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Modulated release of bioactive protein from multilayered blended PLGA coatings

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Abstract

The objective of this study was to develop a poly(D,L-lactic-co-glycolic acid) (PLGA)-based coating system for producing biologically-inspired delivery profiles. Protein-loaded microspheres were made from PLGA (50:50) terminated with carboxylic acid groups (PLGA-2A) blended either with more hydrophobic PLGA (50:50) having lauryl ester endcaps (PLGA-LE) or with the more hydrophilic Pluronic F-127 (PF-127). Dense coatings were formed by pressure-sintering the microspheres. Altering hydrophobicity changed the water concentration within coatings, and consequently the time to onset of polymer degradation and protein release was modulated. After blending up to 8% Pluronic, degradation by-products began accumulating immediately upon incubation in saline, whereas, degradation was delayed for up to 14 days with blending of up to 30% PLGA-LE. Primary protein release peaks from one-layer coatings could be created from 7 to 20 days using 8% PF-127 or 30% PLGA-LE blends, respectively. Multilayered coatings of different blends generated several release peaks, with their temporal occurrence remaining approximately the same when layers of other hydrophobicity were added above or below. To allow design of coatings for future use, results were used to construct a model based on Fourier analysis. This polymer blend system and model can be used to mimic temporally varying profiles of protein expression.

Keywords: Polymer blending; PLGA; Protein release; Multilayered; Biodegradable

1. Introduction

The goal of many drug delivery devices has been to obtain zero-order release kinetics. For applications such as treatment of some systemic or chronic diseases, this approach, which is similar to continuous intravenous administration, may be desirable. However, wound healing is a dynamic process involving numerous biomolecules that trigger chemotaxis, proliferation, and differentiation of several cell types. For example, analysis of growth factor expression in callus during bone fracture healing has revealed a complex sequence of several biomolecules, including platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), transforming growth factor (TGF), and bone morphogenetic protein (BMP) (e.g. (Bolander, 1992; Bourque et al., 1993; Bostrom et al., 1995; Yu et al., 2002)). Constant delivery of a single type of molecule may alter cell and tissue behavior, but it does not optimally coordinate appropriate responses. Additionally, sustained exposure, high concentrations of biomolecules, and/or delivery at inappropriate times can desensitize cells as they downregulate expression of receptors or lead to adverse cell behavior (e.g. (Heldin et al., 1982; Assoian, 1985; Border and Ruoslahti, 1992; Lee et al., 1997)). Delivery of growth factors with release profiles inspired by those of natural wound healing may enhance healing and improve integration of implants in bone. Ideally, devices also would be able to deliver multiple molecules with different times to onset of release, similar to those found during bone repair.

In previous work, we developed a multilayered gelatin system for the combined or sequential release of multiple osteotropic biomolecules (Raiche and Puleo, 2001, 2004a,b). By heterogeneously loading and crosslinking the gelatin, different release profiles could be achieved. Osteoblastic responses could be modulated by releasing BMP-2 and IGF-I individually, together, or in sequence. Alkaline phosphatase activity and matrix mineralization were greatest with release of BMP-2 followed by IGF-I or by

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BMP-2 + IGF-I. Recurrent concerns about glutaraldehyde used as a crosslinking agent, however, stimulated interest in developing alternate delivery systems based on synthetic polymers.

Biodegradable polyesters of lactic and glycolic acids have enjoyed remarkably broad medical application for nearly 30 years. Despite potentially negative side effects resulting from acidic degradation products, poly(D,L-lactic-co-glycolic acid) (PLGA) devices have been shown to be biocompatible for orthopedic, soft tissue, non-load-bearing, and load-bearing applications (reviewed in Athanasiou et al. (1996), An et al. (2000), Peltoniemi et al. (2002)). PLGA microspheres and films have been widely used for controlled drug release. Both small molecules, such as opioid analogs and hormones, and large proteins, such as growth factors, have been delivered from PLGA devices (e.g. (Yolles et al., 1975; Wang et al., 1996; Cleland et al., 1997; Burton et al., 2000; Charlier et al., 2000; Elkheshen and Radwan, 2000; Pean et al., 2000; Cleland et al., 2001; Han et al., 2001; Lu et al., 2001; Meinel et al., 2001; Woo et al., 2001; Kim and Burgess, 2002)).

Several approaches have been used to modulate release of drugs from PLGA. Techniques include colyophilizing amphiphilic polymers with the protein to be released (Morita et al., 2001), addition of surfactants to the aqueous, drug-containing phase of a double emulsion (Blanco and Alonso, 1998; Rojas et al., 1999), copolymerizing with poly(ethylene glycol) (Penco et al., 1996; Bae et al., 2000), blending with different molecular weights of the same polymer (Bodmeier et al., 1989; von Recum et al., 1995), or blending with polymers of different hydrophobicity/hydrophilicity (Cha and Pitt, 1990; Park et al., 1992; Yeh et al., 1996; Cleek et al., 1997; Ravivarapu et al., 2000). These approaches cannot only change the release kinetics over a period of days to years, but the bioactivity of encapsulated proteins also may be protected.

The present studies sought to build on the previously referenced work by blending hydrophilic and hydrophobic polymers to change the equilibrium water content within biodegradable coatings and therefore to change the time to onset of protein release. Specifically, the objectives of this project were to: (a) develop multilayered PLGA-based coatings that mimic time-dependent concentrations of growth factors present during natural wound healing, and (b) develop a model capable of describing drug release by manipulation of the independent variable, hydrophobicity.

