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Citation: Hsu, Bryan B., Samantha R Hagerman, Kelsey Jamieson, Jovana Veselinovic, Nicholas O'Neill, Eggehard Holler, Julia Y. Ljubimova, and Paula T. Hammond. "Multilayer Films Assembled from Naturally-Derived Materials for Controlled Protein Release." *Biomacromolecules* 15, no. 6 (June 9, 2014): 2049–2057.

As Published: <http://dx.doi.org/10.1021/bm5001839>

Publisher: American Chemical Society (ACS)

Persistent URL: <http://hdl.handle.net/1721.1/101181>

Version: Author's final manuscript: final author's manuscript post peer review, without publisher's formatting or copy editing

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HHS Public Access

Author manuscript

Biomacromolecules. Author manuscript; available in PMC 2015 April 07.

Published in final edited form as:

Biomacromolecules. 2014 June 9; 15(6): 2049–2057. doi:10.1021/bm5001839.

Multilayer Films Assembled from Naturally-Derived Materials for Controlled Protein Release

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Abstract

Herein we designed and characterized films composed of naturally derived materials for controlled release of proteins. Traditional drug delivery strategies rely on synthetic or semi-synthetic materials, or utilize potentially denaturing assembly conditions that are not optimal for sensitive biologics. Layer-by-Layer (LbL) assembly of films uses benign conditions and can generate films with various release mechanisms including hydrolysis-facilitated degradation. These use components such as synthetic polycations that degrade into non-natural products. Herein we report the use of a naturally-derived, biocompatible and degradable polyanion, poly(β -l-malic acid), alone and in combination with chitosan in an LbL film, whose degradation products of malic acid and chitosan are both generally recognized as safe (GRAS) by the FDA. We have found that films based on this polyanion have shown sustained release of a model protein, lysozyme that can be timed from tens of minutes to multiple days through different film architectures. We also report the incorporation and release of a clinically used biologic, basic fibroblast growth factor (bFGF), which demonstrates the use of this strategy as a platform for controlled release of various biologics.

Keywords

Drug delivery; layer-by-layer; growth factor; sustained release

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SUPPORTING INFORMATION AVAILABLE

Growth curves of (polycation/PMLA)_n films, ionic titration of polyplexes, potentiometric titration of PAA, fractional releases of (chitosan/PMLA/lys/PMLA)_n and (chitosan/PMLA/bFGF/PMLA)_n films, loadings and thicknesses of (chitosan/PMLA:PAA/lys/PMLA:PAA)_n films, proliferative and cytotoxicity of (chitosan/PMLA)_n films, and dose-response of bFGF on proliferation are available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

Controlled release from biodegradable matrices offers attractive possibilities in creating personalized medicine for on-demand and/or pre-programmed care with dynamic tunable delivery^{1, 2}. Topical application, implantation or injection of these materials, whether alone or as a coating, can administer localized dosages of therapeutics and significantly reduce complications from systemic toxicity^{3, 4}. In addition, with bolus release, biologics like growth factors are underutilized because of their rapid degradation and clearance *in vivo*. In addition, only nanogram levels are necessary to elicit biological response and hence a controlled low-dose release would enhance not only the therapeutic efficiency, but also practical factors such as increasing patient compliance through fewer applications, reduced cost from less growth factor needed, and minimal upkeep from a preprogrammed release profile. There are numerous mechanisms explored for controlling drug release ranging from dissolution, degradative, or diffusive mechanisms⁵, to triggered release via external stimuli such as electrical or photonic means⁶. For such delivery devices, biocompatibility and biodegradability are desirable and would eliminate concerns of systemic toxicity and complications from released polymer byproduct.

A prominent, well-studied degradable polymer for controlled release is poly(lactic-*co*-glycolic acid) (PLGA), which has a number of clinical uses for drug delivery^{7, 8}. Although processing of PLGA for drug encapsulation is amenable for certain small molecules, it is potentially denaturing for sensitive biologics^{8, 9}, as it may require organic solvent and/or heat conditions for processing. Furthermore, the relative loading of biologic drugs into PLGA is defined in part by the thermodynamics of polymer mixing, and typically implies low fractions of drug, thus requiring significantly more polymer carrier for a given net amount of drug. The resultant drug release is a complex interplay between the drug's diffusivity and the PLGA carrier's degradation; heterogeneous drug distribution, pore formation, water ingress/swelling, and polymer structural stability, among other factors can lead to burst and/or multi-phasic release profiles that cause undesirable fluctuations in drug release¹⁰.

An alternative approach is the aqueous based layer-by-layer (LbL) assembly of polymer thin films that encapsulates biologic drugs such as proteins and nucleic acids by taking advantage of their charged state and/or other secondary interactions^{1, 2, 11}. Instead of using organic solvent to form a water-insoluble hydrophobic polymer blend, LbL assembly utilizes electrostatic complexation between polyelectrolyte components under aqueous conditions to form insoluble, electrostatically-crosslinked matrices. When these films are assembled, they resist dissolution in water and can be designed to release therapeutic cargo through various diffusional, triggered, and degradative mechanisms¹². The latter has shown capability for tunable release of sensitive biologics like growth factors while retaining their biological activity¹³⁻¹⁷. An extensive library of natural biomaterials such as polysaccharides (*e.g.*, hyaluronic acid, chondroitin, heparin, and chitosan) and polypeptides (*e.g.*, poly(L-lysine), poly(L-arginine), poly(L-glutamic acid), and poly(L-aspartic acid) are available for use in LbL assembly; however, to control release, synthetic polycations such as poly(β -amino ester)s (PBAs) are primarily used. While these were originally synthesized for gene delivery¹⁸ they have subsequently been used in LbL films as a hydrolytically degradable

component^{19–21} and sustained hydrolytic release still primarily relies on these synthetically-derived PBAEs.

Herein we investigate the use of completely naturally-derived materials for LbL assembly of controlled release films. To achieve tunable degradation, we replace the commonly used synthetic PBAE with a naturally derived polyanion, poly(β -L-malic acid) (PMLA), which can be extracted in linear, high-molecular weight from *Physarum polycephalum* and branched, low molecular weight from *Aureobasidium* sp., functioning as a DNA polymerase inhibitor in the former²². It has demonstrated excellent biocompatibility with tolerance by mice of up to 1.6 g/kg intravenously and 6 g/kg intraperitoneally^{22–24}, in addition to eliciting no immunogenic response^{22, 24}. The degradation product, L-malic acid, is a metabolite in the Krebs cycle and can be found naturally in high abundance, yielding a “Generally Recognized As Safe (GRAS)” status by the FDA. We also include use of chitosan as an additional component to stabilize film growth and robustness. This naturally-derived polycation has been extensively investigated for its numerous positive biological properties²⁵ and has also received GRAS status by the FDA. We demonstrate that the chitosan-PMLA scaffold is a viable and effective means for controlled delivery of a model protein, lysozyme, and a therapeutically relevant growth factor, bFGF.

