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K. J. L. Burg
Carolinas Medical Center

M. LaBerge
Clemson University

D. L. Powers
Clemson University

S. W. Shalaby
Clemson Unviersity

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IMAGE ANALYSIS AND THE EFFECT OF MOLECULAR ORIENTATION ON DEGRADING LACTIDE POLYMER FILMS

K.J.L Burg[1*], M. LaBerge[2], D.L. Powers[2] and S.W. Shalaby[2,3]

[1]Department of General Surgery Research, Carolinas Medical Center, Charlotte, NC
[1,2]Department of Bioengineering, Rhodes Engineering Research Center, Clemson University, Clemson, SC
[3]Poly-Med Inc., Center for Applied Technology, Pendleton, SC

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Abstract

Absorbable polylactide films were studied in order to observe the effect of orientation on the resulting hydrolytic degradation pattern. Two types of polylactide films with two levels of molecular orientation were studied, during a nine month exposure to a phosphate buffered solution at 37°C. The films were sectioned and quantified as to void area, birefringence area, and intensity of birefringence using a microscope and an image analysis software package. The results, obtained using an analysis of variance, showed that the orientation played a significant role in the degradation pattern of the polylactide films, either decreasing or causing no change in the development of voids, increasing birefringence intensity, and causing an unpredictable response in the amount of birefringence.

Key Words: Absorbable, birefringence, degradation, image analysis, microscopy, orientation, polarized, polyester, polylactide, solid state uniaxial orientation.

*Address for correspondence and current address:
K.J.L. Burg
Department of Bioengineering
501 Rhodes Engineering Research Center
Clemson University
Clemson, SC 29634-0905
Telephone number: (864) 656-6462
FAX number: (864) 656-4466
E-mail: kburg@clemson.edu

Introduction

Bioabsorbable polymers have found an important niche in the medical field as transient devices which need no retrieval surgery. They have been successfully used in a variety of applications from drug delivery to tissue engineering [2, 4, 6, 8, 16, 21, 27]. The most common absorbable materials, the polyesters, have been scrutinized with particular attention to their degradation behavior and subsequent effect on surrounding tissue. It has been proposed that a heterogeneous degradation phenomenon occurs in the absorption of polyester materials; i.e., the aqueous medium infiltrates the specimen, begins to degrade the center, leaving carboxylic end groups which further accelerate the degradation process [10, 13, 24]. In the last stage of degradation, when the mass loss progresses to the exterior of the material, the acidic products which had not until this point been able to move through the surface, are released from the center of the material into the surrounding environment. This could be detrimental to the tissues if a large release occurs.

The heterogeneous degradation has been correlated to a series of chemical events, based on the chemical structure of the polylactide material [3, 9, 11, 12, 13, 14, 15, 19, 24, 28]; however, the link to processing determined morphology has yet to be established. Molecular orientation has been used as a means of enhancing the mechanical properties, e.g. increasing the modulus and strength of rods and films, for specific applications [25, 26]. However, none of the earlier studies have systematically addressed the effect of orientation on the degradation pattern. Solid state uniaxial orientation (SS-UO) was used in this study to orient two types of polylactide films [22]. The purpose of the present study was to determine if and to what extent the orientation of two types of polylactide films, a poly-L-lactide and a poly-L-lactide-*co*-D,L-lactide, will change their degradation pattern.

Measurements and observations were made on vari-

ous cross sections along the length of a particular film and at various locations within each cross section in order to determine if the degradation pattern was uniform or heterogeneous. The degradation of an absorbable material is most obviously monitored by recording the void volume within the material, starting with zero voids in the initial film. This analysis may be conducted by sectioning the material and measuring the void area. Both the amount of voids and their location within the material will assist in describing the degradation pattern. The degradation of the material may also be monitored by calculating both the area and intensity of birefringence, defined as the ability of the material to rotate the plane of polarized light. Birefringence can be associated with the presence of crystalline regions, ordered amorphous regions, and from occurrences such as stresses, imperfections, or voids. Therefore, as the void content and molecular order of the material change with time in aqueous solution so too will the birefringence. The area of birefringence will pinpoint where and the extent to which reorganization and degradation is occurring whereas the intensity of birefringence will give an idea as to why these changes are taking place.

