20, 30s respectively) in deionized water, followed by 5 minutes in PSS and three deionized water washes (10, 20, 30s) for 10 repetitions. On top of the base layers, tetralayers were stepwise built stepwise, adding a layer of PolyX (+), polyanion (−), lysozyme (+), and polyanion (−) to the surface to create the following architecture: (PolyX/polyanion/Lysozyme/polyanion)_n where the PolyX is either Poly1 or Poly2, the polyanion is either heparin or chondroitin, and n refers to the number of tetralayers deposited on the substrate. A typical dipping protocol is 10 minutes in a solution of Poly1 dissolved in 100 mM sodium acetate buffer, pH 5.1, 3 washes (10, 20, 30 s) in deionized water, 7.5 minutes in heparin dissolved in 100 mM sodium acetate buffer, pH 5.1 with 3 washes (10, 20, 30s) in deionized water, 10 minutes in lysozyme in 100 mM sodium acetate buffer with 2 washes (20, 30s) in deionized water and 7.5 minutes in heparin in 100 mM sodium acetate buffer with 3 deionized water washes. Films were controlled to be 0.75 inches × 0.25 inches in size, or roughly 1.2 cm². Note that by changing the contents of a dipping bath, different molecular species can be loaded in different layers of the film, allowing sequential release. Once a film is constructed, placement in phosphate buffered saline triggers hydrolytic degradation of the poly(β-aminoester), allowing release of protein.

Film Characterization—Varying numbers of tetralayers were dipped onto clean quartz substrates pre-treated with 10 base layers. Three techniques were used to analyze buildup. The thickness of the resulting films was measured by scoring the samples to the base of the film with a razor blade and measuring the step height using a profilometer (P10 Surface Profiler) with a 2μm tip radius stylus. Protein incorporation in the film was measured by UV-Vis spectroscopy. A profile was taken of each sample on a Cary 6000i spectrophotometer from 200–800 nm. Proteins absorb light at 280 nm due to tryptophan, tyrosine, and cystine, and intensity of absorption can then be correlated to protein buildup within the film. The absorbance value at 320 nm (baseline) was subtracted from the absorbance value at 280 nm and plotted according to number of tetralayers. Instantaneous release was used to quantify the total protein concentration in the film by using 1 mL of 1M NaOH for 1 hour, which disrupts film architecture and releases all incorporated protein. A 50 μL sample was quenched in 1X PBS and read using a micro-BCA Protein Assay Kit (Pierce Biotechnology, Rockford IL). Briefly, bicinchoninic acid can be used to quantify protein concentration by detecting a reduction of copper by the protein of interest in an alkaline environment. A color change can be monitored and compared to a constructed standard curve. Triplicate 100 μL aliquots of standards and samples were run in 96 well plates according to the manufacturer's protocol and read on a microplate reader (PowerWave XS, BioTek, Winooski VT).

Release Characterization—Samples were released into phosphate buffered saline pH 7.4 at either room temperature or 37 degrees Celsius in a microcentrifuge tube containing 1 mL of PBS. At a series of different time points, 0.5 mL of sample was removed and 0.5 mL of fresh PBS was introduced to the sample container. Samples were frozen at −20C until analyzed. Two

methods were used to detect release of lysozyme into the solution. A micro-BCA Protein Assay Kit (Pierce Biotechnology, Rockford IL) was used to detect the total amount of protein released during a time point. To track the functionality of the released protein, bacterial lysis experiments were performed[22]. A cloudy suspension of bacteria lysed by lysozyme will lead to clearing of the solution, which can be tracked as a decrease in absorbance at 450 nm. This work is done as a kinetic plate reading assay, in which the slope of the graph of [absorbance at 450 nm versus time] calculated for samples and standards is proportional to the concentration of enzyme present in the solution. A standard curve of [concentration vs slope] from 200–0 μM is constructed (which is linear over this concentration range), and the concentrations of the unknown samples interpolated based on the slope of each individual sample. In these tests, 290 μL of a 0.25 mg/mL solution of *Micrococcus lysodeikticus* was mixed with 10 μL of samples and standards performed in triplicate and read in a 96 well plate at 450 nm every 15 seconds for a total of 10 readings. The readout from the assay is a concentration of enzyme present based on the lysing ability of the sample, and thus represents the concentration of functional protein present in the sample. Error bars for all figures in this paper represent a 99% confidence interval calculated from triplicate repeat samples of 2 independent trials (total of 6 data points).

