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Evaluation of *In Vitro* Drug Release, pH Change, and Molecular Weight Degradation of (Poly(L-lactic acid) and Poly(D,L-lactide-co-glycolide) Fibers

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ABSTRACT

Biodegradable fibers of poly(L-lactic acid) (PLLA) and poly(D,L-lactide-co-glycolide) (PLGA) that encapsulated a water-soluble drug were created by a patented technique consisting of wet-spinning a water-in-oil emulsion. These fibers are 2.4% by mass drug, which is slowly released, making these fibers potential candidates for implantation as drug delivery devices and/or tissue-engineering substrates. Drug release kinetics and changes in molecular weight were investigated over time. This study demonstrated that drug release rates and molecular weight degradation are a function of the amount of aqueous phase added as an emulsion during fabrication. The type of polymer used (PLLA or PLGA) determines the molecular weight degradation rates, but has little effect on drug release kinetics.

INTRODUCTION

At the most basic level of most tissue-engineering applications is the scaffold on which cells are to grow, proliferate, and ultimately function. In our laboratory, we have attempted to develop a new generation of fiber-based scaffolds for tissue engineering. Our ultimate goal is to be able to load sensitive biological molecules, such as growth factors, enzymes, and so on, into the fibers at the time of fabrication. These biological molecules will then be slowly released over time. When these fibers are then used as scaffolding for cells to grow on in tissue-engineering applications, the cells that grow on or near these fibers will receive the growth factors, enzymes, and so on, that are slowly released from them. This allows drug delivery to be localized to individual fibers within a scaffold rather than broadly applied to the entire system. The importance of this study is that by using this technology, it may now be possible to create heterogeneous fiber scaffolds where drug delivery is spatially controlled within a few hundred microns.

In this preliminary study, we use only bovine serum albumin (BSA) as a simple analog to the growth factors that will eventually be loaded in other studies. BSA is commonly used as both a surfactant and a model protein for drug release studies involving polymeric microspheres, and one should refer to Cohen, Jain, Lu, Crotts, and many others[1–3] for further information regarding the use of this agent in studies of this nature. In addition, BSA is often incorporated as a protective protein into microspheres containing growth factors, and therefore its additional use as a surfactant in such applications can be beneficial.[4]

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Typical fiber extrusion methods require high temperatures or exposure to harsh organic solvents, or both, which would destroy the biological activity of any growth factor. Therefore, our laboratory has spent the past 6 years developing and ultimately patenting[5] the composition and

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method of creating a fiber scaffold at room temperature, which is capable of slowly releasing sensitive biological molecules such as growth factors, and so on, with demonstrated retained biological activity. Our technology expands the basic drug delivery microsphere concept into a fiber-based construct yielding similar therapeutic agent loading and release properties and capabilities. However, there may be differences in kinetics due to surface area-to-volume differences between a fiber and sphere.

These "drug" (where drug can now mean small-molecule drugs, proteins, growth factors, enzymes)-loaded fibers have utility in a number of applications. With this technology, both physical and pharmaceutical support comes from the same fiber. In nature, there is a dynamic exchange of growth factors and other molecular signals between cells and the extracellular matrix in/on which they grow. This technology is a first approximation to that model, as the cells growing on these fibers can receive these same molecular signals from the fiber on which they are growing. Therefore, the scaffold itself can modulate cellular behavior by the drugs released by individual fibers within the scaffold. Applications of this technology allow heterogeneous drug delivery within the same matrix simply by weaving fibers with different factors into different regions of the scaffold and achieving drug delivery within a spatial accuracy of 10's to 100's of microns. We believe this may be the first step to creating heterogeneous, anatomically accurate three-dimensional scaffolds capable of tissue regeneration.

Our patented technique of fiber extrusion involves using an aqueous emulsion in a modified wet extrusion process.[5] The key point of using an emulsion is that the sensitive biological molecules, such as proteins, growth factors, cytokines, enzymes, and so on, are exposed to an aqueous, biologically friendly environment. This step is identical to the solvent evaporation method of making microspheres, and has been repeatedly shown to maintain the biological efficacy of many different drugs and proteins.[2,3,6–8]

In summary, our fibers are made predominantly from inexpensive, Food and Drug Administration (FDA), approved polymers such as poly(L-lactic acid), and demonstrate both *in vitro* and *in vivo* prolonged delivery of sensitive biological molecules with retained biological activity. This retained activity is due to our unique fabrication process that provides nearly complete protection of the loaded biological molecule.