Materials and Methods

Polymers

A poly-L-lactide-*co*-D,L-lactide (90:10) was obtained in pellet form (Resomer LR 909, molecular weight = 1,300,000 Daltons, polydispersity = 2; Boehringer Ingelheim, Ingelheim, Germany) and a poly-L-lactide was obtained from PURAC Biochem (molecular weight = 270,000 Daltons, polydispersity = 12; Gorinchem, The Netherlands), also in pellet form. The polymers were placed under vacuum at 50°C for 24 hours in order to purge the system of volatiles.

Film processing

Films of regular thickness (0.8 ± 0.09 mm) and uniform appearance were processed on a Carver Laboratory Single Column Press (Fred S. Carver, Inc., Wabash, IN). Poly-L-lactide (PL) in the amount of 22.3 g was placed on a 2.0-mm thickness steel plate covered with polytetrafluoroethylene release paper (Airtech International, Carson, CA) and placed on the lower press platen. A 0.5-mm thickness steel frame (151-mm by 151-mm outside dimensions and 91-mm by 127-mm inside dimensions) and a 2.0-mm thickness steel plate (151 mm by 151 mm), also covered with coated paper, were added to the top. The press platens were heated to a temperature of 190°C and maintained with no applied pressure for 10 minutes. Subsequently, a 6,800-kg force was applied and the platens were quick quenched with cooling water to room temperature with cooling water, in order to reduce the crystallinity [14, 18]. After 10 minutes, the press was released and the films were removed from the press. Poly-L-lactide-*co*-D,L-lactide (PL-DL) in the amount of 15.7 g was similarly processed using a platen temperature of 150°C and slow cooling for 20 minutes, in order to maintain some crystallinity, to room temperature. Films were cut into 60.0×3.2×0.8 mm dimensions using an Isomet Low Speed Saw (Buehler, Lake Bluff, Illinois) with a 127-mm diameter, 0.381-mm kerf Vanguard thin kerf diamond coated blade (Rockazona, Peoria, AZ). These were labeled "nonoriented" films since they were not processed using SS-UO. A second set of films was similarly fashioned to a thickness of 1.6 mm, using 30.4 g PL and 22.3 g PL-DL, respectively. These films were dimensioned to 52.0×16.9×1.6 mm and each of this set was oriented to a two times draw SS-UO.

To uniaxially orient the films using SS-UO, a 52.0×16.9×1.6 mm strip was placed in the center of a rectangular cavity mold of width 16.9 mm; a plunger matching the cavity dimensions was placed along the top of the cavity. The mold was inserted in the Carver press, and the unit was heated to just above the glass transition temperature, 60°C, of the polymer. The heating units were immediately turned off and a 6,800-kg force was applied. The PL-DL and PL specimens were removed when the mold had been cooled or quenched, respectively, to room temperature; the result was a longer film of 104.0×16.9×0.8 mm. The films were then cut further into specimens of 60.0×3.2×0.8 mm, matching the dimensions of the nonoriented films. A total of 80 films was manufactured, including 20 of each type, for immediate and monthly analysis over the nine-month time span.

The films were sterilized with ethylene oxide (Anprolene Sterilizing Gas Ampules; H.W. Andersen Products, Inc., Oyster Bay, NY) at room temperature, as they would be before implantation, and aerated under continuous vacuum for 48 hours. The sterilized samples were then placed in a phosphate buffered environment at pH 5.5 and a temperature of 37°C, to emulate an acidic environment. The phosphate buffered solution [17] was made by adding 7.09 grams of Na_2HPO_4 (Aldrich Chemical Company, Milwaukee, WI) and 79.34 grams of NaH_2PO_4 (Fisher Scientific, Fairlawn, NJ) to a small amount of distilled water and diluting this mixture to 4 liters. Each specimen was placed in 0.05 liters of the solution and maintained at 37°C. The specimen containers were shaken with a modified Lab-Line Multiwrist Shaker (Lab-Line Instruments, Inc., Melrose Park, IL) at a frequency of approximately 20 Hz over a 9-month time period in order to allow all surfaces an equal exposure opportunity. Eight specimens, two oriented PL, two oriented PL-DL, two nonoriented PL, and two

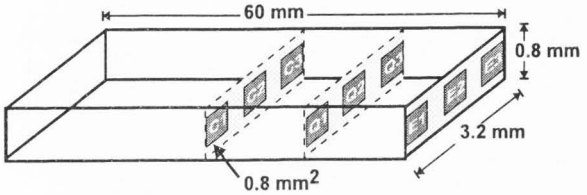

Figure 1. Section locations.

nonoriented PL-DL, were removed and dried under vacuum for 24 hours each month before analysis.