2. Materials and methods

2.1. Polymers and blending

PLGA is generally made by a ring opening polymerization reaction catalyzed by stannous octanoate or zinc (Vert et al., 1998). Use of stannous octanoate and hydrophobic molecules present during purification results in polymer chains terminating with aliphatic chains. One version, which we will designate PLGA-LE (50:50, MW 63,000 Da, T_g 47 °C; Alkermes, Wilmington, OH), requires months to degrade because of its high molecular weight and hydrophobic lauryl ester endgroups.

Current processing and purification techniques allow for manufacture of PLGA terminated with carboxylic acid groups. One such polymer, PLGA-2A (50:50, MW 11,000 Da, T_g 41 °C; Alkermes), degrades after approximately 2 weeks. Its low molecular weight and hydrophilic endgroups are responsible for the relatively short degradation time. Because of its shorter degradation time, PLGA-2A was used as the base polymer for this study.

Pluronic F-127 (PF-127; Sigma, St. Louis, MO) is a triblock copolymer of polyethylene oxide (PEO) and polypropylene oxide with molecular weight of approximately 12,300 Da, 70% of which is PEO (Schmolka, 1972). PEO segments increase the overall hydrophilicity of the copolymer and make it water soluble. Pluronics are widely used for their gel forming, surfactant, and non-ionic interaction properties. They have been incorporated into microspheres as excipients because their non-ionic interactions protect proteins and drugs during double emulsion and spray-freeze drying processes (Carrasquillo et al., 2001; Giunchedi et al., 2001; Morita et al., 2001).

Blending was used to alter hydrophobicity of the coatings consisting primarily of PLGA-2A. Preliminary studies showed that blending up to 30% by mass PLGA-LE into PLGA-2A represented an upper limit to maintaining a homogeneous distribution of both polymers within a single coating layer. Similarly, up to 8% by mass PF-127 was blended in PLGA-2A to increase hydrophilicity.

2.2. Microsphere preparation

Protein-loaded microspheres were made using a conventional W/O/W process. A 1% solution of lysozyme was made in phosphate-buffered saline (PBS), pH 7.4. Lysozyme was selected as a model protein because its size and isoelectric point are similar to those of relevant growth factors. Also, being an enzyme, bioactivity of released protein can be readily measured. To reduce inactivation of encapsulated enzyme resulting from contact with hydrophobic polymer and solvent, 2% gelatin was added to the lysozyme solution. Lysozyme-gelatin solutions were added 1:9 v/v to 10 wt.% total polymer (i.e., PLGA-2A, PLGA-LE, and PF-127) in methylene chloride, were vortexed, and were sonicated at 25 W for 5 s to form the first emulsion. The W/O emulsion was transferred 1:9 v/v to a solution of 1% polyvinyl alcohol (MW 30,000–70,000 Da; Sigma) in deionized water to form the W/O/W emulsion and mixed for 4 h. Microspheres were collected by centrifugation, filtered using an 8 μm membrane, rinsed with deionized water, and dried under vacuum for 48 h. Control (blank) microphores were prepared identically except that lysozyme was omitted from the PBS solution.

2.3. Pressure-sintered multilayered coatings

Dense coatings were made using methods adapted from those originally described by Cohen et al. (1984) and more recently by Nof and Shea (2002). By applying pressure while the polymer is above its glass transition temperature, particles are consolidated. Because the glass transition temperatures of polymer blends in the present work were in excess of 40 °C, microspheres

were plasticized with acetone vapor to allow pressure-sintering below 37 °C. Although coatings can be applied to nearly any substrate, tissue culture plates were used for simplicity. Microspheres were weighed into 48-well plates and then placed in acetone vapor, saturated at 25 °C, for 30 min. Plasticized microspheres were compressed with a Delrin piston at 100 Pa for 5 s to ensure even spreading and consolidation of the coatings. Acetone was removed from the coatings by placing plates in vacuum for 48 h. Layers were added sequentially in the same manner. The thickness of each layer was linearly related to the initial mass of microspheres. A mass of 15 mg of microspheres resulted in 11 μm thick coatings, and 30 mg of microspheres gave 22 μm thick layers. Coating density was 4.28 mg/mm^3; there was no effect of polymer blend ratio on coating density.

2.4. In vitro polymer swelling and degradation and protein release

Single layer coatings of different polymer blends were used to determine swelling. After incubation in 0.15 M PBS, pH 7.4, at 37 °C for increasing time, the coatings were removed and blotted, and their wet weight was measured on an analytical balance. In degradation experiments, samples were incubated under the same conditions, but supernatant was collected and replaced at regular intervals of 24 or 48 h. Degradation of PLGA blend coatings was assessed by quantifying the concentration of oligomers of lactic and glycolic acid in the supernatant using methods adapted from Goddu et al. (1955). In brief, 12.5% hydroxylamine in deionized water, pH 12.0, was added to supernatant samples, 1:3 v/v, and incubated at 37 °C for 30 min. Hydroxylamine reacts with esters, breaking oligomers into monomers with a primary amine substituted for the ether oxygen. The reaction was quenched by adding a 2.75% ferric chloride in 60% perchloric acid (1:7.5 v/v of the reacted hydroxylamine-supernatant solution). Fe^{3+} ions coordinate with the carbonyl oxygen and primary amine, changing their oxidation state. The associated color change produced a concentration-dependent absorption peak at 570 nm. A standard curve was constructed using ethylacetate. Because PF-127 does not contain ester groups and therefore is not detectable by ester saponification, coatings made from PLGA-2A/PF-127 blends were adjusted for the mass fraction of PLGA-2A.