Results and Discussion

Films were constructed using pH 5 sodium acetate baths of heparin, lysozyme, and Poly1 and washed with deionized water to create films with the architecture (Poly1/heparin/lysozyme/heparin). Film buildup was tracked by monitoring thickness, protein incorporation, and instantaneous protein release (Figure 1, Table 1). After a brief induction period where little protein incorporation, film thickness, or protein release is achieved (approximately 10–20 tetralayers), all three methods indicate that the films build and incorporate protein in a roughly linear fashion.

An induction period for multilayer growth is typical of many LbL systems and has been reported in the literature[23,24]. In this initial period, surface effects are hypothesized to influence the buildup of the LbL film until complete surface coverage is achieved after several adsorption cycles. In comparing the two techniques which measure protein content (UV-Vis, and BCA) and that which measures total film buildup (Profilometry), it appears that protein incorporation becomes linear after approximately 10 tetralayers, whereas the thickness increase becomes linear at approximately 20 to 30 tetralayers. The thickness per tetralayer repeat unit in the linear growth regime is approximately 0.42 μm , which is large in comparison with typical electrostatic multilayer systems that exhibit 0.01 to 0.03 μm per bilayer pair, and suggests that although protein incorporation is substantial after just 10 tetralayers, the polymer composition in the film may be changing in a nonlinear fashion during this initial period. This regime of superlinear growth is hypothesized to be due to intermolecular interdiffusion of macromolecules into the film during the adsorption process, which leads to increasingly thick films[24,25] until a constant repeat unit thickness is ultimately achieved[23]. However, unlike asymptotic growth films, eventually a steady state, linear growth trend is established.

This second, linear regime is hypothesized to be created by a “front” in which diffusion of polymers into the bulk film is possible (vastly increasing the amount of protein able to be incorporated in each dip step compared to the initial steps when the film and therefore the front are thinner), underneath which is a “reorganized” layer which is impermeable to diffusion, retaining the linearity of the film[23–27]. The diffusive character of the front and therefore of the film are affected by a number of factors including hydrophilicity of the polymer backbone, charge density of the polyions, and molecular weight of the polymers involved. The buildup characteristics seen in Figure 1 are very consistent with the growth patterns of other films containing poly(β -aminoesters) and polyanions such as heparin[19].

In general, there is strong agreement between the three measurement techniques, indicating excellent protein incorporation in the films, with linear build-up after an initial induction period. This linearity makes it simple to predict the additional amount of protein incorporated by adding subsequent tetralayers, as the amount incorporated increases linearly with the additional number of tetralayers dipped. For [Poly1/heparin/lysozyme/heparin]80 films, a UV-Vis signal of 2.064 units at 280 nm corresponded with a profilometry thickness of 23.78 μm or approximately 1137.5 $\mu\text{g}/\text{cm}^2$ of loaded lysozyme.

Once it was established that films could be built with excellent protein incorporation, the release characteristics of the films were examined. Films were released at 37° C in PBS to approximate physiologic conditions and analyzed using a micro-BCA kit. In Figure 2A, the release curves show that lysozyme can be released with a linear trend over a period of approximately 14 days, releasing approximately 650 $\mu\text{g}/\text{cm}^2$ of incorporated protein. Although caution must be taken with extending the utility of these results too far, since these experiments are idealized simulations of body conditions that lack enzymes, cellular reactions, and complex mass transfer profiles likely to be encountered *in vivo*, the linear release trend is encouraging from multiple perspectives. First and foremost, if this release trend was extrapolated to *in vivo* conditions, it would be very desirable for the drug delivery applications it is designed for, because it allows for constant, low levels of protein to be released from the surface. Compared to burst release profiles in which most of the protein is released instantly and is therefore lost to a greater body volume and cleared before therapeutic action can take place with only a minority of the protein incorporated being controlled in release, nearly all of the release in these films occurs in a controlled fashion in a constant, easy to predict (and therefore dose) fashion. This controlled delivery has significant advantages over pill or bolus injection methods to keep the concentration at the local site of interest within a therapeutic window between an upper limit of toxicity and a lower limit of effectiveness. This method therefore represents a new modality for the timed local release of proteins within the body where the expense or size of a dose was previously prohibitive. It is simple to load more drug by increasing the number of tetralayers used and also simple to predict the additional amount incorporated. It is also encouraging that, even in a midrange number of built tetralayers (films from 10 to 80 tetralayers were examined) very large amounts of protein can be incorporated while most growth factor applications require only nanogram/mL concentrations of protein. This suggests that these films have copious loading capacity for the applications of tissue engineering and drug delivery.