MATERIALS AND METHODS

Materials

The biodegradable polymer fibers used in this study were fabricated by a wet spinning technique described herein, with further information and examples provided in Nelson *et al*.[9] The poly(L-lactic acid) (PLLA) pellets (average M_w, 40–70 kDa; intrinsic viscosity, 0.80–1.20) were obtained from Polysciences (Warrington, PA). The 50:50 poly (D,L-lactide-co-glycolide) (PLGA) pellets (average M_w, 40–75 kDa; intrinsic viscosity, 0.6–0.8) were purchased from Sigma (St. Louis, MO). All other chemicals used were purchased from standard laboratory material suppliers unless otherwise noted.

Polymer solution preparation

The polymer solutions were prepared by dissolving 1.0 g of the respective polymer (PLLA or PLGA) into 5.0 mL of methylene chloride in a 10-mL glass vial containing a magnetic stir bar. Methylene chloride is a commonly used solvent for PLLA and PLGA, and is often used in the preparation of drug-containing microspheres.[10] The vial was sealed with Saran Wrap and tightly capped with a nylon plug, and the contained volume was noted. The mixture was magnetically stirred (Thermolyne Nuova II; Barnstead International, Dubuque, IA) for 1.5 h to achieve a uniform solution. No volume loss was apparent after dissolution of the polymer.

BSA solution preparation

Bovine serum albumin (BSA, fraction V; Sigma-Aldrich, St. Louis, MO) was used as a model drug for the release studies cited herein, and as a surfactant to stabilize the emulsion in this study. This protein is often used in the preparation of drug delivery microspheres, and therefore its release characteristics, surfactant capabilities, and degradation due to processing are well defined. Stock solutions of 5 and 10% (w/v) were prepared by dissolving BSA powder in reverse osmosis/deionization (RO/DI)-treated water. The solutions were sterile filtered (pore size, 0.2 μm) in an aseptic environment before use.

Polymer fiber fabrication

Emulsification. Water-in-oil emulsions were prepared by adding either 500 μL of the 5% (w/v) BSA solution or 250 μL of the 10% (w/v) BSA solution to 5 mL of the polymer solution and sonicating, using a probe sonicator (Vibra-Cell; Sonics & Materials, Newton, CT) at 100 W for approximately 60 s. An ice bath was used during sonication to minimize solution heating. Therefore, all emulsions had the same total mass of BSA, but two different volumes of dispersed aqueous phase. This ensured that polymer type and aqueous load carried by the fibers were the only treatment variables under study. PLLA and PLGA fibers with no incorporated aqueous phase (and, therefore, no BSA) were also used as controls in this study.

Fiber spinning. The emulsion and control solutions were loaded into 5-cm^3 glass syringes (gas tight; Hamil-

ton, Reno, NV), using a clean, blunt-tip, 18-gauge needle, and were allowed to degas at room temperature for 10 min before use. The syringes, in turn, were mounted in a syringe pump (KDS200; KD Scientific, Holliston, MA), with Viton tubing used to connect the syringe to a blunt-tipped, stainless steel, 22-gauge needle (Small Parts, Miami Lakes, FL) that served as the spinneret. The spinneret was ultimately pierced through a rubber septum used to hold the coagulation bath in the extrusion tube (20×2.22 cm [ID] glass tube). The bottom end of the extrusion tube was immersed in the coagulant reservoir. In this case, the coagulation fluid used was hexane, and the reservoir was a polypropylene dish. The syringe pump was turned on and adjusted to flow at a constant rate of 0.05 mL/min. As the emulsion flowed into the coagulation bath, a fiber began to form. The fiber was removed from the bath and wrapped around a rotating bobbin that wound the fiber at a constant linear rate of 16 m/min.

The winding speed of the bobbin was monitored with a hand-held optical tachometer (Monarch Instrument, Amherst, NH). The mean linear velocity of the emulsion in the spinneret was 0.385 m/min, whereas at the bobbin it was 16 m/min; giving a draw ratio of 41. In other words, we noted a 41-fold increase in mean linear velocity between the spinneret and the bobbin. This draw ratio induces significant mechanical strain on the forming fiber.