Release experiments were conducted by incubating samples in 0.15 M PBS, pH 7.4, at 37 °C. Supernatant was collected and replaced at regular intervals of 24 or 48 h. Using lysozyme as a model molecule allowed assessment of activity retention throughout the microsphere formation and pressure-sintering processes to be determined. Lysozyme acts on the β1,4 linkages of *N*-acetylmuramic acid to *N*-acetylglucosamine disaccharide commonly found in cell walls of gram-positive bacteria, such as *Micrococcus lysodeikticus*. To reduce non-specific adsorption and enhance sensitivity of the assay, microtiter plates first were precoated with 1% bovine serum albumin in PBS. Supernatant samples were added 1:1 v/v with 0.5 mg/ml *M. lysodeikticus* in 0.05 M acetate buffer, pH 5.0. Absorption at 450 nm was measured after 0 and 10 min. Lysozyme concentration was determined by comparison of reaction rates with a set of known standards.

2.5. Modeling and Fourier transform

Because drug delivery profiles vary in time, they can be treated as time-dependent signals (Brazel and Peppas, 2000; Tzafriri, 2000; Narasimhan, 2001). Fourier analysis can be used to describe time-dependent functions as the sum of a set of sine or cosine waves. Fourier transforms converts data from a 'time-domain' into 'frequency-domain':

$$X(\omega) = \int_{-\infty}^{\infty} x(t)\, e^{-j\omega t}\, dt \tag{1}$$

where $x(t)$ is the time-dependent function. $X(\omega)$ is generally a complex number. Frequencies were determined by:

$$\varpi(n) = \frac{0.5 \sum_{k=0}^{i} n_k - 0.25 n_i}{i} \tag{2}$$

where i is the number of elements in $x(t)$ and n_k is the number of a particular element. Phase was determined by:

$$\phi(\omega) = \arctan\left(-\frac{\mathrm{im}[X(\omega)]}{\mathrm{real}[X(\omega)]}\right) \tag{3}$$

where im[$X(\omega)$] and real[$X(\omega)$] are the imaginary and real parts, respectively, of the Fourier transform solution for a particular element n. Magnitude was determined by taking the square root of the sum of the squares of im[$X(\omega)$] and real[$X(\omega)$]. Active lysozyme release data was analyzed for all experimental cases. Release profiles were reconstructed by magnitude-weighted summation of sine waves of predominant frequencies with related phase shift as determined by Fourier analysis. After verifying that reconstructed profiles correlated strongly with experimental profiles, existence of trends in frequency, magnitude, and phase were examined with respect to independent variables of coating fabrication.

2.6. Statistical analysis

Samples sizes of 4–6 were used for the experiments. Error bars are not shown in the degradation and release figures because they obscure viewing of the multiple data sets plotted. Standard deviation ranged from 12 to 27% of the mean. Statistical analysis was performed using INSTAT3 software (GraphPad Software, San Diego, CA). Differences in swelling, degradation, or amount of protein released were analyzed for statistical significance using one-way ANOVA followed by post-hoc comparison using the Tukey test.

3. Results

3.1. Polymer blending

As shown in Fig. 1, water absorption was strongly affected by blending PGLA or PF-127 into a base polymer of PLGA-2A to increase hydrophobicity or hydrophilicity, respectively.

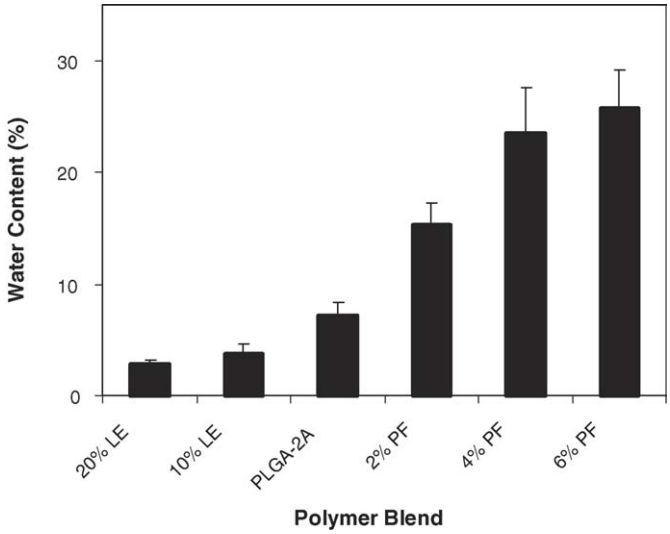

Fig. 1. Effect of polymer blending on water uptake in single layer coatings composed of PLGA-2A blended with either PF-127 or PLGA-LE.

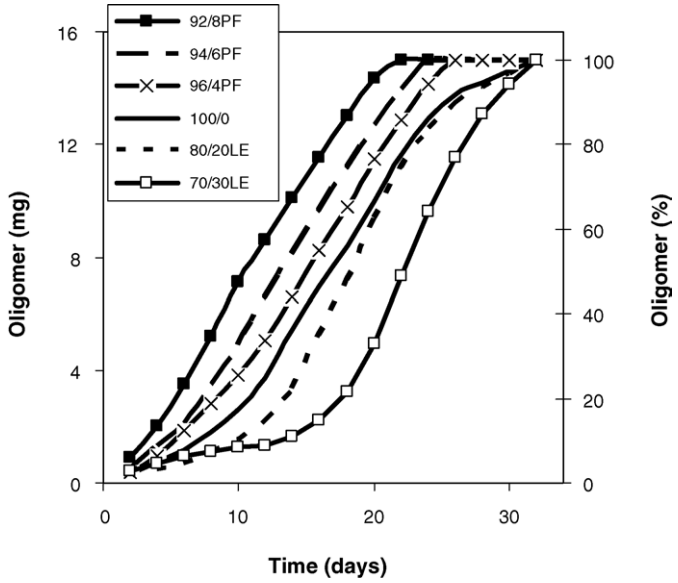

Fig. 2. Effect of polymer blending on oligomer release from single layer coatings composed of PLGA-2A blended with either PF-127 or PLGA-LE.