MATERIALS AND METHODS

All materials were used without further purification unless otherwise noted. The polyelectrolytes used in this study were obtained from various sources: Poly(L-Lysine) (PLL, 30–70kDa, Sigma-Aldrich), fluorescein-labeled PLL (30–70kDa, Sigma-Aldrich) linear polyethylenimine (LPEI, 25kDa and 250kDa, Polysciences), chitosan (15 kDa, Polysciences), polyallylamine hydrochloride (PAH, 60 kDa, Polysciences), poly(sodium-4-styrenesulfonate) (SPS, 70 kDa, Sigma-Aldrich), poly(acrylic acid) (PAA, ~50 kDa, Polysciences). Poly(β -L-malic acid) (PMLA, 40 kDa) was cultured from *Physarum polycephalum* as previously described²². Hen-egg lysozyme, 3 M sodium acetate, and all other materials were obtained from Sigma-Aldrich. Phosphate-buffered saline (Dulbecco’s PBS 10 \times) was obtained from Invitrogen and diluted to 1 \times concentration before use. Recombinant human basic fibroblast growth factor (bFGF) was obtained from Biologend. Cell culture medium consisted of Dulbecco’s modified eagle medium (DMEM) supplemented with L-Glutamine, antibiotic-antimycotic, and heat-inactivated fetal bovine serum (FBS), which were obtained from Invitrogen and used at 1 \times concentrations. All solutions involving H₂O used MilliQ purified water.

Polymer degrees of ionization were determined by potentiometric titration, similarly to as described previously²⁶. After bubbling solutions with N₂, 15 mL solutions 0.5 mg/mL of PMLA or PAA in H₂O were titrated with 0.2 M HCl or NaOH and normalized to titration of pure H₂O. The pK_a was taken as the pH at which half of the monomer side chains are ionized.

In a 96-well plate, 40 μ L of 10 mg/mL polycation (or lysozyme) solution was combined with 40 μ L of polyanion solution and 70 μ L of a diluted NaCl solution, each prepared in 10 mM sodium acetate, pH 5.0. Optical density at 450 nm was normalized to the maximal

absorbance after blank (buffer) subtraction. Chitosan-PMLA polyplexes formed intractable pastes so 5-fold diluted solutions were used.

Unless otherwise noted, polymer or proteins were formulated at 1 mg/mL concentrations and films were assembled using programmable slide strainers (Carl Zeiss). Silicon wafers were pre-cleaned with methanol and water, irradiated with plasma (Harrick PDC-32G) and coated with a baselayer of (LPEI/SPS)₁₀ as described previously²⁷.

Films of (polycation/PMLA)_n with non-proteinacious polycations (*i.e.*, without lysozyme or bFGF) were assembled from 100 mM sodium acetate, pH 5.0, solutions with programmed cycles of immersion for 15 min in polycation solution, 10 s, 20 s, and 30 s of wash in buffer, then 15 min in PMLA solution and 10 s, 20 s, and 30 s of wash in buffer. Analogous films containing protein in a (protein/PMLA)_n or (chitosan/PMLA/protein/PMLA)_n architecture were assembled from 10 mM sodium acetate, pH 5.0 solutions with identical incubation times of 15 min in polymer/protein solution and 10 s, 20 s, and 30 s washes in H₂O. Solutions of bFGF were formulated at 50 µg/mL. After assembly, films were dried under house vacuum at room temperature overnight.

Thickness was measured from razor-scored films by determining the average step-height difference between six measurements with a 2.5 µm tip (Dektak 150 Profilometer). Release studies of films of 1 cm² area were performed by incubation in physiological conditions of 500 µL of PBS, pH 7.4 at 37°C. Solutions were periodically replaced with fresh aliquots of PBS pre-warmed to 37°C and returned to incubation. Concentration of PLL^{Fluor} was determined by comparison of absorbances at 494 nm for release aliquots to standard curves. Quantification of lysozyme released from the film using bicinchoninic acid (BCA) assay was found unacceptable, because of background signal originating from chitosan and PMLA film components. Therefore we used a lysozyme-specific enzymatic assay to determine the concentration of active lysozyme. Mixtures of 200 µL of 0.3 mg/mL *Micrococcus lysodeikticus* in PBS and 50 µL of lysozyme-containing sample or standard in PBS was monitored at 450 nm and 37°C in a 96-well plate format. The reduction in turbidity of sample solutions was compared to a standard curve to determine lysozyme concentration. bFGF concentration was measured by ELISA and performed according to manufacturer instructions (Peprotech).

To determine the effect of film components released into solution on cell viability, we incubated films in 1 mL of cell culture medium with 10% FBS at 37°C, similarly to as described for the release studies. NIH3T3 cells were seeded in a 96 well tissue culture plate at 10,000 cells/well in cell culture medium with 10% FBS and after an overnight incubation, media was replaced with 100 µL of sterile-filtered film release media. The cells were incubated overnight and then their viability was determined by MTS assay (Promega) and performed according to manufacturer's directions.

The activity of bFGF released was determined by proliferative assay with NIH3T3 (ATCC) fibroblast cells. Cell culture was composed of Dulbecco's modified eagle medium (DMEM) supplemented with fetal bovine serum, and the assay was adapted as previously described¹⁴. Cells were seeded at 1000 cells/well in 100 µL of 10% FBS culture medium in a 96-well

tissue culture plate and incubated for 2 d after which cells were serum starved in 100 μ L of 0.5% FBS culture medium. After overnight incubation the media was replaced with 100 μ L of PBS containing film release media (from the above mentioned release studies) that was combined with 900 μ L of 0.5% FBS culture medium and then sterile filtered. After 2 d of incubation, cell numbers was quantified by MTS assay (Promega) according to manufacturer's directions. Proliferative activity was determined by proportional increase in absorbance as normalized to a PBS control.

RESULTS AND DISCUSSION

LbL Assembly and Degradation

A number of mechanisms have been used for controlled release from LbL films; harnessing tunable degradation of at least one component in a multi-component, drug-laden, film is an important approach for releasing various therapeutics ranging from small molecules to biomacromolecules¹. PMLA offers an innocuous and naturally-derived alternative to the commonly used synthetic polyesters like PBAEs and PLGA. With potentiometric titration, we measured PMLA's pH dependent ionization, which is an important characteristic when considering electrostatic or hydrogen-bonded LbL film assembly. Figure 1 shows that the pH at which PMLA reaches 50% ionization (pK_a) is 4.58 ± 0.02 with roughly 68.2% and 99.3% of the carboxylic acid groups ionized at pH 5.0 and 7.4, respectively. These degrees of ionization may also tend to be higher in salt solutions due to electrostatic screening effects^{28, 29}. Therefore assembly of films at pH 5 or above will have substantial electrostatic interactions.