Embedding protocol

EMbed 812 (Electron Microscopy Sciences, Fort Washington, PA), is an epoxy resin, replacing the earlier Epon 812. Mixture A was made by mixing 62 ml of EMbed 812 with 100 ml of dodecenylsuccinic anhydride (DDSA), according to manufacturer recommendations. Mixture B was made by combining 100 ml of EMbed 812 with 90 ml of nadic methyl anhydride (NMA). A 1:1 mixture of A and B was combined with the accelerator in the amount of 1.5% of the total volume of A and B. The mixture was stirred thoroughly and poured in a peel away plastic mold. The resin was partially polymerized in a 37°C environmental chamber over a 24-hour period. Four films were placed flat on the top of the partially polymerized embedding resin. Resin was poured over the top of the specimens to complete the embedding. The blocks were cured in a 37°C environmental chamber for 48 hours.

Sectioning

The blocks were first faced using an EXAKT Cutting System (EXAKT Medical Instruments, Inc., Oklahoma City, OK), just exposing the 3.2×0.8 mm cut edge of the film. The block was then glued to a plastic cube specimen mount with the faced area up. The sectioning was performed using the Reichert-Jung Polycut-E sliding microtome (Leica Inc., Deerfield, IL) with a 40 degree tungsten carbide D profile blade.

The blade angle on the sliding microtome was 3 degrees with a speed of 3.5 mm/sec to face and 3 mm/sec to section. While facing each block, the microtome was set to continuous stroke, 10-μm thickness. Once an even cut was achieved, the microtome was set to single stroke, and the front and back stops were set as close to the blade as possible. Sections were taken at 10 μm. As a section was cut, it was gently removed with tweezers and placed on a droplet of deionized water on S/P® Brand Superfrost™ Plus Microscope charged slides (Baxter Diagnostics, Inc., Deerfield, IL). A square of plastic Glad® Clingwrap (First Brands Corporation, Danbury, CT) was placed on top of the section and a roller was gently applied along

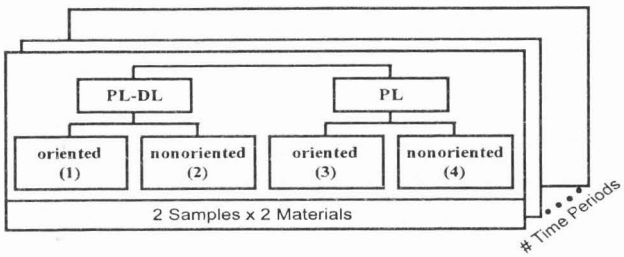

Figure 2. $2 \times 2 \times 11$ factorial design.

the plastic surface to flatten the section [1]. A paper towel square was placed on top of the plastic and the slides were stacked under a weight to dry. Several sections were taken at location *C* (see Figure 1), the block was cut with the EXAKT cutting system to expose the specimen at the quarter length, *Q*, and the procedure was repeated. Finally, the block was cut with the EXAKT cutting system to expose the specimen at Location *E* and the sectioning procedure was repeated. The slides were dried overnight at room temperature.

Microscopic analysis

The image analysis system consisted of a Zeiss Large Universal Research Microscope (Carl Zeiss, Inc., Thornwood, NY), a Macintosh Quadra 900 (Apple Computer, Inc., Cupertino, CA) computer using adapted NIH Image 1.40 software [23], and a CCD AVC-D7 video camera (Sony Corporation, Tokyo, Japan) that displays the microscope image on an attached TV monitor. The computer captures still images from the video camera for analysis, and the software identifies areas of similar visual appearance which can then be quantified.