PLGA-2A absorbed approximately 7% water by mass at 3 h. Addition of 10 and 20% PLGA-LE reduced hydration to 4 and 3%, respectively. Although the water content of the more hydrophobic blends was significantly lower than that of PLGA-2A ($p < 0.05$), the results for the PLGA-LE blends did not differ from each other. Adding PF-127 significantly increased hydration of PLGA-2A ($p < 0.001$). There was a blend-dependent increase between 2 and 4% PF-127 ($p < 0.001$), but the water content of the 4 and 6% blends was comparable. Physically, PLGA-2A blends with 6% PF-127 underwent considerable swelling and became very soft. PLGA-2A blends with 20% PLGA-LE increased in opacity as the small amount of water was absorbed, but the layers remained hard.

3.2. Single layer coatings

One-layer, 15 mg (11 μm thick) coatings first were used to examine the effects of blending on degradation and protein release. Polymer blending strongly affected oligomer release from coatings ($p < 0.01$; Fig. 2). For 100% PLGA-2A coatings, there was lag, manifested as a toe region, through about 10 days before oligomer release significantly increased with degradation by-products accumulating in the supernatant at a linear rate of approximately 0.78 mg day^{-1} through approximately day 22. Coatings consisting of 92% PLGA-2A and 8% PF-127 began degrading immediately at about 0.75 mg day^{-1} and continued approximately linearly for 20 days. As PF-127 content decreased, the linear region of degradation shifted later in time, beginning at approximately 7 and 10 days and ending at approximately 22 and 24 days for blends containing 6 and 4% PF-127, respectively. The rate of degradation was comparable among the Pluronic blends and PLGA-2A. When 20% PLGA-LE was added, the toe region increased, and the lag time before linear accumulation of oligomers was increased to nearly 14 days. Degradation by-products amassed at a rate of approximately 0.92 mg day^{-1} through 22 days. When 30% PLGA-LE was added, the linear phase of accumulation of oligomers was delayed until almost day 17 and continued to 32 days. Similar blend-dependent behavior was observed for the thicker 22 μm (30 mg) coatings (data not shown).

Protein release behavior followed from the polymer degradation profiles. Some early loss of protein, peaking at 3–4 days, was noted for all coatings (Fig. 3a). The loss increased with hydrophobicity ($p < 0.01$), with 8, 10, and 13% of the total amount lost for the 6% PF-127, 100% PLGA-2A, and 30% PLGA-LE blends, respectively. Following the lag in accumulation of oligomers and the subsequent shortening of polymer chains to a critical length for solubilization, the major peak in release of enzymatically active lysozyme was observed. Release peaks of 500–600 ng for 6% PF-127, 100% PLGA-2A, and 30% PLGA-LE occurred at approximately 10, 14, and 17 days, respectively. Smaller amounts of protein were released after the primary peak, as degradation of the coatings was completed. Release of lysozyme from 30 mg coatings compared well with delivery from 15 mg coatings (data not shown). Release peaks not only were centered approximately in the same temporal location, but they also had similar width. The one difference was that less early loss occurred from the thicker coatings. Fig. 3b shows cumulative release of protein as a function of time. The effect of blending can be seen in the lag before the rapid rise in release, which corresponds to the location of the major peak in instantaneous release.

3.3. Multilayered coatings

Two- and three-layer coatings composed of protein-loaded and/or unloaded layers of different polymer blends were used to examine the ability of the materials to deliver one or more molecules at different times or a single molecule for an extended period of time. Fig. 4 shows representative results for degradation of two-layer coatings consisting of 100% PLGA-2A over 70% PLGA-2A/30% PLGA-LE. The behavior of each layer