In exploiting PMLA's significant negative charge, we examined its film growth behavior with various polycations that have been well studied in polyelectrolyte multilayers. As shown in Figure 2, each (polycation/PMLA)_n film reveals a concurrent growth with the number of bilayers deposited, exhibiting the tunability in thickness and loading traditionally observed in multilayer films. A figure with the compiled growth curves is shown in Figure S1. Based on reported pK_a values for poly-L-lysine (PLL, $pK_a \sim 9.9$ ³⁰), linear polyethylenimine (LPEI, $pK_a \sim 7.9$ ³¹), polyallylamine (PAH, $pK_a \sim 8.8$ ³²), and chitosan ($pK_a \sim 6.5$ ³³), each polycation bears significant positive charge under the assembly conditions (pH 5.0) thus driving electrostatic film assembly with PMLA.

As sometimes observed in LbL film assembly, there can be variability in growth behavior depending on the film components. Similarly, we observed that each (polycation/PMLA)_n film grows exponentially to different degrees. Films composed of strong polyelectrolytes, or weak polyelectrolytes in highly charged states, can exhibit nanoscale growth that increases linearly with number of layers^{34–36}, typically the slope of the growth curve is in the range of nanometers to a few tens of nanometers per bilayer pair. On the other hand, when polyelectrolytes are weakly charged they may be highly diffusive in the LbL film, which can lead to exponential growth with bilayer thicknesses approaching hundreds of nanometers. Nonlinear growth is theorized to be a result of “in and out” diffusion where some polyelectrolytes can readily diffuse into and within the LbL film^{37–40}. Therefore the resulting growth behavior is subject to a number of characteristics such as the

polyelectrolyte's degree of ionization⁴¹, charge density⁴², molecular weight⁴³, secondary intermolecular interactions^{42, 44, 45}, and ionic strength of solution⁴⁶.

With these clear examples of multilayer film assembly using PMLA, we studied its role in facilitating film disassembly and component release. The hydrolysis of the ester backbone in PMLA would reduce its molecular weight and consequently destabilize the overall film integrity. We used a fluorescently labeled PLL component in a (PLL^{Fluor}/PMLA)₂₀ film and tracked its elution over time for insight into the release behavior imparted by PMLA-based films. As is shown in Figure 3, we found that PLL^{Fluor} continued to release for 30 hours, with the majority eluting within the first 24 hours. The duration in release is similar to what was observed with previous (polycation/polyanion)_n systems utilizing PBAEs, like (Polymer 1/DNA)_n which showed sustained release in PBS, pH 7.4 at 37°C for 16 to 30 hours^{20, 47}.

Polyelectrolyte Complex Stability

Our previous studies have used lysozyme as a good model for the investigation of protein release from different LbL film architectures¹³ with a size (i.e. 14.3 kDa with a hydrodynamic radius of 1.9 nm⁴⁸) and charge (i.e., isoelectric point, pI of 11⁴⁹) relevant to other therapeutically interesting biologics. While investigating (lysozyme/PMLA)_n film architectures, we discovered the ionic strength and pH of solution was highly influential on film growth; films could be assembled in 10 mM sodium acetate at pH 5.0, but not in 100 mM sodium acetate at pH 5.0, 10 mM sodium phosphate at pH 7.4, or phosphate buffered saline at pH 7.4. The stability of intermolecular crosslinks, especially ionic, is a well known factor in film assembly⁵⁰ and so we suspected the number and density of ionic crosslinks formed between the protein and polyanion may be less numerous than between a typical polycation and polyanion in an LbL film. Although lysozyme exhibits a net positive charge, it is in low number and of patchy distribution across the globular surface⁵¹. In line with what we observed during attempted (lysozyme/PMLA)_n film assemblies at pH 5.0 and pH 7.4, ionic strength at 10 mM was too weak to prevent electrostatic film assembly with lysozyme (net charge +10⁴⁹). In contrast, 100 mM ionic strength disrupted film assembly due to increased ionic shielding. At pH 7.4, lysozyme's charge density is even lower (+7⁴⁹), making it more sensitive to ionic shielding, which is evidenced by a lack of film growth in 10 mM sodium phosphate and PBS solutions.

To probe the stabilities of electrostatic interactions formed in our multilayers, we titrated two-component polyelectrolyte complexes (polyplexes) with increasing concentrations of sodium chloride. Addition of salt disrupts electrostatic crosslinks through ionic shielding and the critical concentration necessary for these polyplexes to reach dissolution (as determined by turbidity) reflects their relative strengths of complexation. This strategy has been fruitfully explored for other (protein/polyanion) film architectures⁵² and has uncovered direct relationships between the stabilities of polyplexes in solution and their analogous LbL films^{53–55}. For insights into the PMLA-based systems, we studied the stability of polyplexes between lysozyme and various polyanionic compounds at pH 5. As shown in Figure S2 and summarized in the first three rows of Table 1, each of the polyplexes are stable up to a critical sodium chloride concentration, [NaCl]_C, beyond which the polyplexes are dissolved. Previous work has shown heparin to be an excellent polyanion for the LbL assembly of

lysozyme¹³ and as such, its polyplex is found to be stable in up to 580 mM of NaCl, agreeing with reported values⁵⁶. Heparin is strongly polyanionic with fully ionized sulfate moieties and in conjunction with its polysaccharide backbone (i.e., capable of hydrogen-bonding) it likely contributes additional stability to these polyplexes over PMLA, a weak polyelectrolyte. By comparison, PAA forms a more stable complex with lysozyme. Potentiometric titration of PAA (Figure S4) indicates it has a pKa of ~6.6 with substantially lower ionization (18.9%) than PMLA. Although it is capable of fewer electrostatic crosslinks at pH 5.0, hydrogen bonds can stabilize polyplex formation, as has been observed in previously assembled bilayer LbL films at pH 4⁵², where PAA is even more poorly charged (<10% ionized). Hydrogen-bonding would be insensitive to ionic shielding effects and to demonstrate this, we titrated lysozyme polyplexes with tannic acid, a polyphenol known to form such complexes with proteins in solution and in LbL assemblies⁵⁷. Shown in Figures S1 and Table 1, we found these complexes were insensitive to ionic titration.

For comparison of the relative stabilities of polycation-polyanion polyplexes, where electrostatic crosslinks would be more numerous, we studied the ionic titration of PLL, chitosan, PMLA, and PAA based complexes. As shown in Figure S3 and summarized in Table 1, each polyplex showed greater stability than lysozyme-based electrostatic polyplexes. This can be attributed to denser electrostatic crosslinks, which are overall less susceptible to salt induced dissolution. The gradual loss of turbidity with increasing ionic strength suggests loosening that progresses towards dissolution, where the high multivalency of crosslinks maintains some semblance of complexation despite high ionic strength. This is in contrast to lysozyme polyplexes, which have a sharp decline in stability after reaching a critical sodium chloride concentration.