The slide to be examined was placed on the microscope stage, the microscope was Köhler Illuminated [20], the image was captured at 200× magnification, and the void sizes were quantified. Sections *C*, *Q*, and *E* in Figure 1 were analyzed at locations 1, 2, and 3; at each location a 0.8 mm^2 area was examined. In this manner, both the longitudinal and cross sectional surface-center degradation was monitored.

A polarizer, analyzer, interference filter, and Sérnamont compensator were added to the Zeiss microscope and the sections were analyzed for birefringence [29]. A polarized image of each section was also captured, and the area of birefringence was similarly quantified using 200× magnification to obtain an average area of birefringence for the sections *C*, *Q*, and *E* at locations 1, 2, and 3 (Fig. 1). The average intensity of birefringence for each entire section *C*, *Q*, and *E* was quantified using 79× magnification.

Statistical analysis

This is a $2 \times 2 \times 11$ factorial experimental design

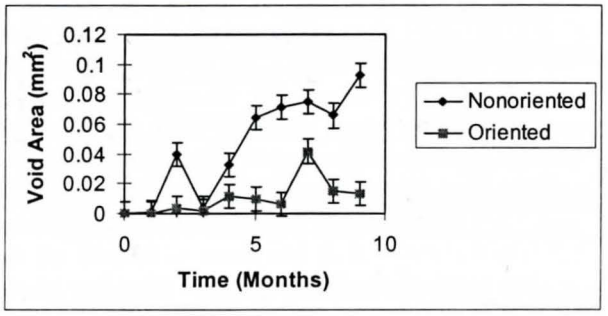

Figure 3. Change in void area with time, PL.

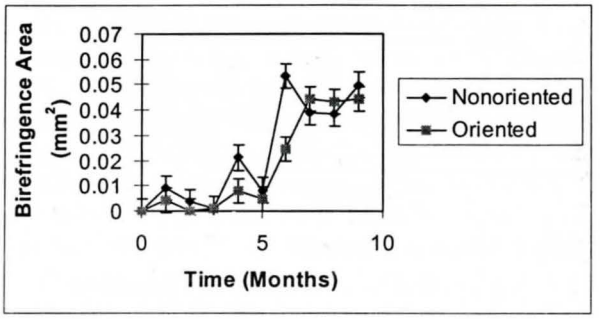

Figure 4. Change in birefringent area with time, PL.

[7],where there are two material types, two levels of orientation, and eleven absorption time periods. The monitored absorption times were at one month intervals for nine months; the remaining two time periods are the initial, as received polymer and the initial polymer film immediately after processing. The experiment was repeated two times, following this design shown in Figure 2. The data was analyzed using the analysis of variance (ANOVA) procedure, specifically with the SAS® (SAS Institute, Cary, NC) statistical software.

Results and Discussion

This study involved the analysis of two types of polylactide materials of differing molecular weights and crystallinities; therefore, very different behaviors were observed over the course of the study. The PL-DL molecular weight is significantly higher than that of the PL, whereas the PL-DL crystallinity is significantly lower. Overall, however, the percent crystallinity and the molecular weight of either oriented material (PL-DL or PL) is not significantly different from that of its corresponding nonoriented material (PL-DL or PL) [5], allowing each nonoriented material to act as the control.

Image analysis

The image analysis included an assessment of void area, birefringence area, and birefringence intensity. The PL-DL samples displayed no changes in void or birefringence area over the time interval studied; therefore the discussion of these two variables only details results from the PL specimens. Figure 3 illustrates the changes in void area with increasing exposure time for the oriented and nonoriented cases. An analysis of variance was used to analyze the void and birefringence data; select results, including significance values, are included in Table 1. Overall, time in solution, a main effect, has a significant effect (significance value of $Pr = 0.0001$) on void area (Table 1); however, Figure 3 demonstrates that the oriented PL is minimally affected by time. The nonoriented PL, in comparison, behaved quite differently and displayed a significant increase in void area over the nine month time interval, thus there is a significant time and orientation interaction (denoted by *Time*Ori* in Table 1). The response of the nonoriented PL is attributed to the ease of the aqueous medium in infiltrating the less dense regions, causing increased degradation throughout the film. The majority of degradation product in the central region collects there and, due to its oligomeric nature and the surrounding relatively intact polymer, it will not leach out of the sample until a later time period. The oriented PL contains tightly packed chains and it is much more difficult for water to infiltrate this system; therefore, there will be less degradation throughout.