In addition to these advantages, the controlled and linear release profile suggests a surface erosion mechanism for release, which is consistent with previous work showing ester hydrolysis to be the driving force behind release from LbL films created with poly(β -aminoesters)[28]. While burst release was also considered, burst release profiles tend to have a power law shape in which most of the release occurs at the beginning, unlike this linear release. Similarly, release can be seen due to ionic interactions with the film when release buffer has high ionic strength (greater than 0.5 M)[28,29], but due to the mild difference in ionic strength of the assembly and release conditions (0.1 vs. 0.2 M) and the release span of months rather than seconds, this mechanism of release is also unlikely.

A second, important characteristic is the change in loaded dose with increasing numbers of layers. Also in Figure 2A, a family of release curves for films with (Poly1/heparin/lysozyme/heparin) architecture and varying numbers of tetralayers is striking for the observation that with increasing numbers of tetralayers, both the total amount of protein released and the time span of release are increased, in good agreement with the hypothesized mechanism of surface erosion. Films with 20 tetralayers released 100 μg of protein over 7 days while films with 43 tetralayers released 650 $\mu\text{g}/\text{cm}^2$ of protein over 14 days. Bulk releasing films would possibly have increased loading with increasing numbers of tetralayers, but release the entire load over the same amount of time independent of the number of tetralayers. This in turn suggests, when

taken with the linear buildup and release of the film, that a surface erosion mechanism of release (rather than bulk diffusion) is responsible, as demonstrated and discussed in previous publications[2, 19]. To allow for comparison of release kinetics, these values have also been plotted as percent of total release (taking release at the last time point to be total release) in Figure 2B. As can be seen in this figure, films with more tetralayers take longer to release their entire payload of protein, again reinforcing the mechanism of surface erosion.

To address the question of fractional release (the fraction of the drug loaded into the film that is released) two sets of films were constructed with the architecture (Poly1/heparin/lysozyme/heparin) and varying numbers of tetralayers. One set of films was released at 37°C and the other was instantaneously released as described in the Materials and Methods section. The total release was calculated in each instance, and plotted in Figure 2C. In this figure, nearly 95% release or higher is observed at low numbers of tetralayers (50 tetralayers and below). At 80 tetralayers, approximately 85% release is achieved. Because this is a fully degradable system, it is anticipated that eventual recovery of the remaining 15% would happen over a long course of time. It is likely that the thickness of the film and incomplete degradation of overlying polymer networks hinders further release of protein from the film.

One of the advantages of using a synthetic erodable polymer is that the drug delivery from the device can be tuned through additional mechanisms over those already discussed by modifying the molecular structure of the polymer used. Poly1 is only one of a large family of poly(β -aminoesters) that can be used in these films; by tuning the composition of the polymers used for this purpose, one can alter the degradability of the ester bond and therefore decrease or increase the time scale over which the film degrades. A second poly(β -aminoester), Poly2, was used to explore the effect of the kinetics of ester hydrolysis on protein release from the constructed multilayers (see Scheme 1). Poly2 differs from Poly1 in that it has an additional two methylene units in the backbone next to the ester bond, making Poly2 more hydrophobic and making the ester bond less susceptible to hydrolysis. This in turn is predicted to decrease degradation and therefore increase time span of release. Thus, characterization and comparison between Poly1 and Poly2 films yields further interesting information on the erosion of these films as well as allowing demonstration of the ability to tune release by tuning the characteristics of the synthetic polymer used.