Controlled Protein Release

Using our insights into the relative stabilities of protein-polyanion and polycation-polyanion polyplexes, we examined (lysozyme/PMLA)_n bilayer systems and (chitosan/PMLA/lysozyme/PMLA)_n tetralayer systems as rapid and sustained release film formulations, respectively. As described earlier, we found the bilayer films were capable of assembly from low salt solutions of 10 mM sodium acetate, pH 5.0. The (lysozyme/PMLA)_n growth, as shown in Figure 4a, reveals an initial slow growth until 20 bilayers, after which growth becomes proportional with the number of bilayers. An initially sluggish growth has been similarly described for other multilayer film architectures^{58, 59} including lysozyme-based films¹³ and was attributed to various factors such as an initially slow period of exponential growth⁵⁹ or island formation of surface-complexes that coalesce to seed later film growth³⁸. After this induction period, we observe a linear growth that has been observed in LbL films composed of strong polyelectrolytes³⁴⁻³⁶ and particle based films where minimal interdiffusion can occur. The film thickness deposited per bilayer in this linear growth regime is 5.1 nm/bilayer, which is roughly 1.3 times lysozyme's hydrodynamic diameter (~3.8 nm⁴⁹). When including PMLA's thickness contribution, the lysozyme deposition appears to be limited to a monolayer adsorption, with minimal interdiffusion, which is likely due to lysozyme's size and globular structure.

When examining the release behavior of (lysozyme/PMLA)₁₂₀ films in physiological conditions (PBS, pH 7.4 and 37°C), we found a rapid lysozyme release as shown in Figure 4b. The combination of higher pH and ionic strength appears to completely destabilize the film within 30 min and coincides with our previously described observations. When calculating the loading density we find that there is 1.22 ± 0.10 mg/mm³ of lysozyme in the film, a density approaching the 1.25 to 1.62 mg/mm³ of protein typically found in dried formulations⁶⁰. This indicates that lysozyme is a substantial component of these films and although PMLA is a minor component (by mass), it plays a major functional role in facilitating assembly into tunable thin films.

For additional control over lysozyme release, we developed a tetralayer architecture of (chitosan/PMLA/lysozyme/PMLA)_n that would impart robustness against pH and/or ionic strength changes and include hydrolytic degradability for controlled release. Ionic titration showed polycation-polyanion polyplexes substantially more robust than lysozyme-PMLA polyplexes (Table 1), and so we integrated a chitosan-PMLA component into the film. Chitosan was chosen as the polycation because of its beneficial therapeutic properties in some applications²⁵. Similar to what we observed in the (chitosan/PMLA)_n growth curve (Figure 2b), Figure 4c shows that this tetralayer architecture exhibits exponential growth that is representative of significant interdiffusion within the film. The film growth in the latter part of super-linearity (>15 bilayers) is 69.2 nm/tetralayer, a quantity greater than necessary for a simple monolayer adsorption mechanism that would lead to surface charge reversal. This difference in growth compared to the bilayer system shows that chitosan can facilitate interdiffusion and consequently more material deposition per adsorption cycle.

When examining the tetralayer's release profile, Figure 4d showed a controlled release for both 20 and 60 tetralayers, equating to roughly 2 days and 5 days, while releasing 30.1 ± 1.1 μg/cm² and 206.0 ± 0.4 μg/cm² of lysozyme, respectively. From our previous investigations into other protein-based LbL films, especially those containing growth factors, these loadings are in far excess of what would be needed for *in vivo* therapeutic activity, as loadings of tens to hundreds of ng/cm² are able elicit biological response^{14, 17}. We found that the incorporation of chitosan allowed for a more sustained degradative release mechanism avoiding the bolus release we observed with the bilayer system. When these release profiles are shown as a fraction of total release (Figure S5), it is clear there is additional sustainment of release from the 60 tetralayer film, which would be expected from the thicker film in which an erosion mechanism would dominate. It should be noted that hydrolysis of the PMLA backbone as well as ionization of the film components contributes to film disassembly, where the PMLA half-life for degradation is 10 h²² under the same conditions of pH 7.4 and 37°C. The commensurate thickness and loading increases of 5.9-fold and 6.8-fold, respectively, of 60 tetralayer films compared to 20 tetralayer films reveal that the interdiffusional effects enhancing film growth also enhance lysozyme loading. At 60 tetralayers, the lysozyme loading density is 0.62 ± 0.02 mg/mm³, almost exactly half that of the (Lysozyme/PMLA)₁₂₀ bilayer film with an equivalent number of total layers (240), which suggests there is minimal competition between lysozyme and chitosan, and that the chitosan/PMLA component has a balanced contribution to film growth. We also tested analogous films of (PLL/PMLA/Lysozyme/PMLA)_n assembled in 10 mM sodium

phosphate, pH 7.4. Although these films showed significant growth, they did not incorporate detectable amounts of lysozyme, and at this pH, the charge density of lysozyme may be too low (+7⁴⁹) for stable electrostatic film incorporation. While ensuring sufficient drug payload is of primary importance, minimizing possibly deleterious effects of pH on drug and film components must also be considered when developing assembly conditions.

With insight from our studies on polyplex stabilities, we hypothesized that gradually replacing PMLA with PAA as the polyanion in (chitosan/polyanion/lysozyme/polyanion)₂₀ films could introduce a tunable release profile. Previous studies have shown that mixtures of two weak polyelectrolytes, PLL and a PBAE, could vary ovalbumin release profiles from multilayer films⁶¹. We assembled films from PMLA:PAA molar mixtures of 100:0, 75:25, 50:50, 25:75, and 0:100 and found that while these film thickness and lysozyme loadings were fairly similar (Figure S6), their release kinetics changed dramatically. As shown in Figure 5, the release can be sustained from ~2 days to more than 3 weeks by increasing the PAA fraction with first-order kinetic fits showing the time for half-maximum lysozyme release ($T_{1/2}$) also increases from 0.5 days to more than 5 days. The intermolecular interactions (e.g., ionic and hydrogen bonding) in the film are significantly affected by the changed nature of the aqueous environment when transitioning from assembly to release conditions; increasing pH (from 5 to 7.4) causes increased ionization in the polyanions and increasing salt concentrations weaken the electrostatic interactions. Based on our potentiometric and ionic titrations, PMLA is significantly charged during both assembly (68% ionized) and release conditions (99% ionized) with those electrostatic bridges with lysozyme being highly sensitive to ionic shielding. In contrast, PAA is weakly charged during assembly (19% ionized) and can form a number of hydrogen-bonding interactions with lysozyme that are salt insensitive. Transition to release conditions causes significant but incomplete ionization (66% ionized), allowing for lysozyme stabilization despite the pH change. These factors in addition to the non-degradability of PAA are likely factors that allow for the sustainment in lysozyme release when increasing PAA fraction is incorporated into these LbL films.