Generally, the longitudinal section location (LSect) has no significant effect ($Pr = 0.027$) on void area, and the cross section location (Csect) is only significant at a probability level of 0.10. Each orientation level was examined separately to further clarify these results. Select areas, representing extreme cases, were compared for both the nonoriented and oriented: i. *C2* was compared with *E1* and *E3*; ii. *C2* was compared with *C1* and *C3*; and iii. *E2* was compared with *E1* and *E3* (refer to Figure 1). No comparisons demonstrated significant differences in the case of the oriented PL; so, considering the average values over all ten time periods, there appears to be an even distribution of voids across the oriented specimen. Both comparisons I and iii were also nonsignificant for the nonoriented PL. However, ii, the most extreme comparison, resulted in a significant difference in void area for the nonoriented PL. Point C2 (Fig. 1) had a much greater void area, on the average, than points C1 or C3, implying that the nonoriented PL may be prone to heterogeneous degradation around month five. This is attributed to the shorter chains being easier to orient and crystallize and any monomer acting as a plasticizer [5].

The orientation, time, and longitudinal section location all have a significant effect ($Pr < 0.05$), on the birefringent area, that is, the quantity. The orientation

Table 1. Statistics[a] for image analysis, PL and PL-DL.

Dependent Variable	Source	DF[b]	F Value[c]	Pr[d] > F
Void Area				
	Orientation	1	55.94	0.0001
	Time	9	10.16	0.0001
	LSect	2	1.30	0.2745
	CSect	2	2.31	0.1013
	Time*Ori	9	4.28	0.0001
Birefringence Area				
	Orientation	1	4.28	0.0395
	Time	9	27.6	0.0001
	LSect	2	3.84	0.0228
	CSect	2	1.10	0.3351
	LSect*Time	18	3.54	0.0001
Birefringence Intensity				
	Orientation	1	16.34	0.0001
	Time	9	13.92	0.0001
	Material	1	1.18	0.2794
	LSect	2	0.81	0.4495
	Orientation*Time	9	5.34	0.0001

[a]Null hypothesis (H_0): The source of variability is zero [b]Degrees of freedom [c]Fisher test statistic
[d]Probability of incorrectly rejecting the null hypothesis and concluding that the source of variability in question is significant

does not influence the area in any consistent manner from time period to time period as can be seen in Figure 4. In fact, this measurement is probably far more informative when comparing between two material types, since the area reflects the inherent chemical structure. The birefringent area increases over the exposure time period as the chains reorganize and crystallize. This increase begins as the PL starts to acquire larger amounts of lower molecular weight material [5]. Comparisons i, ii, and iii were again made, this time comparing the average birefringent area over ten time intervals at different points (as shown in Figure 2). No substantial differences were found in amounts of birefringent area, not totally surprising since the disordered amorphous regions, lacking birefringence, will be the first to degrade.

Birefringence intensity

The intensity measurement can give an indication of packing of the chains, crystal size, voids or imperfections in the material or even in the crystal. This is not to be confused with the birefringent area measurement which is more a measure of the presence of orientation. Although the birefringent areas appear to be evenly dispersed across the material, their intensity is not as uniform. This measurement was unfortunately limited to an average measurement of the intensity of a given longitudinal section *C*, *Q*, or *E*, and not subdivided into cross sectional locations *1*, *2*, and *3*. The average intensity of birefringence was measured for both PL and PL-DL, computed from an analysis of the entire cross section.

The average intensity did not vary from longitudinal location to location. Comparing each section visually, however, it is apparent that the nonoriented PL has much brighter areas around the edges, whereas the oriented PL has parallel, interior bands of crystalline areas (Figs. 5 and 6). Evidently the more tightly packed, oriented material loses its birefringence intensity around the edges as this, the most accessible portion, is preferentially degraded. The nonoriented PL, being more loosely packed, allows water to readily infiltrate the interior of the material. According to the heterogeneous degradation theory, the inner portion remains less bright as the degradation product is retained here.