All fibers were placed in a 630 Torr (25 inch-Hg) vacuum at room temperature overnight in order to dry them and draw out solvents remaining in the polymer. One batch of each fiber type was produced, for a total of six batches; and multiple sections of fiber from arbitrary locations on the bobbin from each batch were used for all studies conducted. Dried fibers were used for release and degradation studies, and thus entrapped water remaining from the emulsification process was not a concern.

Release and degradation studies

Two different experimental designs were used in the study: the first was designed to evaluate the protein release kinetics due to supernatant exposure and subsequent degradation of the subject fibers, and the second was to determine the change in polymer molecular weight as the fibers degraded. For both of these *in vitro* experimental designs, sterile, phosphate-buffered saline (PBS, pH 7.4) containing 0.01% (w/v) thimerosal (an antibacterial agent) was used as the release and degradation media. Solution pH was assayed at each time point before changing the assay buffer in order to monitor the effect of fiber degradation on supernatant pH.

BSA release. The PLLA and PLGA fibers produced for this assay were cut into similar lengths and bundled loosely, in triplicate. Each bundle was then weighed in order to normalize the data. Once weighed, each bundle was dipped into 70% ethanol for 15 s to minimize the potential for bacterial contamination, air dried in a class II, sterile biological hood, and then placed into a sterile, 5-mL polystyrene tube. The tubes were then filled with 3 mL of sterile PBS to completely cover the fibers, capped, sealed with Parafilm to avoid evaporation, and incubated at 37°C in a humidified environment. BSA release and pH measurements were taken on the day 1 after incubation, and thereafter at weekly intervals for 15 weeks. At every data point, the PBS solution in the sample tubes was transferred under sterile conditions to a new tube, which was capped, sealed with Parafilm, and frozen at −20°C for later analysis. The tube containing the fiber bundle was then refilled with 3 mL of fresh, sterile PBS. Before analysis for drug release parameters, the frozen PBS samples were brought to room temperature over a period of several hours.

Protein analysis: Protein analysis of the PBS samples was accomplished according to the micro protocol (working range, 1 to 25 μg/mL) for the Coomassie Plus protein assay reagent kit (Pierce, Biotechnology, Rockford, IL), with absorption analysis performed on an Ultrospec 2000 UV/Visible spectrophotometer (Biochrom, Oxford, UK). A calibration curve for optical density at 595 nm versus protein concentration was first made using the BSA standards provided with the reagent kit. To accommodate the effects of pH on the Coomassie assay, a separate calibration curve of optical density against pH was generated. At each time point, 1.0 mL of the PBS medium was used to assay the total protein concentration. These concentrations were normalized to the respective weight of BSA loaded into each fiber bundle, yielding a percent release for each week over the course of the study.

pH determination: PLLA biodegrades into lactic acid, and PLGA biodegrades into both lactic acid and glycolic acid.[11] *In vivo*, lactic acid enters the tricarboxylic acid cycle and is metabolized and eliminated from the body as carbon dioxide and water, whereas glycolic acid is excreted unchanged in the kidney or it enters the tricarboxylic acid cycle and is eventually eliminated as carbon dioxide and water.[12] The effect on the pH due to the degradation of PLLA and PLGA microspheres and fibers in solution is well documented.[3,13] As such, although pH measurements were performed at each assay time point, the data were used only to determine the range in buffer pH for the various fiber types under test. Measured pH represented the change in supernatant pH over each 1-week sampling period, with total change in solution pH calculated accordingly.[14] Such measurements were performed with the same PBS samples used for the BSA release study using an ÄKTApurifier HPLC pH monitor (Amersham Biosciences/GE Healthcare, Piscataway, NJ). Two hundred microliters of each PBS sample was

pipetted into selected wells of a 96-well tissue culture plate, so as to allow total immersion of the pH sensor tip. Once stabilized for 30 s, the reading for a given sample was taken. The pH sensor was then washed with deionized water and blotted dry before the next reading.