Therapeutic Growth Factor Release

As a demonstrative example for therapeutic release, we replaced lysozyme with fibroblast growth factor-basic (bFGF) in a (chitosan/PMLA/bFGF/PMLA)_n tetralayer architecture. As observed in the growth curve (Figure 6a), these films show concomitant increases in film thickness with number of tetralayers. At 60 bilayers the film is 534 nm, which is considerably thinner than the 3.3 μm for analogous lysozyme-based tetralayers. The weight (16.3 kDa) and hydrodynamic diameter (~5.6 nm⁶²) of bFGF is close to that of lysozyme; however, since only nanogram levels are needed to elicit biological response ($\text{ED}_{50} \sim 1\text{--}4$ ng/mL), we used a considerably diluted protein solution (50 $\mu\text{g/mL}$) during assembly that results in less material deposited per tetralayer. Beyond the initial slow growth period (> 20 tetralayers), we found 10.7 nm/tetralayer deposited, which is roughly 6.5-fold less than the lysozyme-based tetralayer films.

Through examination of the release of these bFGF-based films, we found the desired sustained release profiles. Their duration (Figure 6b) extends beyond 10 days with 9.1 ± 1.5

ng/cm² and 20.1 ± 4.8 ng/cm² of bFGF loaded in 20 and 60 tetralayer films, respectively. Because of the near-linear growth curve, the loading of these bFGF films can be predetermined based on the film thickness, which is controlled by the number of layers deposited as is characteristic of LbL release systems. When examined as fraction of total release (Figure S7), it is clear that the kinetics of bFGF elution is similar and independent of the film thickness to at least 60 tetralayers. Studies of the release kinetics from these films (Figure 6b) found sustained release for more than 10 days. There are two features that are different than observed with the lysozyme-based tetralayer release profiles: both 20 and 60 tetralayers have similar release kinetics (Figure S7) and their duration of release is longer. Previous studies with growth factors and lysozyme in LbL films^{13–15, 17} have shown both kinetic dependence and independence with the number of layers deposited; contributions of film thickness, architecture, component composition, and types of intermolecular interactions can have substantial influence on overall film morphology and stability.

As LbL films can be assembled under benign aqueous conditions and is based on reversible intermolecular interactions like electrostatic and hydrogen-bonding, biologics can avoid the denaturing conditions sometimes plaguing other types of controlled release formulations. Upon contact with bodily fluids, the actual *in vivo* concentration is variable with the area of film and contact volume ultimately dependent on the location application. However, we can demonstrate the proliferative activity of the film-released bFGF *in vitro*. Stimulation of cell proliferation by bFGF follows a dose-response relationship and we found 10 ng/mL to be sufficient for maximal effect (Figure S8). We compared this concentration of as-received bFGF with the bFGF released into PBS from 60 tetralayer films and found that its accumulation in each aliquot had significant proliferative activity (Figure 6c) that compares well to previous bFGF-based LbL films¹⁴. In fact, the latter's greater activity over the as-received bFGF may indicate that it is the beneficiary of the co-release of chitosan that stabilizes bFGF activity by protecting it from heat-inactivation and proteolysis⁶³. For comparison, our study of the proliferative activity of the (chitosan/PMLA)_n scaffold showed no significant activity (Figure S9) on its own. In conjunction with the relatively non-cytotoxic nature (Figure S10) of the scaffold, this controlled release formulation can sustainably release therapeutic proteins, like a growth factor, from a completely biodegradable thin film coating.

CONCLUSIONS

In the interest of generating improved biocompatibility in a biodegradable film formulation for controlled drug delivery, we utilized a naturally derived polyanion PMLA, whose degradation products are GRAS by the FDA. We investigated the ability of PMLA to assemble into multilayers with PAH, chitosan, PLL and LPEI and found the characteristic layer-dependent growth and sustained release behavior. Bilayer and tetralayer films using a model protein, lysozyme, showed tunable release from 30 minutes to more than 5 days. We found that the differences in their release kinetics, and overall film assembly could be explained by the relative stabilities of electrostatic complexation. By using a PMLA:PAA blend for the polyanionic component, we were able to tune release out to more than 3 weeks. To explore biomedical applicability of this film architecture, we demonstrated the sustained

release of a growth factor, bFGF, for nearly two weeks and found it retained its biological activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This research was supported (in part) by the U.S. Army Research Office under contract W911NF-13-D-0001 and the Air Force under contract W911NF-07-D-0004. The authors wish to dedicate this paper to the memory of Officer Sean Collier, for his caring service to the MIT community and for his sacrifice.

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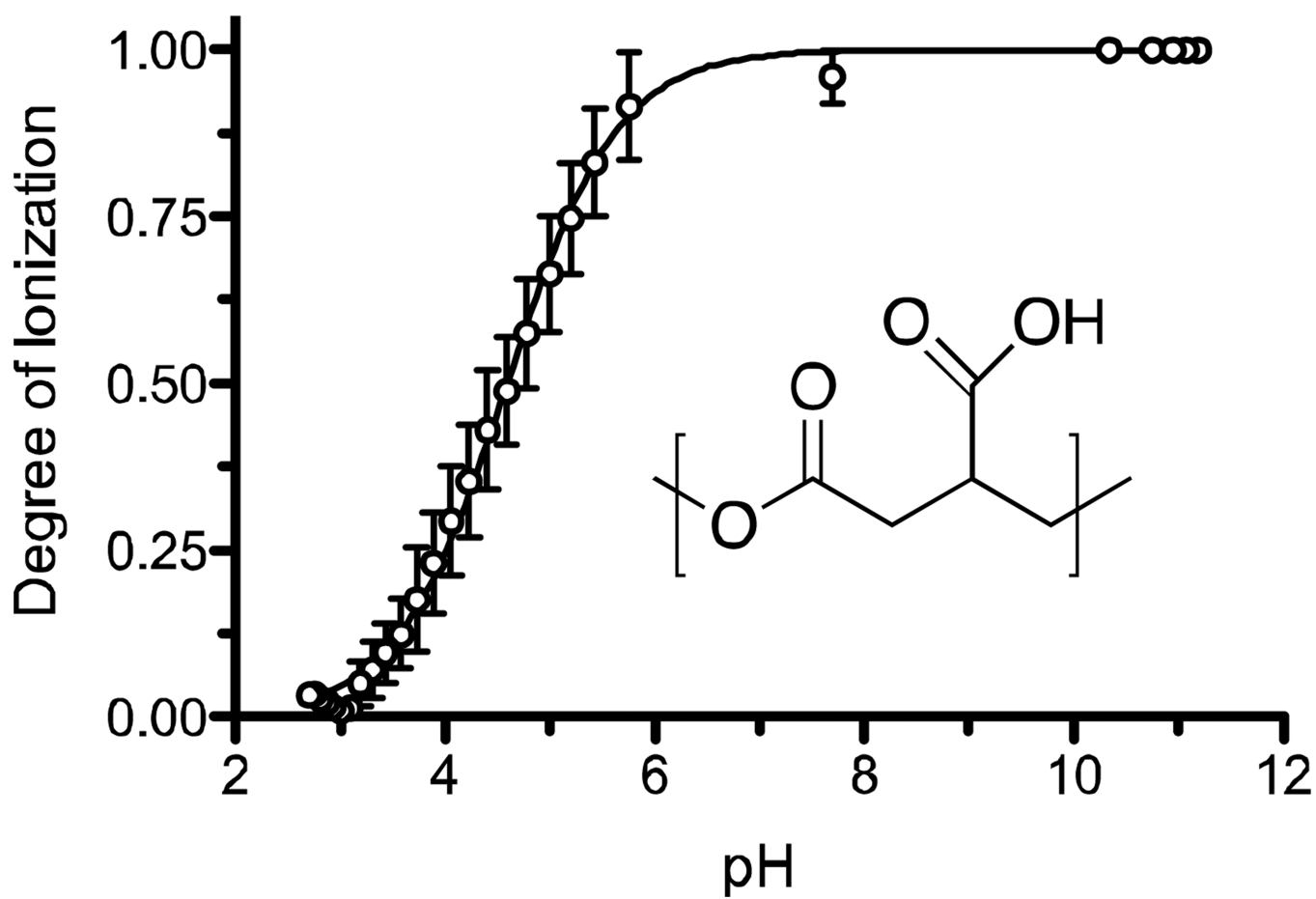