It was found that orientation and time had a significant effect (Pr = 0.0001) on the birefringence intensity (Table 1); that is, for all sections *C*, *Q*, and *E*. Figure 7 shows the change in birefringence intensity over time for the oriented and nonoriented samples, incorporating both PL and PL-DL, and Table 1 displays the statistical results. Overall, the oriented material shows equal or greater birefringence values than the nonoriented. This

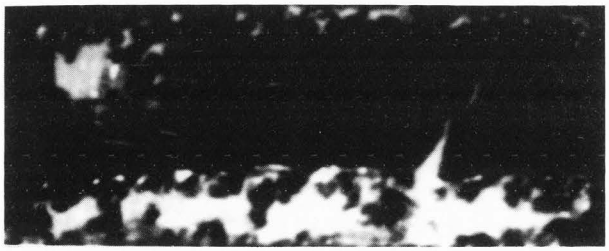

Figure 5. Cross section of nonoriented PL film after 2 months degradation (3.2mm×0.8mm; picture width 3.2 mm).

Figure 6. Cross section of oriented PL film after 8 months degradation (3.2mm×0.8mm, picture width 3.2 mm).

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makes sense as the orientation process essentially anneals the material and allows the crystalline fraction to develop and increase in thickness with this additional processing step. This results in a much brighter specimen in polarized light.

Figure 7 also shows the importance of exposure time. The nonoriented materials, including both PL and PL-DL, undergo a decrease in birefringence, starting after two months exposure, then regain this after 6 months, corresponding to the appearance of lower molecular weight molecules in the materials [5]. The oriented materials show a shorter time span of decreased values; they undergo a decrease after 4 months exposure, and then regain this after 5 months. The molecular chains which are cleaved during processing and during early exposure will solubilize and leach from the system. This will result in a less dense or more disperse crystalline packing scheme which will result in a decrease in birefringence intensity. Once the remaining molecular chains reorganize and become more dense and oriented, the birefringence will increase again. The nonoriented material takes longer to regain birefringence since it will take longer to organize the nonoriented chains.

While the change in birefringence area and void area was nonexistent in the case of PL-DL, there was a change in birefringence intensity. The material type does not make a substantial difference in the birefringence intensity values. Normally, one would expect the longer chained, higher molecular weight PL-DL material

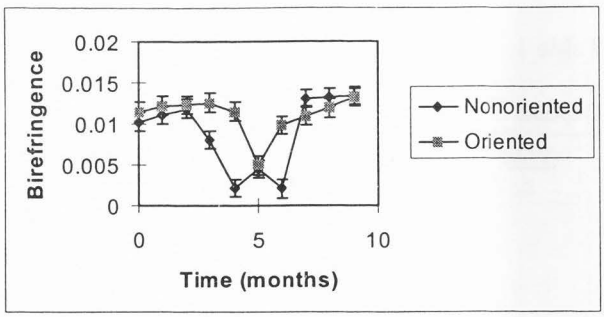

Figure 7. Average change in birefringence intensity with time, PL and PL-DL.

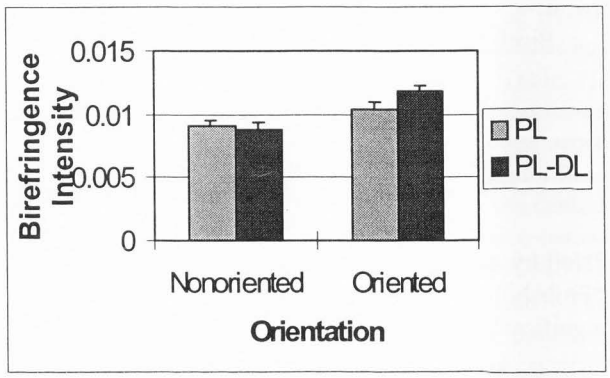

Figure 8. Change in average birefringence intensity with orientation, PL and PL-DL.

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to be slower to orient and therefore be lower in birefringent intensity. However, due to the quench cycle, the fast crystallizing PL material formed many small crystals, while the PL-DL was able to more slowly form fewer, larger crystals, thus accounting for the equality in birefringence intensity. Although, on the average, the material type had a nonsignificant effect ($Pr = 0.28$) on this value, it can be seen from Figure 8 that the birefringence value of the PL-DL oriented material was significantly ($p < 0.05$) larger than the that of the oriented PL. The PL-DL material has a substantial amorphous fraction. When the material is stretched or oriented, the amorphous and, to a lesser extent, the crystalline orientation as well as the strain at the amorphous/crystalline interface will contribute to the birefringence of the system. This will be less prevalent in the PL oriented system, which has a much lower amorphous fraction.