Comparing buildup data of P2 compared to similar films for P1 (Figure 3A), there is similar agreement between the three measurement techniques, suggesting that linear film buildup and incorporation occur around 20 tetralayers. However, compared with similar films containing Poly1 instead of Poly2, two interesting differences are uncovered. In Figure 3B, release of a film with the architecture (P2/heparin/lysozyme/heparin) demonstrates that the time period of release is successfully modified by tuning the degradable polymer used. Over 34 days of release are observed with Poly2 films, indicating a 2x increase in release time using the more hydrophobic polymer. This suggests that surface erosion occurs more slowly due to decreased hydrolysis of the polymer and therefore releasing protein for a longer span of time. The second interesting difference is noted by comparison of thickness and total protein incorporation of Poly1 and Poly2 films in Table 1. The more hydrophobic films are capable of increased protein loading as well as increased time span of release. This can be understood on the nano-level to be due to a modification in the film morphology or architecture. Because of the added two methylene units, the distances between the positively charged units along the backbone increases, leading to lower polycation charge density. This lower charge density, along with the fact that hydrophobic chain segments can yield less extended, more coiled chain conformations in solution and upon adsorption, results in the adsorption of thicker, loopier polyelectrolytes. Thus the Poly2 films are anticipated to be thicker, and therefore have a larger volume with which to store loaded protein and other charged species which interdigitate into the forming film during assembly.

As another modality for changing release behavior, a different polyanion, chondroitin, was explored in films of the architecture [Poly1/chondroitin/lysozyme/chondroitin] with varying numbers of tetralayers. In Figure 4A, profilometry, UV-vis and instantaneous release curves are plotted in manners similar to Poly1-heparin-lysozyme and Poly2-heparin-lysozyme films. Contrary to the film building behavior for heparin, chondroitin buildup is characterized by an extended period of time in the first, superlinear surface modification portion of the buildup regime. Even by 50 tetralayers it is unclear whether the films are beginning to build linearly or not. Values at 50 tetralayers are much lower than those of Poly1/heparin or Poly2/heparin films. UV-vis absorbance at 280 is 0.236 units, the thickness of the film is 0.027 μm , and the film was loaded with approximately 133 $\mu\text{g}/\text{cm}^2$ of protein (values for each film construction are summarized in Table 1). The release from these films reflects the difference in incorporation rates, showing power law dependence, less incorporation and faster time to completion of release (Figure 4B). Kinetic data of the same samples are given in Figure 4C. Because there is a more burst-like release profile, it is not surprising that the kinetic plots overlay each other, showing that the timescale of release is independent of the amount released. However, in alternative applications in which a burst at the beginning is desirable with low levels of sustained release following, these films might be useful. In addition, the same trend of increased loading and time to complete release is seen with these films as is seen in the case of Poly1/heparin and Poly2/heparin films.

The ultimate concern in encapsulating proteins for drug delivery is whether the processing conditions will destroy the activity of the encapsulated component. Layer-by-layer deposition is a likely candidate for the preservation of activity of encapsulated proteins due to the aqueous baths used in the process as well as the mild pH, which can be adjusted to biological conditions. To quantify the functionality of released enzyme, activity assays of lysozyme were performed. Lysozyme's native activity is to cleave bacterial cell walls; one can detect the amount of functional protein in solution by a kinetic reduction in turbidity of a bacterial solution. In Figure 6, results of the micro-BCA assay for typical films are plotted in tandem with the results of a functional assay which reports the concentration of active enzyme present in a given sample. Encouragingly, 80%–100% of activity is preserved within the films throughout the length of the trial. These rates of activity compare favorably with those of protein release from LbL films in the literature. In the case of Derbal et al[30], activity at physiologic pH was possible, but long term activity of the enzyme (over a period of months) dropped to levels of approximately 30% of their original value. In the case of Caruso and Tiourina[9,10], excellent retention of activity was possible, ranging from 70 to 100%; however, the capsules required a high pH to release, which is unattainable for many medical device release applications, and underwent a burst style of release. By combining hydrolytic degradability in a polyion directly with the protein of choice in LbL assembly, it is possible to protect and retain protein for long periods of time while sustaining the ability for extended release at biologically relevant conditions.