Figure 1. Potentiometric titration of PMLA in water at room temperature. The symbols represent measured values of PMLA (*inset*) and the solid line is a four-parameter fit, giving a measured pK_a of 4.58 ± 0.02 .

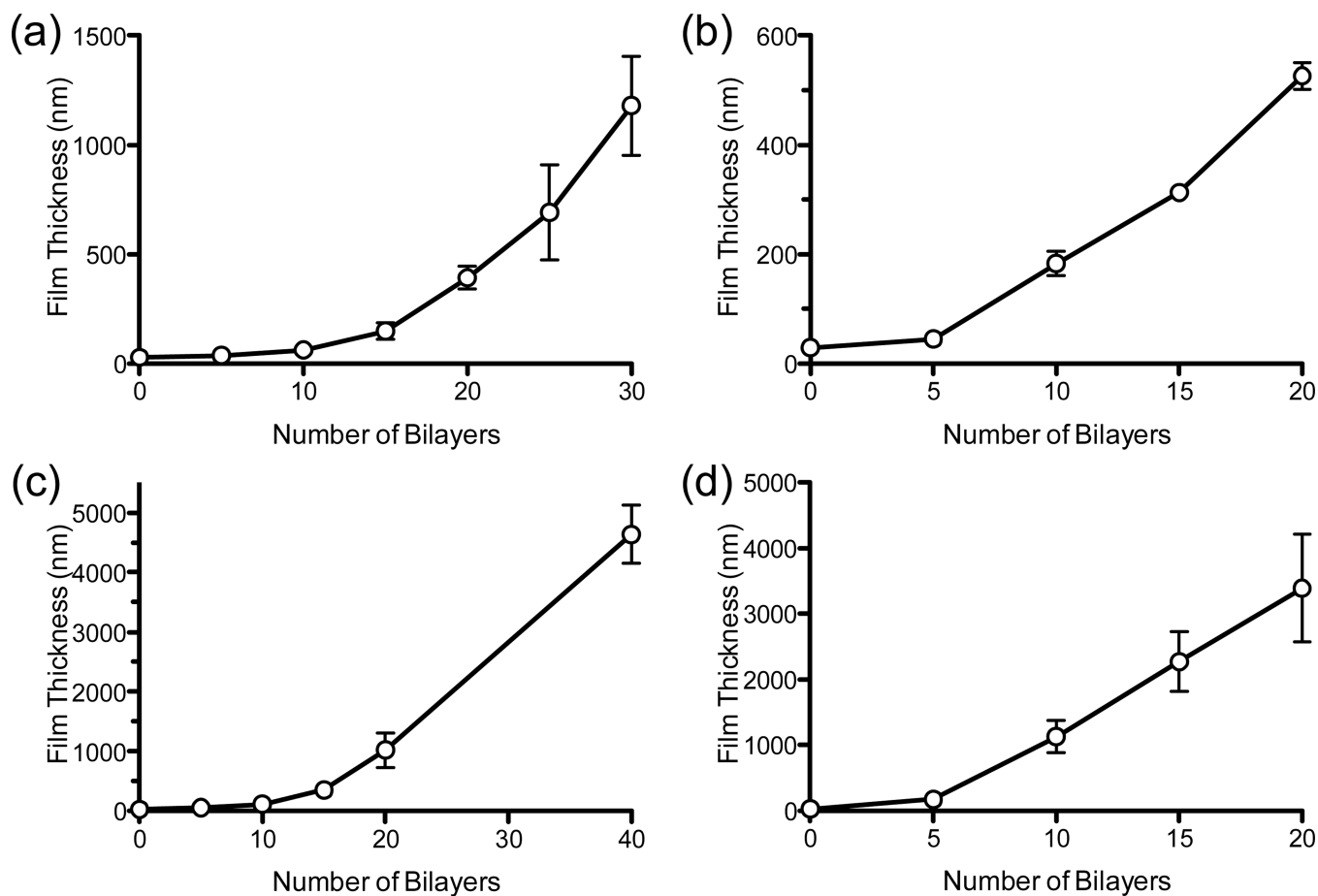


Figure 2. Film growth of (polycation/PMLA)_n films. Growth curves represent film assembly with polycations of PAH (a), chitosan (b), PLL (c), and LPEI (d).