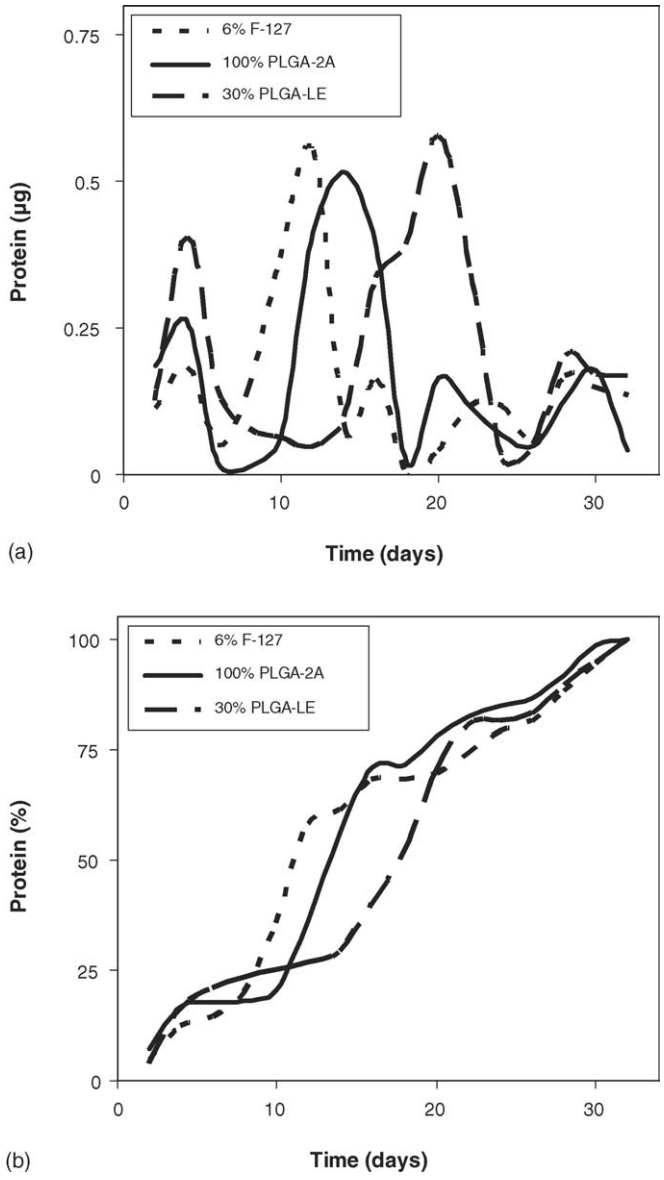

Fig. 3. Effect of polymer blending on (a) instantaneous and (b) cumulative release of bioactive lysozyme from one-layer coatings composed of PLGA-2A blended with either PF-127 or PLGA-LE.

follows from Fig. 1. Compared to the 100% PLGA-2A layer, the 30% PLGA-LE blend layer showed a longer lag before the linear rise in degradation ($p < 0.01$). Overall, a lag of 6–7 days was observed, after which oligomers accumulated in the supernatant at a rate of approximately 1.2 mg day^{-1} until day 26.

Using the same order of layers, that is 100% PLGA-2A over 70% PLGA-LE blend, release of enzymatically active lysozyme from two-layer coatings is shown in Fig. 5; either the top layer, bottom layer, or both layers were loaded. All coatings showed an early loss of protein around 3–4 days. The effect of the order of loading was manifested in the location and width of main release peak ($p < 0.05$). Loading of only the bottom, 30% PLGA-LE layer resulted in a long lag period, with release occurring between 14 and 24 days. With only the top, 100% PLGA-2A layer loaded, the lag was slightly shorter, and release occurred between 11 and 20 days. When both layers contained

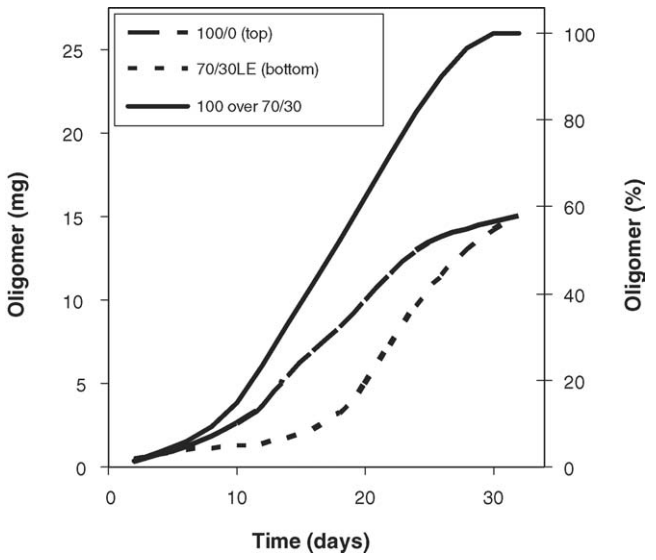

Fig. 4. Oligomer release from two-layer coatings comprising 100% PLGA-2A over 70% PLGA-2A/30% PLGA-LE blend.

lysozyme, contribution from both layers was apparent, with prolonged release beginning at day 10 and continuing through day 24.

Representative results for three-layer coatings are shown in Fig. 6. The top layer was 8% PF-127 blend, the middle layer 100% PLGA-2A, and the bottom layer 30% PLGA-LE blend. Again early loss was found with all coatings, with those containing a greater number of loaded layers showing a higher loss ($p < 0.001$). When only the bottom layer was loaded, the primary release peak was between days 18 and 24. Additional smaller peaks were found at 8 days and at 15 days. When the middle and bottom layers were loaded with lysozyme, a bimodal peak representative of two overlapping major release peaks was observed between days 8 and 24. The two contributing peaks were centered at days 14 and 20. When all three layers were loaded, three

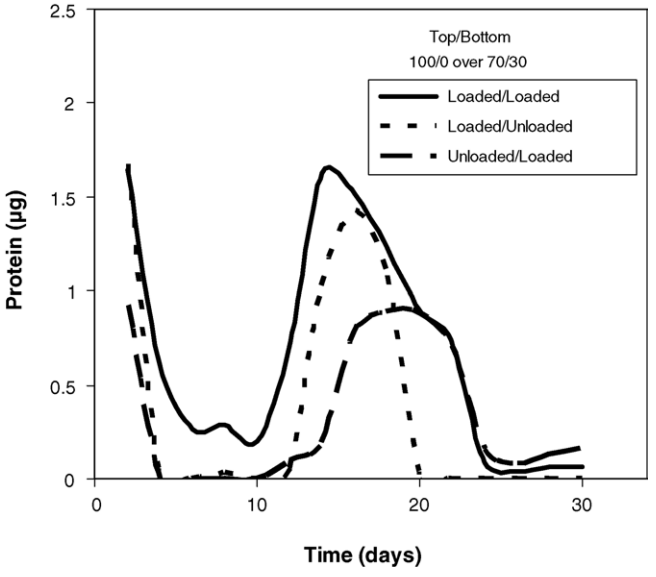

Fig. 5. Active lysozyme release from two-layer coatings comprising 100% PLGA-2A over 70% PLGA-2A/30% PLGA-LE blend.