Conclusions

In this paper, a new method of protein encapsulation is described using layer by layer deposition to release active proteins in a method that is applicable to objects of any desired geometry and size scale. The protein rate and timescale of release from the film are tunable by choosing the anion to be used, the properties of the degradable polymer, as well as the number of tetralayers that are deposited upon the surface of interest. Additional film properties such as anticoagulant activity or providing matrix material for cell proliferation can be chosen through the polyanion used. In particular, creating films with heparin leads to a linear release profile that is sustainable over 34 days of release at body temperature with the ability to tune release time span and loading. This system combines the useful characteristics of other approaches including high functionality of released enzyme, as well as the ability to release at physiological pH and

temperature, making it a widely applicable system that can be tailored to a growing number of release applications.

Acknowledgments

The authors would like to thank Yoon Yeo for expertise in lysozyme functionality assays, Dr. David Lynn for helpful and synergistic discussions with the authors, and Dr. Langer for his constant support. Two undergraduate researchers, Nicole Brenner and Girma Endale, contributed to preliminary experiments that helped make this publication possible. Financial support for this work is gratefully acknowledged from the Deshpande Center grant 009216-1 and the National Institutes of Health grant 1-R01-AG029601-01. M. Macdonald is thankful for a National Science Foundation Graduate Research Fellowship. This work made use of MRSEC Shared Facilities supported by the National Science Foundation under Award Number DMR-0213282.

References

1. Decher G. Fuzzy nanoassemblies: Toward layered polymeric multicomposites. *Science* 1997;277(5330):1232–1237.
2. Vazquez E, Dewitt DM, Hammond PT, Lynn DM. Construction of hydrolytically-degradable thin films via layer-by-layer deposition of degradable polyelectrolytes. *Journal of the American Chemical Society* 2002;124(47):13992–13993. [PubMed: 12440887]
3. Decher G, Eckle M, Schmitt J, Struth B. Layer-by-layer assembled multicomposite films. *Current Opinion in Colloid & Interface Science* 1998;3(1):32–39.
4. Hammond PT. Form and function in multilayer assembly: New applications at the nanoscale. *Advanced Materials* 2004;16(15):1271–1293.
5. Fu K, Pack DW, Klibanov AM, Langer R. Visual evidence of acidic environment within degrading poly(lactic-co-glycolic acid) (PLGA) microspheres. *Pharmaceutical Research* 2000;17(1):100–106. [PubMed: 10714616]
6. Berg MC, Yang SY, Hammond PT, Rubner MF. Controlling mammalian cell interactions on patterned polyelectrolyte multilayer surfaces. *Langmuir* 2004;20(4):1362–1368. [PubMed: 15803720]
7. Chung AJ, Rubner MF. Methods of loading and releasing low molecular weight cationic molecules in weak polyelectrolyte multilayer films. *Langmuir* 2002;18(4):1176–1183.
8. Berg MC, Zhai L, Cohen RE, Rubner MF. Controlled drug release from porous polyelectrolyte multilayers. *Biomacromolecules* 2006;7(1):357–364. [PubMed: 16398536]
9. Caruso F, Trau D, Mohwald H, Renneberg R. Enzyme encapsulation in layer-by-layer engineered polymer multilayer capsules. *Langmuir* 2000;16(4):1485–1488.
10. Tiourina OP, Sukhorukov GB. Multilayer alginate/protamine microsize capsules: encapsulation of alpha-chymotrypsin and controlled release study. *International Journal of Pharmaceutics* 2002;242(1–2):155–161. [PubMed: 12176238]
11. Etienne O, Schneider A, Taddei C, Richert L, Schaaf P, Voegel JC, Egles C, Picart C. Degradability of polysaccharides multilayer films in the oral environment: an in vitro and in vivo study. *Biomacromolecules* 2005;6(2):726–733. [PubMed: 15762636]
12. Picart C, Schneider A, Etienne O, Mutterer J, Schaaf P, Egles C, Jessel N, Voegel JC. Controlled degradability of polysaccharide multilayer films in vitro and in vivo. *Advanced Functional Materials* 2005;15(11):1771–1780.
13. Benkirane-Jessel N, Lavalle P, Hubsch E, Holl V, Senger B, Haikel Y, Voegel JC, Ogier J, Schaaf P. Short-time timing of the biological activity of functionalized polyelectrolyte multilayers. *Advanced Functional Materials* 2005;15(4):648–654.
14. Lynn DM, Langer R. Degradable poly(beta-amino esters): Synthesis, characterization, and self-assembly with plasmid DNA. *Journal of the American Chemical Society* 2000;122(44):10761–10768.
15. Lynn DM, Langer R. Degradable poly(beta-amino esters): synthesis, characterization, and self-assembly with plasmid DNA. *Journal of the American Chemical Society* 2000;122:10761–10768.
16. Akinc A, Anderson DG, Lynn DM, Langer R. Synthesis of poly(beta-amino ester)s optimized for highly effective gene delivery. *Bioconjugate Chemistry* 2003;14(5):979–988. [PubMed: 13129402]