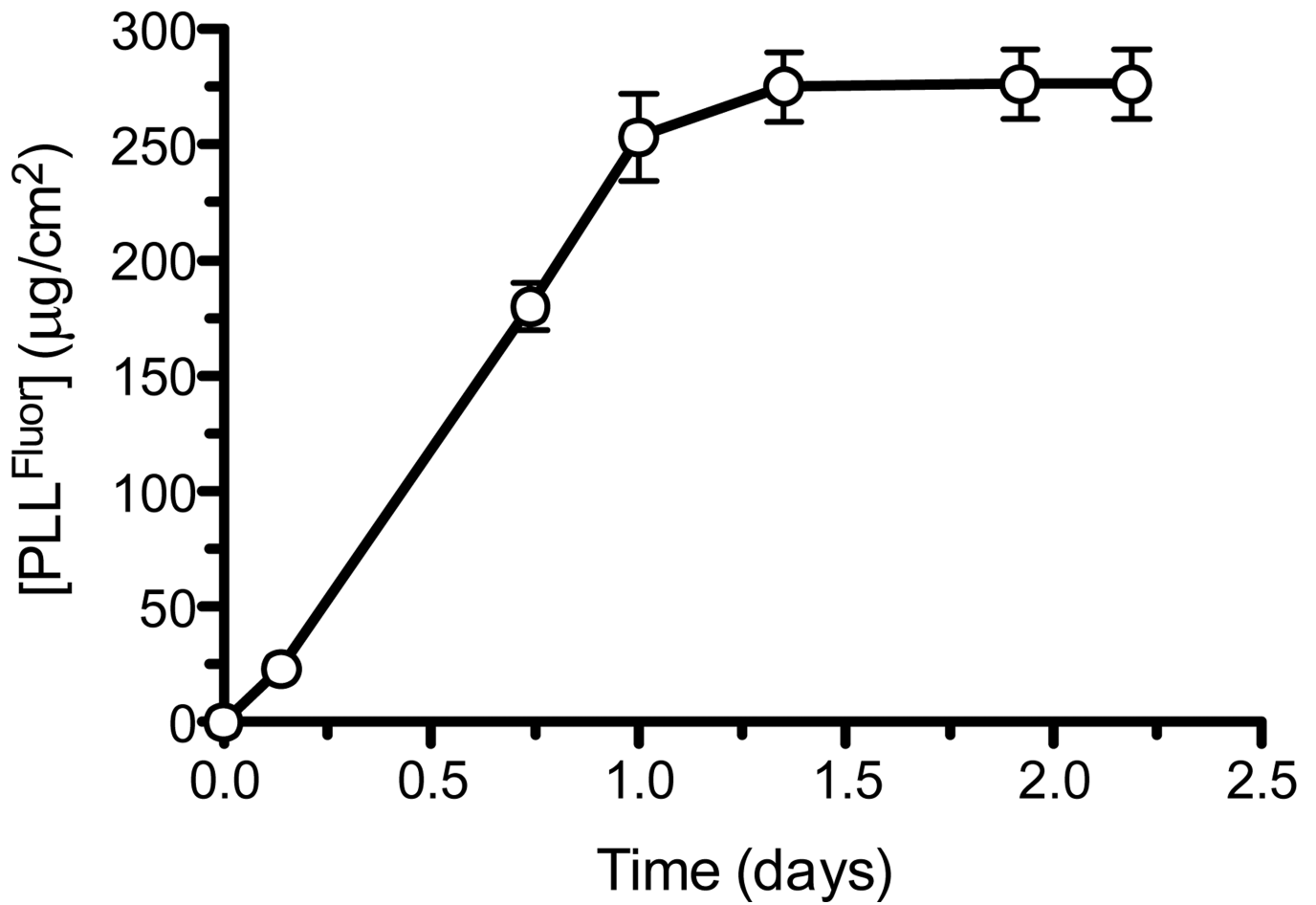


Figure 3. PLL^{Fluor} release profile from (PLL^{Fluor}/PMLA)₂₀ films incubated in PBS, pH 7.4 at 37°C.

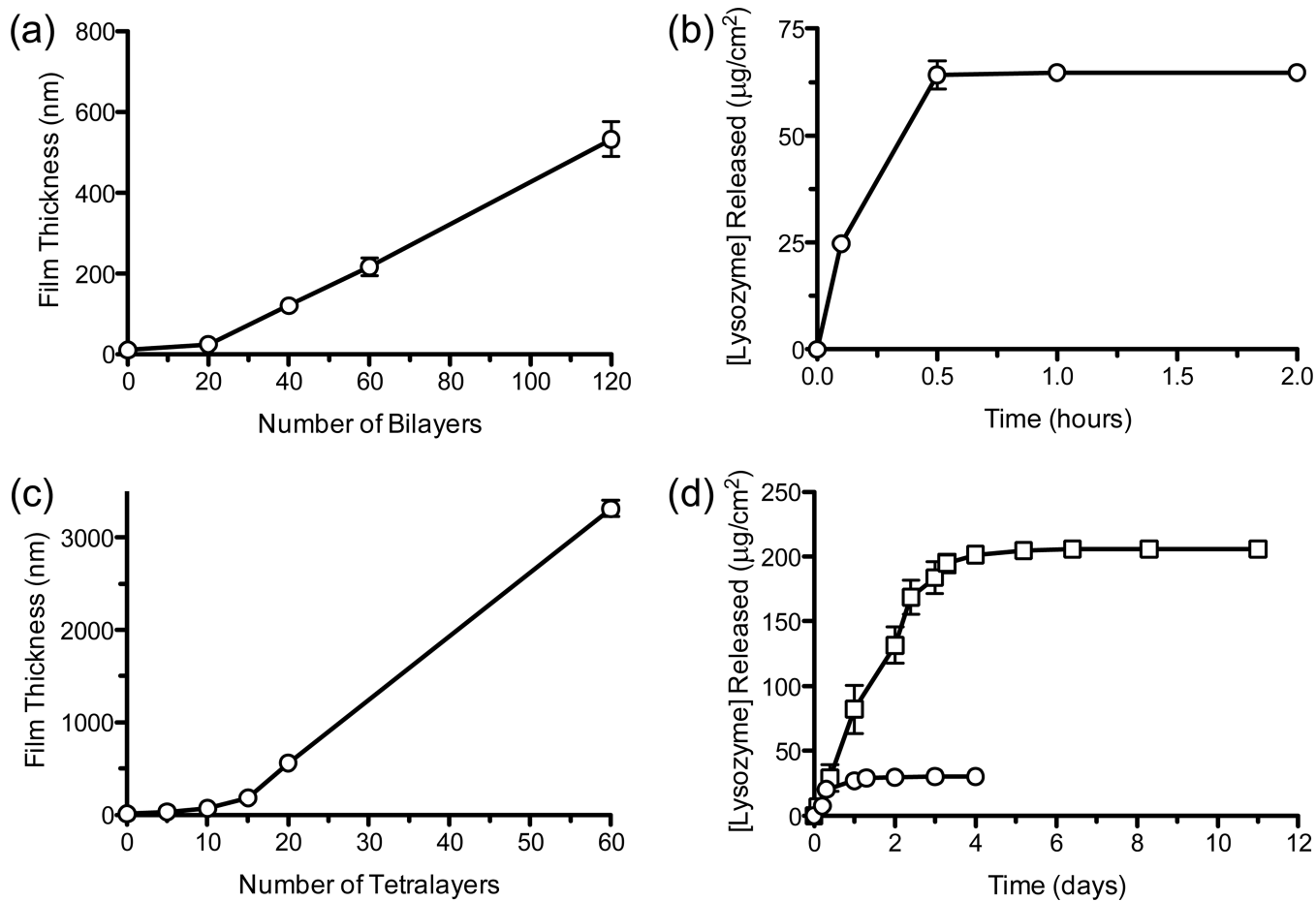


Figure 4.

Film growth curves (a,c) and lysozyme release profiles in PBS, pH 7.4 at 37°C (b,d) for (lysozyme/PMLA)_n films (a,b) and (chitosan/PMLA/lysozyme/PMLA)_n films (c,d). Release data were from 120 bilayer films (b), and 20 (*circles*) and 60 (*squares*) tetralayer films (d).