Molecular weight degradation. Degradation studies are destructive; so for this experimental design, therefore, an additional 288 samples representing 3 replicates of each of the 6 different fibers for 0 (day 1) to 15 weeks of study, were prepared. Similar lengths of each fiber sample were placed into sterile Eppendorf tubes containing 1 mL of PBS. As in the release studies, all tubes were capped, sealed, and placed in a humidified oven at 37°C, and at weekly intervals the PBS solution in the Eppendorf tubes was discarded and replaced with fresh solution, ensuring that all assays were conducted on fibers being subjected to similar conditions. Fibers for molecular weight analysis for a particular week were removed from the PBS bath, blotted dry, and stored at −20°C until the time of gel permeation chromatography (GPC) analysis. For the week 0 data point, samples of dry fibers were taken and stored in the freezer until time of analysis.

Samples for each fiber at each test point were analyzed by GPC and compared with a polystyrene calibration curve created over the range of 520 to 220,000 Da, using methylene chloride as a solvent. An ÄKTApurifier high-performance liquid chromatography (HPLC) machine with UV monitor and a Shodex RI-71 refractive index detector (Showa Denko/Shodex Chromatography [Shoko], Kawasaki, Japan) were used with an Asahipak GPC guard column (model GF-1G7B; Phenomenex, Torrance, CA) and TSK gel column (model G2000-HHR; Supelco).

Weekly fiber samples stored in the freezer were slowly brought to room temperature, and residual surface moisture was removed by blotting. The fiber was then dissolved in 3 mL of methylene chloride to ensure a dilute concentration. This solution was then injected into the HPLC circuit, and the GPC was run for approximately 15 min. Using the Unicorn software controlling the HPLC and attached devices, the number average and weight average molecular weights of the samples were calculated from the UV chromatogram.

RESULTS AND DISCUSSION

Drug release characteristics

To determine the amount of model drug released from the fibers at each data point, the theoretical weight percentage of the protein loaded into the fibers was calculated. The calculation was based on the assumptions that a homogeneous emulsion and fiber were produced, and that the aqueous phase and residual solvents were removed before weighing. In addition, the calculations assumed no loss of loaded drug during the creation of our emulsions or during extrusion of the fibers. For one set of fibers, 0.2 g of BSA was dissolved in 2 mL of water to make a total volume of 2.1 mL (considering volume increase on the addition of BSA to water); thus, the amount of BSA per milliliter was 0.095 g. For the 5% BSA fibers, 250 μL of this solution was added to 5 mL of the polymer–methylene chloride solution. Therefore, the amount of BSA added to the polymer solution was 0.0238 g. Thus, the weight percentage of BSA to the total weight of BSA (0.0238 g) plus PLLA (1.0 g) was 2.32%. For the other set of fibers, 0.1 g of BSA was dissolved in 2 mL of water to make a total volume of 2.05 mL; thus, the amount of BSA per milliliter was 0.049 g. For the 10% BSA fibers, 500 μL of this solution was added to 5 mL of the polymer–methylene chloride solution. Therefore, the amount of BSA added to the polymer solution was 0.0244 g. Thus, the weight percentage of BSA to the total weight of BSA (0.0244 g) and PLLA (1 g) was 2.38%. The calculated protein amounts for each fiber used in the release kinetic study are listed in Table 1.

With the percent protein release calculated, data for the fiber–aqueous phase combinations was then plotted as percent released versus time for the length of the study. The data are normalized to the weight of each respective fiber bundle studied. The graphical depiction of the normalized cumulative release data is plotted in Fig. 1. As is readily apparent from the graphic, complete release of the theoretical maximum protein load in the various fiber types was not achieved over the course of the study. In fact, the greatest cumulative release, evident from the 5% emulsion-loaded PLGA fiber, was only slightly over 60% after 11 weeks of study. Could the study have been continued, it is likely additional BSA would have been released from the fibers, but it is also likely the cumulative release from any type would not have reached the theoretical maximum. Most likely, protein losses during emulsification and loading and during fiber production, as well as loss due to adsorption onto the fiber and the study vessel, yielded the apparent decrease in cumulative release. Further study will be accomplished to determine the sources of protein loss during the manufacturing process, as well as the true drug loading within these fiber types.