17. Little SR, Lynn DM, Ge Q, Anderson DG, Puram SV, Chen JZ, Eisen HN, Langer R. Poly-beta amino ester-containing microparticles enhance the activity of nonviral genetic vaccines. *Proceedings of the National Academy of Sciences of the United States of America* 2004;101(26):9534–9539. [PubMed: 15210954]
18. Wood KC, Chuang Helen F, Batten Robert D, Lynn David M, Hammond Paula T. Controlling interlayer diffusion to achieve sustained, multiagent delivery from layer-by-layer thin films. *PNAS* 2006;103(27):10207–10212. [PubMed: 16801543]
19. Wood KC, Boedicker JQ, Lynn DM, Hammond PT. Tunable drug release from hydrolytically degradable layer-by-layer thin films. *Langmuir* 2005;21(4):1603–1609. [PubMed: 15697314]
20. Jewell CM, Zhang J, Fredin NJ, Lynn DM. Multilayered polyelectrolyte films promote the direct and localized delivery of DNA to cells. *J Control Release* 2005;106(1–2):214–223. [PubMed: 15979188]
21. Jessel N, Oulad-Abdeighani M, Meyer F, Lavallo P, Haikel Y, Schaaf P, Voegel JC. Multiple and time-scheduled in situ DNA delivery mediated by beta-cyclodextrin embedded in a polyelectrolyte multilayer. *Proceedings of the National Academy of Sciences of the United States of America* 2006;103(23):8618–8621. [PubMed: 16735471]
22. Puleo DA, Kissling RA, Sheu MS. A technique to immobilize bioactive proteins, including bone morphogenetic protein-4 (BMP-4), on titanium alloy. *Biomaterials* 2002;23(9):2079–2087. [PubMed: 11996050]
23. Porcel C, Lavallo P, Decher G, Senger B, Voegel JC, Schaaf P. Influence of the polyelectrolyte molecular weight on exponentially growing multilayer films in the linear regime. *Langmuir* 2007;23(4):1898–1904. [PubMed: 17279672]
24. Porcel C, Lavallo P, Ball V, Decher G, Senger B, Voegel JC, Schaaf P. From exponential to linear growth in polyelectrolyte multilayers. *Langmuir* 2006;22(9):4376–4383. [PubMed: 16618190]
25. Jourdainne L, Arntz Y, Senger B, Debry C, Voegel JC, Schaaf P, Lavallo P. Multiple strata of exponentially growing polyelectrolyte multilayer films. *Macromolecules* 2007;40(2):316–321.
26. Lavallo P, Gergely C, Cuisinier FJG, Decher G, Schaaf P, Voegel JC, Picart C. Comparison of the structure of polyelectrolyte multilayer films exhibiting a linear and an exponential growth regime: An in situ atomic force microscopy study. *Macromolecules* 2002;35(11):4458–4465.
27. Schlenoff JB, Dubas ST. Mechanism of polyelectrolyte multilayer growth: Charge overcompensation and distribution. *Macromolecules* 2001;34(3):592–598.
28. Zhang JT, Fredin NJ, Lynn DM. Erosion of multilayered films fabricated from degradable polyamines: Characterization and evidence in support of a mechanism that involves polymer hydrolysis. *Journal of Polymer Science Part a-Polymer Chemistry* 2006;44(17):5161–5173.
29. Dubas ST, Schlenoff JB. Polyelectrolyte multilayers containing a weak polyacid: Construction and deconstruction. *Macromolecules* 2001;34(11):3736–3740.
30. Derbal L, Lesot H, Voegel JC, Ball V. Incorporation of alkaline phosphatase into layer-by-layer polyelectrolyte films on the surface of Affi-gel heparin beads: Physicochemical characterization and evaluation of the enzyme stability. *Biomacromolecules* 2003;4(5):1255–1263. [PubMed: 12959592]

Figure 1.