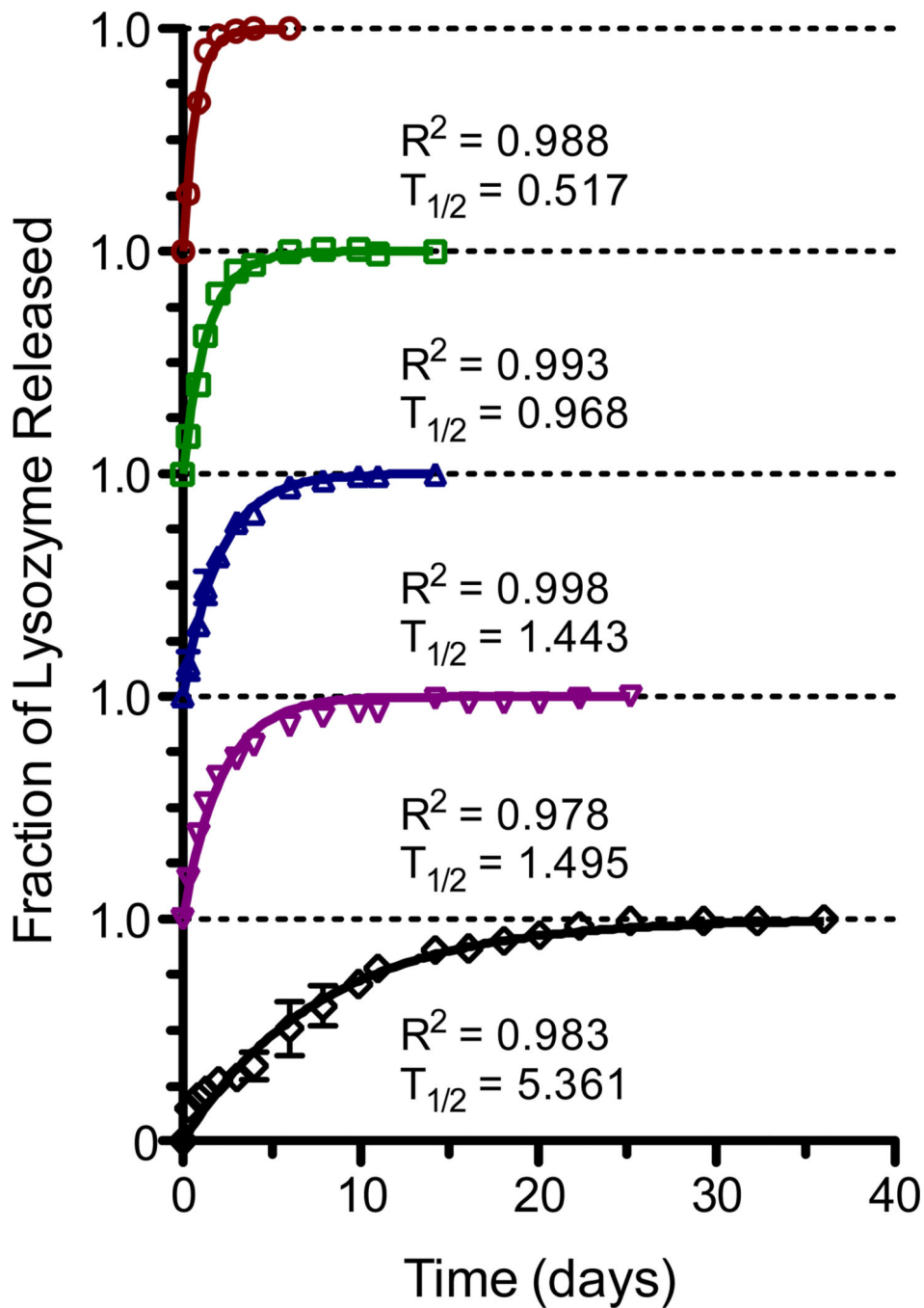


Figure 5. Release of lysozyme into PBS, pH 7.4 at 37°C from (chitosan/polyanion/lysozyme/polyanion)₂₀ films of 100:0 (red circles), 75:25 (green squares), 50:50 (blue triangles), 25:75 (purple inverted triangles), and 0:100 (black diamonds) PMLA:PAA molar ratios. Symbols represent measured data and solid lines are their first-order fits.

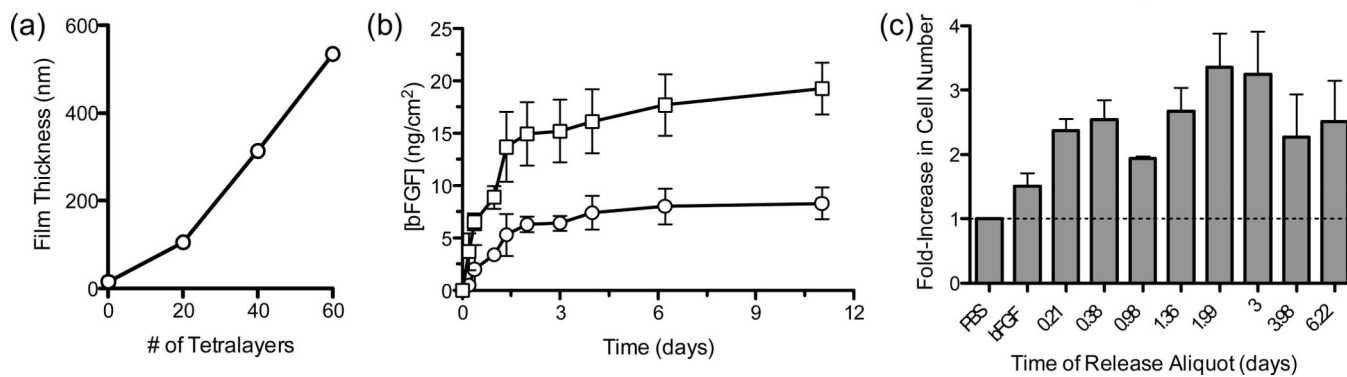


Figure 6. Film characteristics of (chitosan/PMLA/bFGF/PMLA)_n films including its growth curve (a), release profiles in PBS, pH 7.4 at 37°C for 20 (*circles*) and 60 tetralayers (*squares*) (b), and proliferative activity of PBS, 10 ng/mL of as-received bFGF and film-released bFGF on NIH3T3 cells (c).

Table 1

Ionic titration of polyplexes and polyionic species at pH 5.0 and at room temperature. The critical sodium chloride concentration at which complete polyplex dissolution is achieved is defined as $[\text{NaCl}]_c$.

Polyelectrolyte Complex	Type of Interaction	$[\text{NaCl}]_c^*$
Lysozyme - Heparin Sulfate	Electrostatic	0.58 M
Lysozyme - PMLA	Electrostatic	0.29 M
Lysozyme - PAA	Electrostatic	1.17 M
Lysozyme - Tannic Acid	Hydrogen-Bonding	Unaffected
PLL - PMLA	Electrostatic	2.3 M
Chitosan - PMLA	Electrostatic	> 3.4 M
Chitosan - PAA	Electrostatic	> 3.4 M