Linear mixed models analysis (using PROC MIXED from SAS for Windows; SAS, Cary, NC) of the time point release data addressed the comparison of weekly and cumulative mean release:

1. Between fibers of each polymer type (PLLA versus PLGA), separately for each aqueous phase load (5 and 10%),

TABLE 1. PROTEIN LOAD WITHIN FIBERS

Fiber type[a]	*Weight (mg)*	*Aqueous phase (%)*	*Protein aqueous phase (%)*	*Protein (wt%)*	*Total protein (μg)*
PLGA 5	24.8	5	9.52	2.32	575.36
	27.6	5	9.52	2.32	640.32
	28.7	5	9.52	2.32	665.84
PLLA 5	44.0	5	9.52	2.32	1020.80
	60.3	5	9.52	2.32	1398.96
	61.82	5	9.52	2.32	1434.22
PLGA 10	31.0	10	4.88	2.38	737.80
	39.1	10	4.88	2.38	930.58
	51.3	10	4.88	2.38	1220.94
PLLA 10	25.0	10	4.88	2.38	595.00
	31.5	10	4.88	2.38	749.70
	37.55	10	4.88	2.38	893.69

[a]PLGA 5, PLGA fiber containing 5% aqueous load; PLLA 5, PLLA fiber containing 5% aqueous load; PLGA 10 and PLLA 10, PLGA and PLLA, respectively, containing 10% aqueous load.

2. Between fibers of each aqueous phase load (5 versus 10%), separately for each polymer type (PLLA and PLGA)

For analysis 1, only the data for the first 11 weeks of observation were used, because release data for the PLGA fibers were not available after week 11 due to complete degradation of the samples. Each of these analyses controls the family-wise (within each analysis separately) type I error rate to 0.05 by using the Scheffé method of multiple comparisons.

Results for analysis 1. For the 5% fibers, there is no significant difference between PLLA and PLGA for weeks 1 through 5, in either the time point or cumulative mean release. However, a transition occurs at week 6, due to PLGA becoming significantly higher than PLLA in both weekly and cumulative drug release

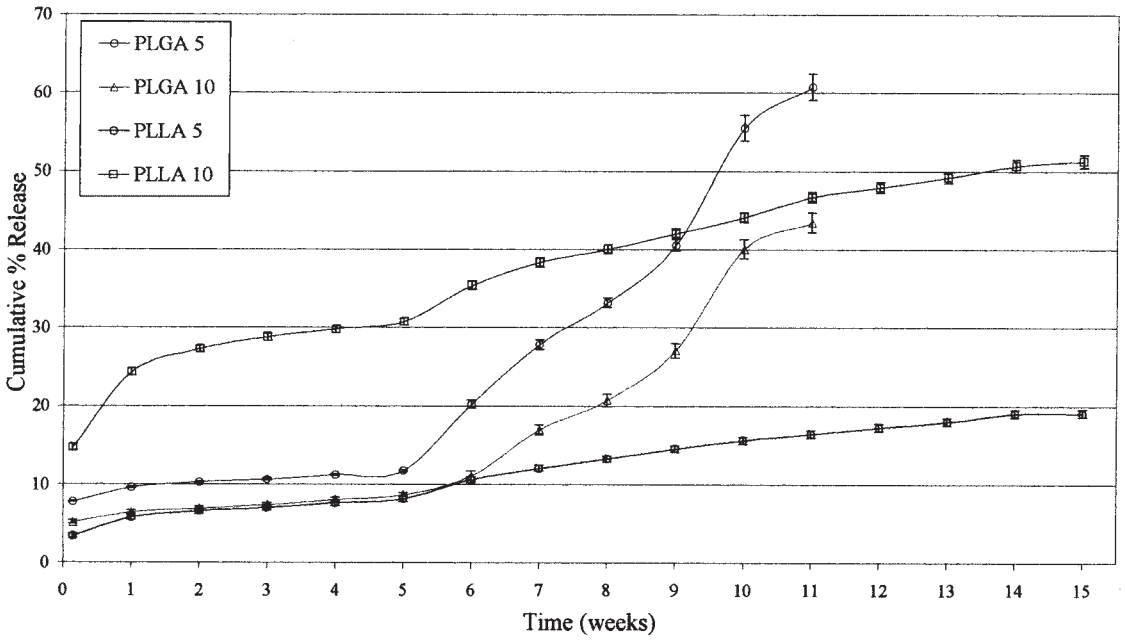

FIG. 1. Cumulative percent drug release as a function of time for PLLA and PLGA fibers carrying 5 and 10% aqueous phase loads. PLGA 5 is a poly(D,L-lactide-co-glycolide) fiber containing a 5% aqueous phase load. Similarly, PLLA 5 is a poly(L,-lactic acid) fiber containing a 5% aqueous phase load. PLGA 10 and PLLA 10 represent the respective fibers with 10% aqueous loads.

through week 11. These findings are consistent with Fig. 1.