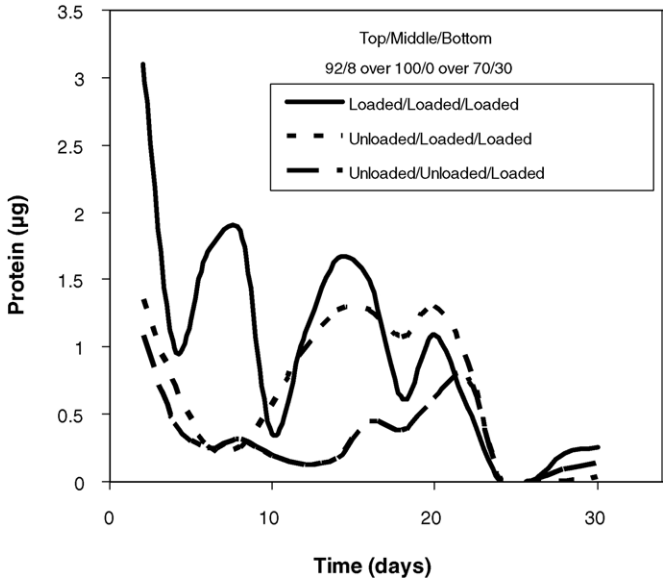

Fig. 6. Active lysozyme release from three-layer coatings, with the top layer containing 92% PLGA-2A/8% PF-127, the middle layer containing 100% PLGA-2A, and the bottom layer containing 70% PLGA-2A/30% PLGA-LE blend.

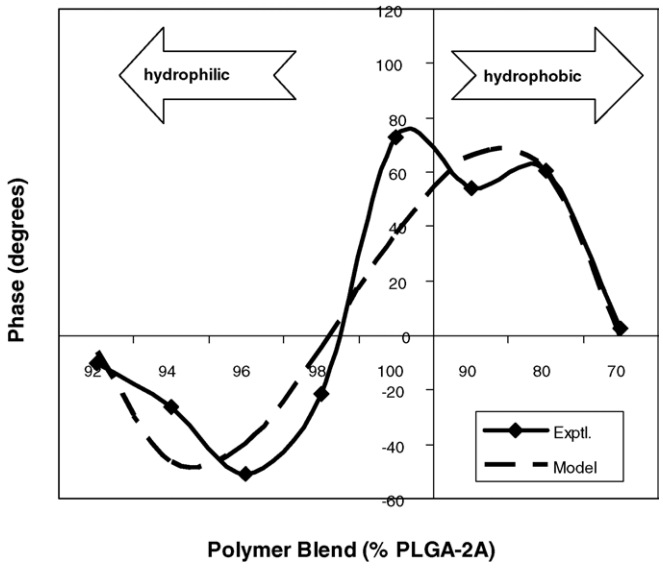

Fig. 7. Phase angles for frequency of 0.0625 (day^{-1}) as a function of polymer blend.

distinct release peaks were observed and were centered at days 7, 14, and 20.

3.4. Fourier analysis and model

Fourier analysis of release data, such as shown in Figs. 2, 3, 5 and 6, revealed the same distinct frequencies, but the phase and magnitude varied as a function of polymer blend ratio (Tables 1 and 2). Plotting phase angle (Fig. 7) or magnitude as a function of polymer blend for each frequency shows a relatively smooth transition between blends. Using the phase and magnitude for each sine wave, release profiles were reconstructed and compared to experimental release profiles. Fig. 8 shows representative model and experimental profiles for one-layer coatings using two polymer blends. The model predicts the measured release data reasonably well.

4. Discussion

4.1. Polymer blending

A variety of inorganic and organic compounds have been blended into poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) to alter degradation and/or release kinetics. Additives have included metal salts (Agrawal and Athanasiou, 1997; Ara et al., 2002), PLA or PLGA with different molecular weight or encaps (Cleek et al., 1997; Ravivarapu et al., 2000), other biodegradable polymers (Cha and Pitt, 1990), or Pluronics (Park et al., 1992;

Table 1
Magnitude of dominant frequencies from Fourier analysis of active lysozyme release from coatings of different polymer blends

Wave	Period (days)	Frequency (day^{-1})	PF-127 ← Blend (% PLGA-2A) → PLGA-LE							
			92	94	96	98	100	90	80	70
1	0	0	207	268	139	202	82	65	60	117
2	32	0.03125	172	199	132	182	74	17	81	38
3	16	0.0625	121	194	63	150	61	78	120	101
4	11	0.09375	111	87	93	98	77	26	130	72

Table 2
Phase of dominant frequencies from Fourier analysis of active lysosyme release from coatings of different polymer blends

Wave	Period (days)	Frequency (day^{-1})	PF-127 ← Blend (% PLGA-2A) → PLGA-LE							
			92	94	96	98	100	90	80	70
1	0	0	0	0	0	0	0	0	0	0
2	32	0.03125	−13	−1	148	58	−41	−69	−154	124
3	16	0.0625	−10	−26	−51	−21	73	54	61	3
4	11	0.09375	−14	7	−44	17	−33	−71	−41	−58

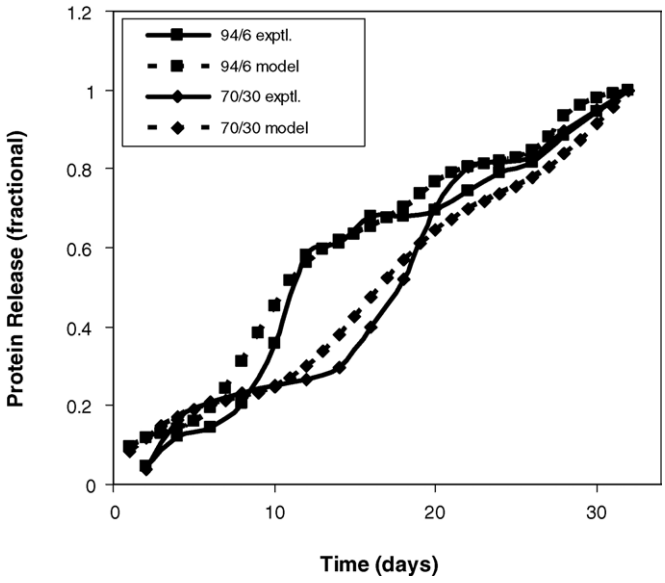

Fig. 8. Comparison of reconstructed protein release with experimental data from 94% PLGA-2A/6% PF-127 and 70% PLGA-2A/30% PLGA-LE blend single layer coatings.