Conclusions

To summarize, orientation, exposure time, and cross

section have a significant effect on the amount of void volume. Specifically, an increase in orientation causes a decrease in the rate of void production in PL due to aqueous environment while time causes an increase in voids. There is a heterogeneous degradation pattern in nonoriented PL as can be seen by increased voids in the center of this material. Orientation and time have a significant effect on the area of birefringence. Time causes an increase in the area of birefringence of the PL and orientation causes an inconsistent response in birefringent area. An increase in orientation and increase in time both cause an increase in intensity of birefringence, the PL-DL oriented system having greater intensity than the PL oriented system. The edges of the PL nonoriented material are brighter whereas there are birefringent bands within the oriented PL. It is therefore possible to modulate the absorption rate and mechanism by carefully controlling the level of orientation in a given material.

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Discussion with Reviewers

M. Deng: Can authors explain why the orientation affect PL-DL and PL differently during aging study?
Authors: The molecular weights of the two materials are very different, also the crystalline content, both of which play a large role in relative ease of chain mobility and therefore degradation.

M. Deng: I am not sure how the cutting would affect the morphology of the polymer. According to my own experience, PL is very hard at room temperature, even non-degraded polymer samples will be affected by cutting.
Authors: Yes, this is indeed a limitation of histological methods - there will be cutting artifact. Due to the relatively high molecular weights and early absorption times analyzed, the sectioning process was not problematic, hopefully resulting in minimal artifact. The extent of artifact in future work investigating more sensitive systems could potentially be resolved using confocal microscopy or magnetic resonance microscopy.

Reviewer II: It is very difficult to believe that the more amorphous PL-DL material did not exhibit any changes in void area or birefringence. The authors fail to specify the starting molecular weight or crystallinity of the materials. This is very disconcerting. Without this information they may be comparing apples and oranges. The authors have not offered any explanations for the PL-DL material. Thus, effectively the paper is based on only one material - the PL form. Here also they have failed to use the more rigorous techniques for quantifying degradation such as molecular weight, viscosity, crystallinity, glass transition temperature etc. Although the parameters they have chosen to use are valid, they by themselves provide only meager infor-mation that needs to be supplemented. For example, as the authors themselves write, birefringence can be affected by several parameters such as crystallinity, stresses, imperfections etc. The present study does not control any of these properties. Although the manus-cript strives to make an important point by trying to illustrate that orientation affects degradation, the authors need to perform a more closely controlled study.
Authors: Molecular weights, thermal transitions, and mechanical analyses were measured in a separate study and have been published as such. The authors did not wish to republish this data and have, rather, referenced it (Burg and Shalaby, 1997). This study involves observations of two material types, different in molecular weight and crystallinity, where each oriented material was paired with a nonoriented material of the same type. The nonoriented material therefore served as the control polymer to ensure an appropriate comparison.

Reviewer IV: I was very disappointed to find out (only when reading the **Results/Discussion**) that the PL-DL material did not result in any data. The entire set-up of your paper (comparing different films for their degradation behaviour) is herewith destroyed. I would insist upon either continuing your study and gathering the PL-DL data or completely re-write this paper to be a 9 month degradation study of PL. Was the time interval of 9 months long enough?
Authors: Three measurements were made - void volume, birefringence intensity, and birefringence area. There was indeed no change in PL-DL birefringence area or void volume with time. There was, however, a measured change in PL-DL birefringence intensity, as explained in the text and shown in Figure 5. Furthermore, absence of change does not mean that there is no data collected, and the fact that the PL-DL birefringence area and void volume remained constant with time is extremely important.

Reviewer IV: In theory, the PL-DL variant should degrade faster, not slower, due to the fact that cristallinity will be reduced. How do you explain your findings?
Authors: The crystallinity of the PL-DL system is indeed lower; however, the molecular weight of the PL-DL is substantially higher.