Film characterization of (Poly1/heparin/lysozyme/heparin) tetralayers by UV-Vis spectroscopy, profilometry and instant dissolution methods. Percent of value at 80 tetralayers is plotted vs. number of tetralayers to allow comparison of curves. Agreement is seen in the three methods of detecting film building indicating that the films are growing in thickness and incorporating protein.

Figure 2.

Total release and release time span are affected by number of tetralayers. 2A) Replicate samples were dipped with the architecture (Poly1/heparin/lysozyme/heparin) $_n$ and released at 37°C. Total release in $\mu\text{g}/\text{cm}^2$ is plotted vs. time in days. Note that both the amount of protein incorporated and the time to total release are increased with increasing numbers of tetralayers, suggesting surface erosion as a mechanism of release. 2B) The signal value for each timepoint was taken as a percentage of the signal at the last timepoint to allow comparison of release kinetics. Percent of total value is plotted vs. time in days. 2C) Fractional release of lysozyme is 85–95%. Films of various tetralayers of (Poly1/heparin/lysozyme/heparin) films were either

instantaneously released as described in materials and methods or allowed to elute into PBS at 37°C. Total protein loaded or released protein are plotted versus number of tetralayers.

3A)

3B)

Figure 3.

Poly2 Film Characterization 3A) The signal recorded at each point of construction is taken as a percent of the signal at 50 tetralayers and plotted against the number of (Poly2/heparin/lysozyme/heparin) tetralayers to allow comparison of curves. By comparison of curves, it is possible to see the transition from exponential to linear building regimes. 3B) When Poly2 is layered in the architecture [(Poly2/heparin/lysozyme/heparin)₅₀], and released at 37°C, release of over 34 days is achieved, showing the tunability of this system in response to a designed synthetic polymer. Total release in $\mu\text{g}/\text{cm}^2$ is plotted vs. time in days.

Figure 4.

Chondroitin film characterization. 4A) The signal recorded at each point of construction is taken as a percentage of the signal at 50 tetralayers and is plotted against the number of (Poly1/chondroitin/lysozyme/chondroitin) tetralayers to allow comparison of the curves. It is possible to see agreement of different measurement techniques and an exponential pattern of growth. 4B) Release of films constructed with lysozyme and chondroitin. Total release in $\mu\text{g}/\text{cm}^2$ is plotted vs. time in days. Note increased loading with increased numbers of tetralayers. 4C) The signal value for each timepoint was taken as a percentage of the signal at the last timepoint to allow comparison of release kinetics between films with different numbers of tetralayers. Percentage of total release is plotted vs. time in days. Results at 12 and 20 tetralayers included

large error in measurements corresponding to very low release rates and were therefore omitted from the plot.

Figure 5.

The total amount of protein detected using the micro-BCA kit (total protein) was plotted with the total amount of protein detected using the kinetic functional lysozyme assay (functional protein) to elucidate the functionality of the released protein. The amount of total protein released (ug) and functional protein released (ug) is plotted against time in days. This [(Poly1/heparin/lysozyme/heparin)₄₃] film shows nearly 100% maintenance of enzyme activity.

Polymer1 (Poly1)

Polymer2 (Poly2)

Scheme 1.

Structural characteristics of the synthetic polymers used. Note that Poly1 and Poly2 differ only by two methylene units in the backbone, increasing the hydrophobicity of the region next to the ester bond.

Table 1

Comparison of film characteristics of the three types of film discussed herein. Thickness of the films was measured using profilometry, and film protein incorporation was measured indirectly with UV-Vis measurement of absorbance at 280 and directly through instantaneous dissolution of films and quantification of the released protein.

	Film thickness in μm	UV-Vis absorbance of film at 280 nm	Total protein incorporated ($\mu\text{g}/\text{cm}^2$)
(Poly1/chondroitin/lysozyme/chondroitin) ₅₀ film	2.73	0.24	133
(Poly1/heparin/lysozyme/heparin) ₅₀ film	7.92	1.24	650
(Poly 2/heparin/lysozyme/heparin) ₅₀ films	13.23	1.52	800