Yeh et al., 1996). By altering the hydrophobicity/hydrophilicity of the material, the water content within the device can be altered. Similarly, the initiator used during manufacture of the polymer affects hydrophobicity and water uptake (Vert et al., 1998). PLGA with octanoate-terminated chains absorbs less water than if chains are terminated with acidic groups. Because the rate of water diffusion into devices is generally greater than the rate of hydrolysis, changing the rate of absorption has little, if any, effect. To change the rate of hydrolysis, the equilibrium water content must change.

In the present work, blending miscible polymers into a base polymer of PGLA-2A was used to alter absorption of water. Addition of a small amount, even just 2 wt.%, of hydrophilic PF-127 caused a dramatic change in water uptake. However, a plateau in water absorption was reached quickly. Addition of 6–8% PF-127 did not further increase water absorption, and consequently, the rate of hydrolysis did not significantly increase with greater percentages blended into PLGA-2A. Park et al. (1992) and Yeh et al. (1996) previously blended up to 75 wt.% PF-127 into PLGA to alter protein release behavior, whereas, only up to 8 wt.% was used in the present studies. Neither paper reported alteration in the onset to degradation and release. A concern about using large fractions of PF-127 is that domains of Pluronic might form. In an aqueous environment, the water-soluble PF-127 domains will easily dissolve, leaving a porous PLGA-2A device. This is undesirable because of the likelihood of greater release resulting from easier escape of encapsulated biomolecules.

Addition of hydrophobic PLGA-LE reduced water uptake into the coatings. As with PF-127 blends, however, a plateau became apparent, and including more than 30% PLGA-LE in PLGA-2A coatings did not further decrease the water content in the coatings. Furthermore, too high of a loading may lead to segregation within the microspheres and therefore to inhomogenous layers.

4.2. Single layer coatings

Following uptake of water, hydrolysis of ester bonds, autocatalyzed by carboxylic chain ends, begins (Li et al., 1990). As hydrolysis proceeds, soluble oligomers eventually are formed and diffuse out of the polymer. Consequently, oligomers accumulate in the supernatant.

Blending greatly altered polymer degradation and oligomer release from coatings. An increasing toe region was apparent as hydrophobicity of the polymer increased. A small amount of oligomer began accumulating for all coatings from the onset of the experiment. However, oligomer release from coatings made from PF-127 blends continued unabated from the beginning of immersion for approximately 20 days. When 30% PLGA-LE was incorporated, linear oligomer release was delayed until approximately 17 days. A slightly higher rate of oligomer release was observed for the PLGA-LE blends. Because degradation by-products having a lauryl ester encap will be less soluble than those with acid termini, their retention would enhance autocatalysis of the polymer and thereby increase the overall rate of degradation. The time required for coating erosion was between 15 and 20 days from the onset of linear polymer release. Because the thickness of all coatings tested was well less than 100 μm, obvious heterogenous degradation resulting from entrapment of acidic by-products was not observed (Grizzi et al., 1995).

Release of encapsulated biomolecules is related to polymer degradation. Initially, when devices are immersed in a physiological environment, early loss of loaded protein may occur as water diffuses in. Similarly, during the initial phase of random chain scission, when minimal chain shortening has occurred, loaded molecules are released in a diffusion-controlled manner. When oligomers begin to accumulate in the supernatant, loaded biomolecules are released in a degradation-controlled manner. The three phases comprise the triphasic release profile.

All samples exhibited an early loss of protein, which occurs because of adsorbed/segregated proteins on microsphere surfaces forming channels when sintered. This early loss of protein increased in proportion to hydrophobicity of the coating. Lysozyme is a very hydrophilic protein. As hydrophobicity of the polymer blend increased, lysozyme may have become non-uniformly distributed toward the surfaces of microspheres and of the coating after consolidation. Lysozyme on the surfaces of microspheres subsequently would have been more easily lost following immersion in saline. Readily desorbed or diffusible lysozyme was quickly lost, but the rest remained to be released in a degradation-controlled manner.

As desired, alteration of water content and subsequent modulation of onset of polymer degradation by polymer blending also controlled release of bioactive protein. The beginning of the primary release peak correlated well with the onset of linear accumulation of oligomer in the supernatant. The longer the toe region, with greater lag time found for more hydrophobic blends, the later in time the release peak was shifted. The timing of release peaks could be selected based on varying the polymers (more hydrophobic or hydrophilic) and amounts blended. With one-layer coatings, peaks could be obtained between 7 and 20 days.

4.3. Multilayered coatings

Wound healing occurs by action of multiple growth factors expressed at different times (e.g. (Bolander, 1992; Bourque et al., 1993; Bostrom et al., 1995; Yu et al., 2002)). Creating regions of different hydrophobicity across a surface would allow delivery of identical or different molecules to different places and at different times. To deliver multiple or single molecules to the *same* place but at different times, regions of different hydrophobicity, with different times to onset of release, must be stacked perpendicular to the surface. Multilayered devices could be made by laminating, in which individual layers are formed and later bonded together by applying solvent between the laminae. However, with thin layers having different composition, rapid penetration of the solvent can redissolve and intermix the layers.