For the 10% fibers, PLLA has significantly higher release than PLGA for the first few weeks, but this is pattern is reversed from week 6 through the end of observation.

Results for analysis 2. For the PLLA fibers, the cumulative mean release was clearly (from Fig. 1) significantly higher for the 10% fiber than for the 5% fiber. As is also clear from Fig. 1, this pattern is generally true for the weekly release, although statistical significance did not hold for every week.

For the PLGA fibers, as indicated by Fig. 1, the cumulative release is statistically the same for the 5 and 10% fibers for weeks 1 through 5, and then becomes significantly different—with the 5% fiber having higher cumulative release than the 10% fiber for 2 weeks, and then returns to statistical equivalence until the end of the study. The same general trend is followed for the weekly release data.

As evident in Fig. 1, all the fibers show initial burst release of their contained drug load ranging from approximately 3 to 15% of the total drug load for the fiber. The PLLA fiber containing 5% aqueous load (PLLA 5) fiber has the lowest burst, whereas the PLLA fiber containing 10% aqueous load (PLLA 10) has the highest. Analysis of the burst release to verify the significant difference evident between the treatments was performed separately from the weekly release data, but still used PROC MIXED in SAS for Windows. With $\alpha = 0.05$, tests of all the fixed effects (fiber, drug load, and fiber × drug load) found them to be statistically significant ($p = 0.0005$, $p < 0.0001$, and $p < 0.0001$, respectively). In other words, the amount of the burst release is not a simple sum of components attributable to fiber type and drug load separately, but depends on the specific combination of these factors.

Molecular weight degradation characteristics

Weight average molecular weight was determined for the fiber samples, and fiber degradation for the various fiber–aqueous phase combinations is presented graphically in Fig. 2. Data were tracked throughout the study, beginning 1 day after immersion of the fiber bundle into the PBS supernatant. As evident in Fig. 2, there appears to be a significant difference in the degradation of the PLLA and PLGA fibers, although the effects of aqueous phase incorporation are not readily apparent. We were unable to obtain degradation data for the PLGA fibers after week 7 because of complete dissolution of the fiber bundles. This is another apparently significant difference between the PLGA and the PLLA fibers, which yielded degradation data through week 15 of this study.

Linear mixed models analysis was used to analyze the molecular weight data over the course of the study. Specifically, mean molecular weight was compared:

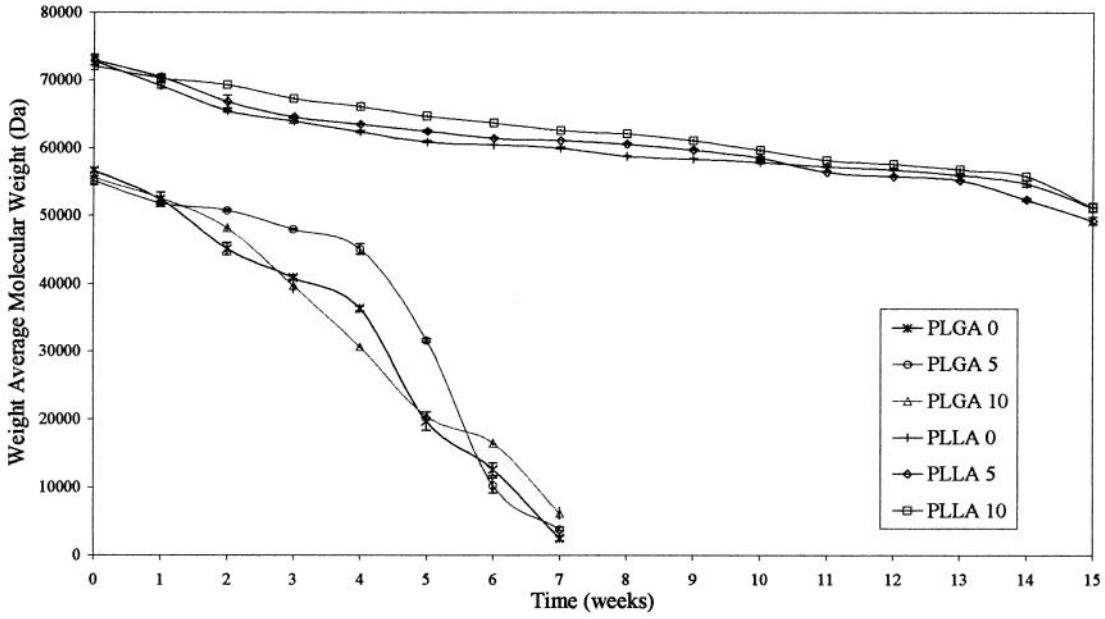

FIG. 2. Weight average molecular weight degradation over the course of the study for all fiber types tested. PLGA 0 is a poly(D,L-lactide-co-glycolide) containing a 0% aqueous phase load. Similarly, PLLA 0 is a poly(L-lactic acid) fiber containing a 0% aqueous phase load. PLGA 5 and PLLA 5 represent the respective fibers with 5% aqueous loads, and PLGA 10 and PLLA 10 represent the respective fibers with 10% aqueous loads.

1. Between fibers of each polymer type (PLLA versus PLGA), separately for each aqueous phase load (0, 5, and 10%)
2. Between fibers of each phase load (0, 5, and 10%), separately for each polymer type (PLLA and PLGA)

For analysis 1, only the overlapping data were used for analysis. Each of these analyses controls the family-wise (within each analysis separately) type I error rate to 0.05 by using the Scheffé method of multiple comparisons.

Results for analysis 1. Here, the statistical analysis merely confirms what is obvious from Fig. 2: regardless of the incorporated aqueous load, the PLLA fibers had dramatically less degradation than did the PLGA fibers. Hence, polymer choice significantly affects the degradation profile of the fiber.

Results for analysis 2. For the PLLA fibers, the differences across the three aqueous loads in molecular weight were statistically significant for most weeks, but the differences were generally small in magnitude (on the order of 5% of the molecular weights being compared). Generally, the molecular weight degradation slightly increased with increasing aqueous load.

For the PLGA fibers, the results are essentially those given by the degradation plots in Fig. 2. Where those curves are close, there is no significant difference between the loads. Where the curves deviate substantially, the differences are statistically significant. However, as indicated in Fig. 2, the molecular weights of the PLGA fibers vary greatly over the 7 weeks of available data.

General pH characteristics

The pH of the assay supernatant was tested at each time point in order to track the relative changes in pH for each fiber type with respect to a blank. Data were not available after week 9 for the unloaded PLGA (PLGA 0), and after week 11 for the other two PLGA combinations (PLGA 5 and PLGA 10), but were collected through week 15 for all of the PLLA fiber replicates. This lack of data for the PLGA fibers was a result of accelerated degradation of the test bundles, which made it impossible to collect further data. Briefly, solution pH over the course of this study ranged from approximately pH 7.0 for all fiber types at the first time point, to approximately pH 5.7 for the PLLA fibers and pH 3.6 or below for the PLGA fibers at their respective last assay point. It is assumed that the degradation of the PLGA fibers that resulted in a significantly lower solution pH at all sample points beyond week 3 also affected release of the model drug (BSA). However, this was not specifically tested in this study. It is apparent, however, that the increased rate of degradation of the PLGA fibers (yielding acid production and a decrease in pH) after week 4 (see Fig. 2), significantly impacted the drug release rate (see Fig. 1).

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CONCLUSION

Testing of the drug release and molecular weight degradation of drug-loaded PLLA and PLGA fibers has indicated that the polymer type and the amount of aqueous phase incorporated into the polymer solution through emulsification are both important to how the fibers respond in an *in vitro* environment. Polymer type significantly affected drug release and molecular weight degradation, which are evident in Figs. 1 and 2. Aqueous phase incorporation significantly affected molecular weight degradation for PLLA, but less so for PLGA, and results in a significant difference in drug release rates for both fiber types.

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AU5

CROW

AU1
Degrees?

AU2
No Cohen in Refs. 1-3?

AU3
“PLLA fiber containing 5% aqueous load” OK? or amend

AU4
“Fig. 2” OK? or amend

AU5
“Ph.D.” OK? or amend