With the methods used, it was not possible to determine from which layer PLGA degradation by-products originated. However, by comparing the oligomer release profile of the two-layer coatings with release from single layers of the same blends, an estimation of which layer contributed to oligomer release was obtained. For example, overlaying oligomer release for 100% PLGA-2A with that for 30% PLGA-LE blend, it was apparent that the early part of oligomer release, from 10 to 20 days could be accounted for by degradation of the upper 100% PGLA-2A layer, and later release, from 20 to 32 days, could be attributed to the bottom 30% PLGA-LE layer. These observations suggest two conclusions. The heterogeneous distribution of hydrophobicity was retained during microsphere plasticization and consolidation. Secondly, multilayered coatings continued to undergo homogeneous degradation; degradation in top or bottom layers was not advanced or delayed by stacking.

Just as with oligomer release, contributions to release of protein from top and bottom layers of two-layer coatings were readily apparent. When three-layer coatings were tested, three peaks were observed. For both the two- and three-layer coatings, not only did the temporal location of the peaks correlate well with delivery from single layers, but the timing also corresponded to that predicted from the hydrophobicity/hydrophilicity of the blend. After the early loss, lysozyme release profiles appeared to result from degradation-controlled release even when coatings were multilayered. Including the initial loss, release peaks were observed at 3, 7, 14, and 20 days.

4.4. Fourier model

One of the objectives of this project was to develop a flexible system for delivering therapeutic biomolecules at desired times. To facilitate translating a desired delivery time into a device, a mathematical model based on independent variables is useful. Focusing on independent variables has two benefits. It removes the difficulty in quantifying parameters, such as tortuosity, swelling, and diffusivity that can vary dramatically over duration of release. Additionally, modeling based on independent variables outlines a direct translation from what is directly manipulated in the laboratory to release of loaded biomolecules.

Fourier analysis was used to successfully describe release of biologically active protein from polymer blend coatings. Critical to the use of Fourier analysis to model drug delivery profiles are the roles diffusion and hydrolysis play in controlling release. A combination of sine and cosine waves can be converted to an exponential function:

$$e^{j\omega t} = \sin(\omega t) + j\cos(\omega t) \quad (4)$$

Both concentration change due to diffusive loss and hydrolysis of ester groups can be described as exponential functions. Therefore, the two processes can be represented by a sum of two or more exponential functions that could be described as the sum of a set of sine waves. The coefficients of magnitude and phases for contributing sine waves were determined from Fourier transform analysis.

Determining the location of the primary protein release peak could be accomplished by plotting peak timing as a function of polymer blend. However, this would only describe a single point of the active lysozyme release profile. In the present work, only an offset signal (0 frequency) and frequencies with periods of 32, 16, and 11 days were required to reproduce delivery profiles. When waves were plotted individually, phases of waves with a period of 32 days located the primary release peak. Waves of periods of 16 days added to the primary peak as well as described any early loss. Waves with periods of 11 days served to sharpen the rise of the primary lysozyme release peaks. Higher frequencies generally corresponded to harmonics of the sampling frequency. Using four components, the Fourier-based model predicted the experimental data well. Additional frequencies may enable slightly better accuracy, such as between 10 and 20 days when the lag resulting from altered hydrophobicity in the blends is most pronounced. By interpolating values for phases and magnitudes for untested polymer blends, protein release peaks can be designed to occur at any time between 7 and 20 days. An important use of the model would be for predicting behavior of other blends. Although the coefficients of magnitudes and phases will be different for other polymers, the general methodology is still applicable.

4.5. Model comparison and advantages

Many mathematical models for drug delivery have been previously developed by several groups (Marentette and Grosser, 1992; Collins et al., 1997; Brazel and Peppas, 2000; Tzafriri, 2000; Narasimhan, 2001; Siepmann and Gopferich, 2001; Lemaire et al., 2003; Nam et al., 2004). Although models both based on theoretical principles and solely on empiricism are generally designed to focus on intrinsic device properties, such as diffusivity, variability in device application, fabrication, and analysis techniques results in lack of universal model utility. Because this Fourier based model relies only on quantification of released molecules, the ultimate goal of controlled release, and implements common mathematical treatment, an accurate model requiring no extra data collection can be made. Seeking modeling solutions for time-varying series in transforms is not unprecedented. Previous pharmacokinetic modeling has been performed using other similar mathematical tools, such as the Laplace transform (Gillespie, 1997; Weiss, 1998). Laplace

transforms accurately describe decay following an impulse function: loss from a high initial action. This is the most appropriate model for intravenous administration. However, in controlled release, drug delivery may begin at times other than the initial point. The Fourier transform accurately represents this behavior.

5. Conclusion

The goal of this study was to develop PLGA-based coatings capable of creating protein delivery profiles inspired by sequential growth factor expression during wound healing. By stacking layers of polymer blends having different hydrophobicity/hydrophobicity, the temporal occurrence of peaks in protein release can be modulated. These multilayered coatings of non-uniform hydrophobicity were an effective solution to deliver macromolecules at discreet times. This approach should readily extend to delivery of multiple molecules at different times. For example, analysis of callus formation following fracture has shown that platelet-derived growth factor predominates in the first 3 days, fibroblast growth factor from 3 to 6 days, insulin-like growth factor from 6 to 9 days, and transforming growth factor from 9 to 21 days in the wound healing cycle (Bourque et al., 1993).

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